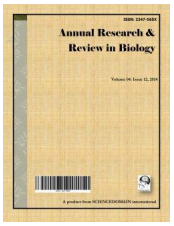

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Inhibitory Effect of Rhizobia Isolated from Several Leguminous against Phytopathogenic Fungi

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Authors' contributions

This work was carried out in collaboration between all authors. Author LL designed the study; author LK performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author SMA managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was carried out to evaluate the antagonistic effect of ten isolated characterized *Rhizobium* sp. and three referenced strains against aggressive phytopathogenic fungi *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., *Humicola* spp. and *Cladosporium* spp. isolated from infected and wilted plants *in vitro* and *in vivo*.
Study Design: First, we have ten strains of rhizobia isolated from leguminous and characterized, after that, some strains of fungi were isolated from infected and wilt plants, such as *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Humicola* and *Cladosporium.* Finally, The investigation of the potential of the isolated rhizobia and three referenced strains was evaluated in dual culture, in pots experiments and on seeds.
Place and Duration of Study: The study was carried out at the Department of Microbiology, Faculty of Nature and Life Sciences, Laboratory of Applied Microbiology, between April and November 2016.

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Methodology: Rhizobia isolates were identified upon their phenotypic traits as: the morphology of the colony, of the physiological characters (growth temperature, salt tolerance, resistance to antibiotics, metabolism of carbon source, generation time…) and also the production of extracellular metabolites as siderophores and proteases. Fungal isolates were identified by their macroscopic and microscopic characters. The antagonistic effect of rhizobia against phytopathogenic fungi was examined *in vitro* by: confrontation in Petri dishes and inoculation of seeds; and *in vivo* by inoculation of plants.
Results: The isolated rhizobia were resistant to penicillin and amoxicillin with generation time ranged from 1.9 to 6.4 h, they