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Rhizoctonia Root Rot of Pepper (*Capsicum annuum*): Comparative Pathogenicity of Causal Agent and Biocontrol Attempt using Fungal and Bacterial Agents

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Abstract

Rhizoctonia root rot of pepper (*Capsicum annuum* L.) is becoming serious in Tunisia. Comparative pathogenicity tests performed for *Rhizoctonia solani* isolates recovered from pepper and potato showed that Rhiz.7 and Rhiz.4 were the most aggressive. They reduced by 53.5%-91.4% the aerial part fresh weight of inoculated cv. Baklouti plants relative to control. Rhiz.7 decreased by 81%-88% the root fresh weight on cvs. Beldi and Baklouti. Various fungal and bacterial agents were tested against *R. solani*. Dual culture trials showed that *Trichoderma harzianum*, *T. viride* and *Glicladium virens* grew and sporulated profusely over *R. solani* colonies and altered its hyphae. *Pseudomonas huttiensis* 69, *P. aureofaciens* 31 and *Burkholderia glathei* 35 reduced pathogen growth by 9.71-12.87%. These bio-agents were tested for their effects on rhizoctonia root rot disease and pepper growth. On cv. Beldi, pre-emergence damping-off, noted after 15 days, was suppressed by 55 (for *G. virens*), 45 (for *T. viride*) and 50% (for *T. harzianum*). This inhibition reached 57.14% using *Bacillus pumilus* 420 and *P. putida* 227. Tested on pepper cv. Altar, all tested fungi decreased by 40% post-emergence damping-off, and significantly increased the plant height of *R. solani*-inoculated and treated plants by 21.13 (for *T. viride*) to 36.34% (for *T. harzianum*) relative to control. *P. aureofaciens* 314 and *P. putida* 227 completely suppressed *R. solani* post-emergence expression. Treatments with *P. aureofaciens* 314, *P. aureofaciens* 31, *Bacillus pumilus* 420, *P. fluorescens* Pf and *P. putida* 227 induced a significant increase in their height compared to control. An improvement of the aerial part fresh weight by 54.54, 48.09 and 47.74%, as compared to control, was induced by *P. aureofaciens* 314, *B. glathei* 35 and *P. huttiensis* 69, respectively.

Keywords: Aggressiveness; Antifungal activity; Biological control; Disease severity; Pepper; *Rhizoctonia solani*

Introduction

In Tunisia, pepper (*Capsicum annuum* L.) is a strategic and economically relevant crop ranked third after tomato and potato in terms of cropped vegetable areas. During the last years, approximately 20000 ha/year were devoted to the growing of open field and protected peppers with an average annual production of about 346000 tons [1]. Furthermore, Tunisia is the third largest pepper producer in Africa, after Nigeria and Egypt and the third largest exporter (in terms of tonnage) after Morocco and South Africa [2].

However, in Tunisia and worldwide, this crop is highly susceptible to many fungal diseases among which damping-off, root rots and wilts are widespread and serious in many pepper-producing regions both in open field and protected cultivation leading to significant plant and crop losses. These diseases can affect pepper at any growth stage and are induced by several soil borne pathogens including *Phytophthora capsici*, *P. nicotianae*, *Rhizoctonia solani*, Fusarium *solani*, *F. oxysporum*, *Verticillium dahliae*, *Pythium* spp. [3-6].

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris*) is a worldwide destructive soil borne pathogen causing various diseases to many economically important crops, under diverse environmental conditions [7]. On pepper, *R. solani* can cause several types of damage at multiple growth stages such as seed decay, pre- and post- emergence damping-off, wire stem, root rot, and hypocotyl or tap root with necrotic spots [8,9].

Several approaches have been adopted to manage diseases caused by *R. solani* involving mainly cultural practices and chemical control. However, due to the pathogen's wide host range, the long-term survival of its resting structures, sclerotia, in the soil and the lack of genetic resistance, yield losses still occur. Moreover, in Tunisia, pepper is grown in short rotation with tomato or potato which are highly susceptible to Rhizoctonia diseases [10,11]. Currently, the use of biocontrol agents, fungi, and bacteria, may offer a potential and viable solution to effectively control this disease.

Among biocontrol agents, *Trichoderma* and *Gliocladium* species are the most widely used antagonists for controlling plant diseases caused by fungi due to their ubiquitous nature, ease with which they can be isolated and cultured and their rapid growth on a variety of substrates [12]. These species-controlled *R. solani* by diverse mechanisms [13-17]. In fact, these species act as competitive hyperparasites, producing antifungal metabolites, whether volatile or not and hydrolytic enzymes that cause structural changes at cell level, such as vacuolization, granulation, cytoplasm disintegration and cell lysis, which have been observed in organisms with which they interact.

Several bacterial species belonging to *Pseudomonas* and *Bacillus* genera have been also used to manage Rhizoctonia diseases [18,19].

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Indeed, strains of *B. thuringensis* were found to be efficient for the biocontrol of *R. solani* of chili pepper based on *in vitro* assays [20]. Moreover, *B. cepacia* was shown able to reduce the severity of Rhizoctonia diseases associated to pepper and tomato [19]. Antibiosis seems to be their principal mode of action [21].

Pseudomonas species were shown capable of markedly inhibiting the growth of *R. solani in vitro* and *in vivo*. Indeed, tomato plants were also highly protected against *R. solani* infestations using this bacterium suspended in water [19]. Moreover, fluorescent *Pseudomonas* species were found to induce systemic resistance in plants as a result of root colonization [18].

Recently, several rhizobacterial isolates and mainly *B. thuringiensis* B2 (KU158884), *B. subtilis* B10 (KT921327) and *Enterobacter cloacae* B16 (KT921429) were found to be efficient for the suppression of *R. solani* radial growth and disease severity and for the enhancement of tomato growth [10].

In Tunisia, *R. solani* is still being a destructive pathogen of pepper and investigations for its biocontrol are lacking. Therefore, the objectives of the current study were: (i) to evaluate the aggressiveness of different *R. solani* isolates involved in damping-off, wilt and root rot of pepper and (ii) to assess the antifungal potential of *Trichoderma* and *Gliocladium* isolates together with bacterial isolates belonging to *Pseudomonas*, *Bacillus* and *Burkholderia* genera against *R. solani* mycelial growth. Their ability to suppress Rhizoctonia Root Rot disease and to enhance growth of infected pepper plants was also evaluated.

Materials and Methods

Plant material

Three pepper cultivars, namely cvs. Baklouti, Beldi and Altar, the most widely grown cultivars in Tunisia, were used in the present study. Seeds were superficially disinfected with 5% sodium hypochlorite for 5 min, rinsed thrice with sterile distilled water (SDW) and allowed to dry at room temperature. Seeds were then sown in 77 cell-trays containing peat previously sterilized at 110°C for one hour and kept under greenhouse conditions for 30 days. Seedlings were watered as needed.

Pathogen culture and inoculum preparation

Nine isolates of *R. solani* recovered from diseased pepper or potato plants showing root rot symptoms and collected from different Tunisian sites were used in the present study (Table 1). Potato-associated isolates were included in this study for comparison since potato is usually short-rotated with pepper. These characterized isolates are held in the Phytopathology laboratory at the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia.

Before use, isolates were grown on Potato Dextrose Agar (PDA) medium amended with streptomycin sulfate (300 mg/L) and maintained in the dark for 7 days at 25°C.

To prepare pathogen inoculum, *R. solani* mycelia were collected from five 7-day-old cultures grown on PDA medium and homogenized in 0.5 L of SDW with an electric mixer for 5 min. The resulting mycelial fragments served for substrate inoculation. Pathogen inocula were added and mixed thoroughly with the culture substrate before planting.

Fungal and bacterial biocontrol agents

Three fungal antagonists, namely *Trichoderma harzianum*, *T. viride* and *Gliocladium virens*, were selected from the collection of biocontrol agents of the Phytopathology laboratory at the Regional Research Centre on Horticulture and Organic Agriculture of Chott-Mariem, Tunisia, to be used in this study. These bio-agents, originally recovered from Tunisian soils, were previously shown effective against several soilborne plant pathogens such as *Verticillium* spp., *Fusarium* spp., *Pythium* [22-24].

Isolate	Original host	Plant cultivar	Original site
Rhiz1	*Capsicum annuum*	Baklouti	Sahline
Rhiz2	*C. annuum*	Baklouti	Sahline
Rhiz4	*C. annuum*	Beldi	Chott-Mariem
Rhiz5	*Solanum tuberosum*	Spunta	Essaïda
Rhiz6	*C. annuum*	Baklouti	Sahline
Rhiz7	*S. tuberosum*	Spunta	Essaïda
Rhiz8	*S. tuberosum*	Spunta	Essaïda
Rhiz9	*S. tuberosum*	Spunta	Kairouan
Rhiz10	*C. annuum*	Chergui	Chott-Mariem

Table 1: *Rhizoctonia solani* isolates used in this study.

Isolate	Bacterial species	Origin
Pf	*Pseudomonas fluorescens*	Tunisia (a reference bacterium)
263	*Bacillus subtilis*	Tunisia
227	*P. putida*	Tunisia
31	*P. aureofaciens*	Tunisia
420	*B. pumilus*	Missouri
35	*Burkholderia glathei*	Missouri
314	*P. aureofaciens*	Missouri
69	*P. huttiensis*	Missouri

Table 2: Rhizobacterial isolates used in this study.

Fungal suspensions were prepared by scraping off mycelium from 7-day-old cultures grown on PDA medium, homogenized with SDW, and then filtered through two-layers of muslin. The resulting conidial suspension was adjusted to 10^7 CFU/mL using a Malassez hemocytometer.

Eight bacterial isolates belonging to *Pseudomonas*, *Bacillus* and *Burkholderia* genera were used in this study (Table 2). They were isolated and identified by Nasraoui et al. [25].

Rhizobacterial stock cultures were maintained on Nutrient Agar (NA) medium supplemented with 40% glycerol and stored at -20°C. Before use, bacterial isolates were grown on NA and incubated at 25°C for 48 h.

Bacterial cell suspensions used for *in vitro* and *in vivo* bioassays were prepared by scraping bacterial colonies, previously grown in NA for 48 h, in SDW and adjusted to 10^6 cells/mL.

Pathogenicity tests

To test the ability of six *R. solani* isolates to cause pre- and post-emergence damping-off disease, disinfected pepper cv. Beldi seeds were sown in cell trays filled with sterilized peat mixed with *R. solani*-infected substrate at the rate of 1:3 (v/v). Seeds sown in non-infected peat were used as uninoculated control. Ten seeds were used for each individual treatment. The percentage of seed germination and seedling emergence were determined after two weeks of incubation under greenhouse conditions.

The six *R. solani* isolates were also tested on pepper seedlings cvs. Beldi and Baklouti for their ability to cause Root Rot disease. Thirty-day old pepper seedlings were inoculated by root dipping for 30 min in the fungal suspensions of each *R. solani* isolate (mycelial fragments) prepared as previously described. Seedlings which roots were dipped in SDW only served as uninoculated control. All seedlings were then transplanted into pots filled with a mixture of peat and perlite (2:1, v/v)

previously sterilized at 110°C for one hour. The inoculated seedlings were grown under greenhouse conditions for 60 days.

At the end of the experiment, pepper plants were uprooted and washed to eliminate the adhering peat and perlite. Plant height and aerial parts and roots fresh weights were recorded. Disease severity was estimated based on the density of *R. solani* lesions formed on collar and roots according to a 0-5 scale, where 0=absence of visible lesions in the collar; 1=1 to 25% of the collar covered with lesions; 2=26 to 50% of the collar covered with lesions; 3=50 to 75% of the collar covered with lesions; 4=large lesions (> 75%) and 5=dead plant.

Pathogen re-isolations were performed from roots and collars of inoculated plants to confirm Koch postulate.

In vitro antagonism assay

Dual culture plate assays were performed in 9-cm Petri plates containing PDA to test the ability of fungal and bacterial agents to inhibit *R. solani* growth. Agar plugs (6 mm in diameter) cut from 7-day-old cultures of *R. solani* were placed each opposite to those of tested fungal antagonists. For bacterial antagonists, 10 µL of each bacterial cell suspension adjusted to 10^6 cells/mL were dropped into a 6 mm-well performed in the Petri plates using a sterile cork borer. Control plates were challenged with pathogen plugs only and bacterial suspension was replaced by a same volume of SDW.

All culture plates were incubated at 25°C for 2 days. Three plates were used for each individual treatment and the whole experiment was repeated twice. The diameter of pathogen colony was measured, and microscopic observations were made to characterize the hyphal pathogen-antagonist interactions.

In vivo biocontrol trials

In order to evaluate the ability of fungal and bacterial agents tested to reduce damping-off and Rhizoctonia Root Rot disease, three biocontrol assays were performed.

Assessment of pre-emergence damping-off suppression ability

Ten pepper cv. Beldi seeds were soaked for 10 min in each antagonist suspension prepared as previously described and sown in cell trays filled with sterilized peat mixed with an aggressive *R. solani* isolate (Rhiz4) at the rate of 1:3 (v/v). Trays were then kept at room temperature (25°C-30°C).

Pre-emergence damping-off percentage was recorded after 15 days of incubation based on the number of non-emerged seeds in relation to the number of total sown seeds.

Assessment of post-emergence damping-off suppression ability

Pepper seedlings cv. Altar (30-day-old) grown in cell trays were treated by root dipping for 30 min in the spore or cell suspension of each fungal or bacterial antagonist, respectively. Treated seedlings were transplanted in cell trays filled with peat infected with an aggressive *R. solani* isolate (Rhiz4) at the rate of 1:3 (v/v). Inoculated and uninoculated control plants were root dipped in SDW and transplanted in pathogen-inoculated and pathogen-free substrates, respectively. Trays were incubated under growth chamber conditions (at 23-26/15-18°C day-night temperatures). Five seedlings were used per each individual treatment.

The parameters, recorded 7 days post-transplanting, were plant height, plant fresh weight, percentage of post-emergence damping-off and disease severity. Post-emergence damping-off (%) was based on the number of plants showing disease symptoms in relation to the total number of emerged seedlings while disease severity was estimated based on the density of *R. solani* lesions formed on collar and roots according to the 0-5 scale detailed above.

Assessment of rhizoctonia root rot suppression ability

Pepper seedlings cv. Beldi (30-day-old) were antagonist-treated and transplanted in pathogen-infected or not substrate, as previously described for cell trays assay. For each antagonistic treatment, five treated plants were separately placed in 17 cm-pot containing a mixture of peat and perlite with the third upper substrate being infected with an aggressive *R. solani* isolate (Rhiz4). Untreated and inoculated or not seedlings were included in the assay. All the seedlings were incubated under the same greenhouse conditions.

Disease severity and plant growth parameters (plant height and aerial part and root fresh weights) were recorded 75 days post-transplanting.

Statistical analysis

The results were subjected to one-way analysis of variance and means separations were carried out using the Student-Newman-Keuls (SNK) test at $P \leq 0.05$. ANOVA was performed using SPSS version 16.0.

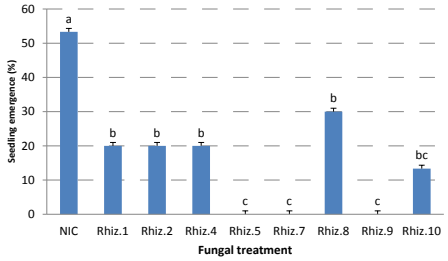
Experiments were conducted according to a completely randomized design for *in vitro* (6 replicates), *in vivo* (5 replications) and in cell trays trials (10 replications).

Results

Comparative pathogenicity of *Rhizoctonia solani* isolates

Comparative ability to induce pre-emergence damping-off: Results given in Figure 1 showed that all tested *R. solani* isolates were pathogenic to pepper cv. Beldi seeds and induced variable pre-emergence damping-off depending on isolates as compared to the uninoculated control. Rhiz.5, Rhiz.7, and Rhiz.9 isolates were found to be the most aggressive ones by inducing complete inhibition of seed germination after two weeks after incubation (ure 1). However, the remaining isolates reduced seed germination by 40 to 80% over control. These results indicated that *R. solani* isolates recovered from potato were more pathogenic on pepper seeds than those isolated from pepper plants.

Comparative ability to induce rhizoctonia root rot: Analysis of variance revealed a highly significant (at $P \leq 0.01$) variation in



NIC: Uninoculated control; Rhiz.1, Rhiz.2, Rhiz.4, Rhiz.8, and Rhiz.10: *R. solani* isolates recovered from pepper plants. Rhiz.5, Rhiz.7, and Rhiz.9: *R. solani* isolates recovered from potato plants.

Figure 1: Effect of seed infection by *Rhizoctonia solani* isolates recovered from pepper or potato on pre-emergence damping-off of pepper cv. Beldi, noted 15 days after inoculation, as compared to the uninoculated control.

Rhizoctonia Root Rot severity recorded, 60 days after inoculation, on pepper plants cv. Baklouti inoculated with different *R. solani* isolates as compared to the uninoculated control. Indeed, the lowest disease index (0.4) was observed on pepper plants inoculated with Rhiz.5 originally recovered from potato and the highest one (4.4) was noted on those challenged with Rhiz.7 associated to potato too (Table 3 and Figure 2). The pepper-associated isolates, namely Rhiz.1 and Rhiz.4, caused a significant disease severity, compared to control.

Rhizoctonia Root Rot disease index recorded on pepper seedlings cv. Beldi, 60 days post-inoculation, varied significantly ($P \leq 0.01$) depending on fungal treatments tested. Rhiz.1-, Rhiz.5-, Rhiz.6- and Rhiz.8-challenged plants showed disease severity indexes ranging from 1.2 to 1.4 which are significantly comparable to that of the uninoculated control. Rhiz.4 and Rhiz.7 isolates induced a relatively severe Rhizoctonia Root Rot disease estimated at 2.5 to 4.2, respectively, and were found to be the most aggressive on pepper plants.

Treatment	cv. Baklouti				cv. Beldi			
	Plant height (cm)	Aerial part fresh weight (g)	Root fresh weight (g)	Disease severity	Plant height (cm)	Aerial part fresh weight (g)	Root fresh Weight (g)	Disease severity
NIC	18.28 a[x]	33.69 a	3.05 ab	0 c	23.98 a	43.71 a	13.28 a	0 c
Rhiz.1	15.1 a	18.09 b	1.69 bc	2 b	17.9 ab	28.05 a	8.24 ab	1.2 bc
Rhiz.4	17.34 a	15.66 b	3.08 ab	1.8 b	17.1 ab	22.41 a	6.27 bc	2.4 b
Rhiz.5	19.36 a	20.36 ab	4.44 a	0.4 bc	22.96 a	36.73 a	11.79 ab	1.4 bc
Rhiz.6	17.74 a	24.96 ab	3.16 ab	1.2 bc	20.54 a	36.44 a	8.81 ab	1.4 bc
Rhiz.7	4.68 b	2.87 c	0.36 c	4.4 a	10.06 b	8.68 b	2.47 c	4.2 a
Rhiz.8	15.72 a	24.29 ab	4.44 a	1.4 bc	19.1a	33.85a	10.45 ab	1.2 bc

[x] Within each column, values followed by the same letter are not significantly different according to SNK test (at $P \leq 0.05$).
NIC: Uninoculated control; Rhiz.1, Rhiz.4, Rhiz.6, and Rhiz.8: *R. solani* isolates recovered from pepper plants. Rhiz.5 and Rhiz.7: *R. solani* isolates recovered from potato plants.

Table 3: Comparative effects of *Rhizoctonia solani* isolates recovered from pepper or potato on Rhizoctonia Root Rot severity and growth parameters of pepper cvs. Baklouti and Beldi plants noted 60 days post-inoculation.

NIC: Uninoculated control; Rhiz.1, Rhiz.4, and Rhiz.8: *R. solani* isolates recovered from pepper plants. Rhiz.5 and Rhiz.7: *R. solani* isolates recovered from potato plants.

Figure 2: Pepper cv. Beldi plants inoculated with different *Rhizoctonia solani* isolates observed 60 days after inoculation as compared to the uninoculated control.

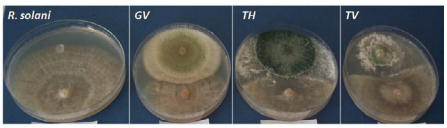
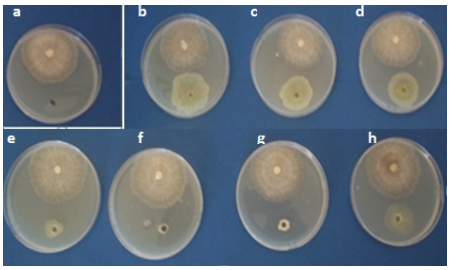
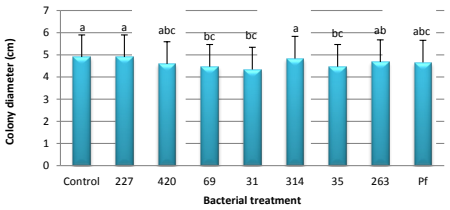


Figure 3: Competitive potential of *Gliocladium virens* (GV), *Trichoderma harzianum* (TH) and *T. viride* (TV) over *Rhizoctonia solani* observed after 5 days of incubation at 25°C compared to control.



a: Control, b: *R. solani* co-cultured with *Pseudomonas putida* 227; c: *R. solani* co-cultured with *P. huttiensis* 69; d: *R. solani* co-cultured with *Burkholderia glathei* 35; e: *R. solani* co-cultured with *P. aureofaciens* 314; f: *R. solani* co-cultured with *P. aureofaciens* 31; g: *R. solani* co-cultured with *Bacillus pumilus* 420; h: *R. solani* co-cultured with *B. subtilis* 263.

Figure 4: Colonies of *Rhizoctonia solani* dual cultured with different rhizobacterial isolates as compared to control observed after 2 days of incubation at 25°C.



Bars sharing the same letter are not significantly different according to SNK test ($P \leq 0.05$)

Control: Untreated control; 227: *Pseudomonas putida*; 420: *Bacillus pumilus*; 69: *P. huttiensis*; 31: *P. aureofaciens*; 314: *P. aureofaciens*; 35: *Burkholderia glathei*; 263: *Bacillus subtilis*; Pf: *P. fluorescens*.

Figure 5: *Rhizoctonia solani* radial growth noted after 2 days of dual culture with bacterial isolates as compared to control.

Data given in Table 3 indicated that the aerial part fresh weight of pepper plants cv. Baklouti differed significantly ($P \leq 0.05$) upon treatments tested. In fact, Rhiz.1, Rhiz.4 and Rhiz.7 were the most aggressive isolates by reducing this parameter by 46.2%, 53.5% and 91.4%, respectively, on inoculated plants relative to control. Pepper plants challenged by the remaining *R. solani* isolates had an aerial part fresh weight comparable to that noted on control plants. On cv. Beldi plants, only Rhiz.7 isolate significantly decreased by 80.14% the aerial part fresh weight, relative to *R. solani*-free control plants.

Pepper root fresh weight, noted 60 days post-inoculation, depended significantly ($P \leq 0.05$) on treatments tested. In fact, plant inoculation with Rhiz.7 reduced this parameter by 81 and 88% on cvs. Beldi and Baklouti, respectively, compared to control. However, plants inoculated by the other *R. solani* isolates showed root fresh weight significantly comparable to control (Table 3).

Fungal treatments tested did not induce a significant adverse effect on plant height of pepper cvs. Baklouti and Beldi plants as compared to control, except the most aggressive isolate Rhiz.7 where this growth parameter was lowered by 74.39% and 58.05%, respectively, relative to pathogen-free control (Table 3).

Biocontrol of *Rhizoctonia solani* by fungal and bacterial agents

***In vitro* antifungal activity of fungal antagonists:** *R. solani* radial growth noted after 2 days of incubation at 25°C did not vary significantly depending on tested fungal treatments. However, after 5 days of incubation, *T. harzianum*, *T. viride* and *G. virens* grew and sporulated profusely over *R. solani* colonies (Figure 3). Microscopic observations of pathogen mycelium at the confrontation zone strong showed hyphal lysis, formation of mycelial cords and coiling of antagonists' mycelia around pathogen hyphae.

***In vitro* antifungal activity of bacterial antagonists:** The diameter of *R. solani* colony, noted after 2 days of incubation at 25°C varied significantly ($P \leq 0.05$) upon bacterial treatments tested. Indeed, the bacterial isolates *P. huttiensis* 69, *P. aureofaciens* 31 and *Burkholderia glathei* 35 reduced pathogen radial growth by 9.71%, 12.87% and 9.71%, respectively, compared to control; whereas the remaining bacterial isolates did not significantly inhibit pathogen growth (Figures 4 and 5).

Microscopic observations of the *in vitro* hyphal interactions at the contact zone between the majority of bacterial and *R. solani* colonies showed strong lysis of pathogen mycelium.

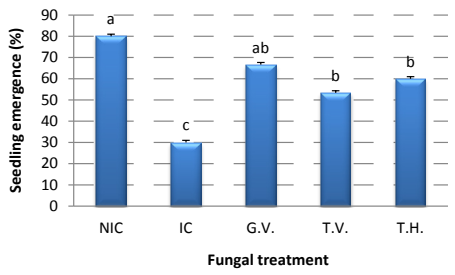
Biocontrol of rhizoctonia root rot using fungal antagonists

Fungal antagonists were evaluated for their ability to suppress disease and to enhance pepper growth under greenhouse conditions.

Suppression of pre-emergence damping-off: Soaking pepper cv. Beldi seeds in *G. virens*, *T. viride* and *T. harzianum* suspensions resulted in an improvement of the percentage of seedling emergence noted after 15 days of incubation. In fact, *R. solani* pre-emergence damping-off was suppressed by 55 (for *G. virens*), 45 (for *T. viride*) and 50% (for *T. harzianum*) as compared to pathogen-inoculated and untreated control (Figure 6).

Suppression of post-emergence damping-off: Tested on pepper cv. Altar, all tested antagonistic fungi decreased by 40% *R. solani* post-emergence damping-off, noted after 7 days of incubation, compared to pathogen-inoculated and untreated control (Table 4).

Disease severity, noted on pepper plants cv. Altar 7 days post transplanting, differed significantly ($P \leq 0.05$) upon tested treatments.



Bars sharing the same letter are not significantly different according to SNK test ($P \leq 0.05$)
NIC: Uninoculated control; IC: Inoculated and untreated control; G.V.: Inoculated and treated with *Gliocladium virens*; T.V.: Inoculated and treated with *Trichoderma viride*; T.H.: Inoculated and treated with *T. harzianum*.

Figure 6: Effect of treatment of pepper cv. Beldi seeds with fungal antagonists on expression of pre-emergence damping-off caused by *Rhizoctonia solani* noted 15 days after inoculation.

Treatments	Damping-off (%)	Plant weight (g)	Plant height (cm)	Disease severity
NIC	0 a[x]	0.39 c	4.7 a	0.00 b
IC	60 c	0.19 b	3.55 b	2.70 a
T.H.	30 b	0.14 ab	4.84 a	1.7 ab
T.V.	30 b	0.08 a	4.3 a	2.3 a
G.V.	30 b	0.13 ab	4.52 a	1.5 ab

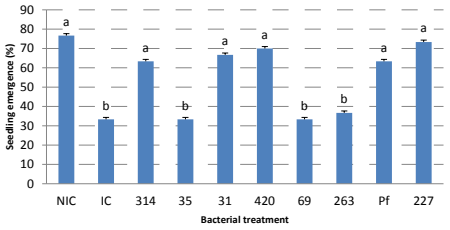
[x] Within each column, values followed by the same letter are not significantly different according to SNK test ($P \leq 0.05$).
NIC: Uninoculated control; IC: Inoculated and untreated control; G.V.: Inoculated and treated with *Gliocladium virens*; T.V.: Inoculated and treated with *Trichoderma viride*; T.H.: Inoculated and treated with *T. harzianum*.

Table 4: Damping-off incidence and severity and growth parameters noted on pepper cv. Altar plants inoculated by *Rhizoctonia solani* and treated by different fungal antagonists as compared to controls noted 7 days after inoculation and treatment.

Treatments	Aerial part fresh weight (g)	Root fresh weight (g)	Plant height (cm)
NIC	27.07 a[x]	5.67 a	33.46 ab
IC	22.65 a	4.52 a	29.68 b
T.H.	25.77 a	4.25 a	31.60 ab
T.V.	25.82 a	4.51 a	34.10 ab
G.V.	28.97 a	4.82 a	37.52 a

[x] Within each column, values followed by the same letter are not significantly different according to SNK test ($P \leq 0.05$)
NIC: Uninoculated control; IC: Inoculated and untreated control; G.V.: Inoculated and treated with *Gliocladium virens*; T.V.: Inoculated and treated with *Trichoderma viride*; T.H.: Inoculated and treated with *T. harzianum*.

Table 5: Growth parameters of pepper cv. Altar plants inoculated by *Rhizoctonia solani* and treated with three fungal antagonists noted 60 post-inoculation as compared to the untreated controls.



Bars sharing the same letter are not significantly different according to SNK test ($P \leq 0.05$).
NIC: Uninoculated control; IC: Inoculated with *R. solani* and untreated control; 227: Inoculated and treated with *Pseudomonas putida* 227; 420: Inoculated and treated with *Bacillus pumilus* 420; 69: Inoculated and treated with *P. huttiensis* 69; 31 and 314:: Inoculated and treated with *P. aureofaciens* 31 and 314; 35: Inoculated and treated with *Burkholderia glathei* 35; 263: Inoculated and treated with *B. subtilis* 263; Pf: Inoculated and treated with *P. fluorescens*.

Figure 7: Emergence of pepper cv. Beldi seedlings inoculated with *Rhizoctonia solan* and treated with different bacterial antagonists, noted 15 days post-inoculation, as compared to controls.

G. virens, *T. harzianum* and *T. viride* based treatments reduced, even insignificantly, disease severity by 44.44%, 37.03% and 14.81% respectively, compared to *R. solani*-inoculated and untreated control (Table 4).

The tested fungal antagonists significantly ($P \leq 0.05$) increased the plant height of *R. solani*-inoculated and treated plants compared to pathogen-inoculated and untreated control ones (Table 5). This increment varied between 21.13 (for *T. viride*) and 36.34% (for *T. harzianum*). In addition, height noted on inoculated and treated pepper cv. Atlar plants was significantly similar to that recorded on the uninoculated and untreated control (healthy plants) (Table 4).

Disease development and growth promotion: Rhizoctonia root rot severity, noted 60 days after transplanting, did not differ significantly between treatments tested. However, plant height varied significantly depending tested treatments where only *G. virens* significantly improved this parameter by 26.41% compared to untreated and inoculated control (Table 5).

Biocontrol of *Rhizoctonia solani* by bacterial antagonists

Suppression of pre-emergence damping-off: All tested bacterial treatments excepting isolates *B. glathei* 35, *P. huttiensis* 69 and *B. subtilis* 263 improved emergence percentage of *R. solani*-inoculated seedlings as compared to pathogen-inoculated and untreated control. This improvement reached 57.14% using isolates *B. pumilus* 420 and *P. putida* 227 (Figure 7).

Suppression of post-emergence damping-off: The eight tested bacterial isolates reduced post-emergence damping-off of pepper

seedlings inoculated with *R. solani* compared to control. Indeed, treatments with *P. aureofaciens* 314 and *P. putida* 227 completely suppressed disease expression, followed by *B. pumilus* 420 which reduced this parameter by 40%. *B. subtilis* 263 and *P. aureofaciens* 31 showed disease suppression ability comparable to the reference strain (*P. fluorescens*) where damping-off was lowered by 30% compared to the untreated control. As compared to the reference strain, isolate *P. aureofaciens* 314 was more effective (Table 6).

Treatments	Damping-off (%)	Plant weight (g)	Plant height (cm)	Disease severity
NIC	0 a[x]	0.54 a	4.7 ab	0.0 b
IC	50 c	0.37 b	3.55 d	2.7 a
314	0 a	0.4 b	4.86 a	0.5 ab
35	30 b	0.27 b	3.87 cd	1.6 ab
31	20 ab	0.34 b	4.63 abc	1.1 ab
420	10 ab	0.37 b	4.3 abc	0.7 ab
69	30 b	0.30 b	3.95 bcd	1.5 ab
263	20 ab	0.33 b	4.23 abcd	1.0 ab
Pf	20 ab	0.38 b	4.4 abc	1.1 ab
227	0 a	0.40 b	4.53 abc	0.6 ab

[x] Within each column, values followed by the same letter are not significantly different according to SNK test ($P \leq 0.05$)
NIC: Uninoculated control; IC: Inoculated with *R. solani* and untreated control; 227: Inoculated and treated with *Pseudomonas putida* 227; 420: Inoculated and treated with *Bacillus pumilus* 420; 69: Inoculated and treated with *P. huttiensis* 69; 31 and 314: Inoculated and treated with *P. aureofaciens* 31 and 314; 35: Inoculated and treated with *Burkholderia glathei* 35; 263: Inoculated and treated with *B. subtilis* 263; Pf: Inoculated and treated with *P. fluorescens.*

Table 6: Damping-off incidence and severity and growth parameters noted on pepper cv. Altar plants inoculated by *Rhizoctonia solani* and treated by different bacterial isolates, noted 7 days after inoculation and treatment, as compared to the untreated controls.

Figure 8: Damping-off severity noted a pepper cv. Altar seedling inoculated with *Rhizoctonia solani* and treated *Pseudomonas aureofaciens* 314 compared to control noted 7 days after inoculation and treatment. **IC:** Inoculated and untreated control

Treatments	Aerial part fresh weight (g)	Root fresh weight (g)	Plant height (cm)	Disease severity
NIC	54.45 bc[x]	9.97 a	33.74 b	1.4 a
IC	49.28 bc	8.97 a	33.02 b	2.0 a
314	76.16 a	6.68 a	41.40 a	1.2 a
227	46.76 c	8.10 a	34.42 b	1.2 a
Pf	52.87 bc	10.20 a	34.34 b	1.2 a
69	72.80 ab	5.89 a	40.98 a	1.6 a
263	51.31 bc	7.87 a	27.40 c	2.6 a
31	49.17 bc	8.90 a	32.98 b	1.0 a
420	36.43 c	7.69 a	34.06 b	1.2 a
35	72.98 ab	9.04 a	36.74 ab	1.8 a

[x] Within each column, values followed by the same letter are not significantly different according to SNK test ($P \leq 0.05$)
NIC: Uninoculated control; IC: Inoculated with *R. solani* and untreated control; 227: Inoculated and treated with *Pseudomonas putida* 227; 420: Inoculated and treated with *Bacillus pumilus* 420; 69: Inoculated and treated with *P. huttiensis* 69; 31 and 314: Inoculated and treated with *P. aureofaciens* 31 and 314; 35: Inoculated and treated with *Burkholderia glathei* 35; 263: Inoculated and treated with *B. subtilis* 263; Pf: Inoculated and treated with *P. fluorescens.*

Table 7: Disease severity and growth parameters noted on pepper cv. Beldi plants inoculated with *Rhizoctonia solani* and treated with different bacterial isolates, noted 75 days after the inoculation and treatment, compared to the untreated controls.

Analysis of variance revealed that plant height, noted 7 days after planting, varied significantly upon treatments tested. Indeed, treatment of pepper cv. Atlar plants with *P. aureofaciens* 314, *P. aureofaciens* 31, *B. pumilus* 420, *P. fluorescens* Pf and *P. putida* 227 resulted in a significant increase of their height compared to *R. solani*-inoculated and untreated control. The highest increment of this parameter, by 36.9% over control, was recorded on inoculated seedlings treated with *P. aureofaciens* 314.

All tested bacterial treatments did not improve pepper cv. Atlar fresh weight compared to the inoculated and untreated control. Moreover, plant weight, noted 7 days after inoculation and treatment, was significantly lower than that noted on the uninoculated and untreated control plants (Table 6).

Data analysis indicated that damping-off severity did not differ significantly between tested treatments. However, plants treated with *P. aureofaciens* 314, *B. pumilus* 420 and *P. putida* 227 showed disease severity scores of 0.5, 0.7 and 0.6, respectively, on a scale from 0 to 5, compared to 2.7 recorded on inoculated and untreated control (Table 6 and Figure 8).

Rhizoctonia root rot suppression: Rhizoctonia Root Rot severity, noted 75 days after planting, did not vary significantly depending on tested antagonistic treatments. However, *P. aureofaciens* 31 reduced disease severity by 50%, even if statistically insignificant, followed by *P. aureofaciens* 314, *B. pumilus* 420 and *P. putida* 227 which reduced this parameter by 40%, compared to inoculated and untreated control (Table 7).

The aerial part fresh weight of pepper plants cv. Beldi, recorded 75 days after planting, varied significantly depending upon the antagonistic treatments tested. Indeed, only treatment with *P. aureofaciens* 314 significantly improved this growth parameter by 54.54%, relative to pathogen-inoculated and untreated control. Also, *P. huttiensis* 69 and *B. glathei* 35 increased this parameter by 47.74 and 48.09%, respectively, compared to untreated controls. Compared with the reference strain *P. fluorescens* Pf, *P. aureofaciens* 314, *P. huttiensis* 69 and *B. glathei* 35 were found to be more effective in increasing aerial part fresh weight (Table 7).

All tested bacterial treatments did not improve pepper cv. Beldi root fresh weight recorded 75 days after planting, compared to both untreated controls.

As shown in Table 7, pepper cv. Beldi plant height, noted 75 days after planting, depended significantly ($P \leq 0.05$) on the antagonistic treatments tested. Indeed, the majority of bacterial agents tested had significantly similar effect on plant height as the two controls excepting *P. aureofaciens* 314, *P. huttiensis* 69 and *B. subtilis* 263 which induced 25% increase in this parameter as compared to controls.

Discussion

The present study investigates the pathogenicity/aggressiveness of different *R. solani* isolates issued from pepper and potato towards two pepper cultivars. These isolates caused pre-emergence and post-emergence damping-off and root rot. These findings are also in agreement with previous studies reporting the pathogenicity of different isolates of *R. solani* isolated from root/hypocotyl of rotted plants (cotton, clover, and common bean) and found that all isolates

were pathogenic and caused seed rot, pre-emergence, post-emergence damping-off and root rot diseases [26,27].

The biocontrol ability of three fungal antagonists (*T. harzianum*, *T. viride* and *G. virens)* against *R. solani* was also studied. In fact, species of the genus *Trichoderma* are the most widely used antagonists for controlling plant diseases caused by fungi due to their ubiquitous nature, ease with which they can be isolated and cultured, their rapid growth on a variety of substrates [12]. The mechanisms by which *Trichoderma* spp. suppress phytopathogens are basically three, i.e. direct competition for space or nutrients [28-30], the production of antibiotic metabolites, whether volatile or not [31,32] and direct parasitism on phytopathogenic fungi [33]. Furthermore, the genus *Trichoderma* possesses good qualities for controlling diseases in plants caused by soil borne pathogens, especially those of the genera *Phytophthora*, *Rhizoctonia* [34,35], *Pythium* [36,37], *Fusarium* [34,38,39] and *Macrophomina* [34].

Results from our study indicated that *R. solani* mycelial growth was slightly inhibited by the antagonists tested. However, microscopic observations at the confrontation zone between *Trichoderma* spp. or *G. virens* and *R. solani* showed a profound change in the pathogen's mycelium: lysis, formation of mycelium cords and a coiling of antagonists mycelium around pathogen; reflecting the mycoparasitism mechanism deployed by these antagonists. Similar effects were induced on *F. oxysporum* f. sp. *tuberosi* by the same antagonists tested in the present study [23]. Additionally, *T. harzianum* used against *F. solani* var. *coeruleum*, *F. roseum* var. *sambucinum* and *F. roseum* var. *graminearum* also caused a significant mycelium lysis [37]. An alteration of the mycelium of *Sclerotium rolfsii* was also induced by *T. harzianum* [40]. Our results are consistent with those of Howell [35] who demonstrated that *T. lignorum* is able to wrap around the mycelium of *R. solani* causing dissolution of the pathogen's cytoplasm. Similar mechanisms (mycoparasitism and lysis) were deployed by *T. harzianum*, *T. viride* and *T. aureoviride* during their *in vitro* interaction with *R. solani* [41]. In addition, many studies have shown that *Trichoderma* species are capable to produce extracellular lytic enzymes [42].

The current study clearly demonstrated that all treatments tested for the control of the post-emergence damping-off performed using the fungal antagonists had significantly increased plant growth. Indeed, treatment with *G. virens* increased plant height by 12.13% and 27.32% compared to *R. solani*-inoculated control in pot and cell trays trials, respectively. Similarly, treatment of tomato plants with *T. harzianum*, *T. viride* and *G. virens* led to an increase by more than 50% of their root and aerial parts fresh weights compared to *V. dahliae*-inoculated and untreated control [22].

A reduction in disease severity on pepper plants was also obtained using *Trichoderma* and *Gliocladium* based-treatments. This reduction reached 44.44% with *G. virens*, 37.03% with *T. harzianum* and 14.8% with *T. viride*, relative to *R. solani*-inoculated and untreated control. Our results are consistent, in part, with those of Sid Ahmed *et al.* [15] who demonstrated that seed treatment and soaking pepper roots with *T. harzianum* led to 44% decrease in root rot caused by *Phytophthora capsici* and to 38% in that induced by *R. solani*.

In the present work, we also noted a decrease in damping-off incidence on pepper seedlings treated with the three tested antagonistic fungi by 40% compared to *R. solani*-inoculated and untreated control. These findings confirm those of Rini and Sulochana [17] who showed that *T. harzianum* is more effective than *P. fluorescens* and *T. pseudokoningii* in controlling *R. solani* in greenhouse and field grown pepper where root rot was reduced by 22.9%. Other previous studies also reported differences in the antagonistic potential of *Trichoderma* species isolated from a suppressive soil and shown active against *V. dahliae* [43,44].

Seed treatment by *G. virens*, *T. harzianum* and *T. viride* also improved the emergence of pepper seedlings by 40, 30 and 20%, respectively, relative to *R. solani*-inoculated and untreated control. The efficacy of *G. virens* in controlling *R. solani*, *P. ultimum*, *S. rolfsii* and *P. capsici* on pepper was also demonstrated [45].

The *in vitro* evaluation of eight rhizobacterial isolates for the control of *R. solani* showed that *P. aureofaciens* 31, *B. glathei* 35 and *P. huttiensis* 69 are the most effective. Microscopic observations made at the contact zone between the tested bacteria and pathogen revealed a radical change in the pathogen hyphae showing a strong lysis and formation of mycelial cords as main stress responses.

In *in vivo* trials, *P. aureofaciens* 314 was found to be the most efficient by suppressing damping-off caused by *R. solani* (100%). Nasraoui *et al.* [25] tested the same rhizobacterial collection and showed that *P. aureofaciens*, *B. glathei* isolated from soil of Missouri and *B. subtilis* isolated from Tunisian soil were the most effective in reducing incidence of take-all of wheat (damping-off) caused by *Gaeumannomyces graminis* var. *tritici*. The other bacteria of the genus *Pseudomonas* tested have also an inhibitory effect on the target pathogen. Indeed, *P. putida* 227 totally suppressed the post-emergence damping-off, compared to the inoculated control, and improved the emergence of pepper seedlings inoculated with *R. solani*.

The present study showed that *P. putida* 227 based treatment increased pepper fresh weight by 67.93%. *P. huttiensis* 69 improved the aerial part fresh weight and the height of the pepper plants cv. Beldi, recorded 75 days after planting, by 47.74 and 24.10%, respectively. De Curtis et al. [19] found that *Pseudomonas* sp. is able to inhibit growth of *R. solani* and *S. rolfsii in vitro* and ensure protection of tomato plants. In addition, isolates of *Pseudomonas* spp. were shown able to inhibit *R. solani* mycelial growth by 83.3% [46]. Additionally, *P. fluorescens* controlled damping-off caused by *P. ultimum* on cucumber seedlings due to its ability to produce antifungal metabolites in the culture such as the fluorescent siderophore (pyoverdin), the pyoluteorin, pyrrolnitrin and cyanide [47]. The enzyme β-1,3-glucanase produced by *P. cepacia* was found to be involved in the suppression of diseases caused by *R. solani*, *S. rolfsii* and *P. ultimum* [48].

In the present study, *B. pumilus* 420 suppressed by 40% pepper damping-off caused by *R. solani*. *B. subtilis* efficiency in controlling damping-off was estimated at 60% compared to *R. solani*-inoculated control. In the same context, treatment of tomato seeds by *B. subtilis* and/or transplanting seedlings in a soil inoculated with the bacterium decreased the severity of *R. solani*-induced disease by secretion of the antibiotic iturin A [49]. Similarly, Mojica-Marín *et al.* [20], working on pepper diseases, showed that *B. thuringensis* is effective in controlling *R. solani in vitro*. In addition, seed treatment and root dipping in *B. subtilis* and *B. licheniformis* cell suspensions reduced the severity of root rot caused by *Phytophthora* by 55% and 50% and that caused by *R. solani* by 44% and 55%, respectively [15].

Conclusion

In the present study, all *R. solani* isolates tested were shown to be pathogenic to pepper plants with variable degree of aggressiveness noted on the two cultivars cvs. Beldi and Baklouti. These isolates were able to induce pre- and post-emergence damping-off and Root Rot Disease as well as plant growth reduction. Further studies are needed to

correlate the aggressiveness of *R. solani* isolates recovered from pepper with their anastomosis group.

In an attempt to biologically control this disease, fungal and bacterial isolates were tested *in vitro* and *in vivo* against *R. solani*. Our results demonstrated that some of the tested biocontrol agents, applied at different pepper growth stages, were able to suppress disease and to improve plant growth. Their effectiveness will be further evaluated under field conditions, in naturally infected soils, and against the other pepper phytopathogenic fungal species.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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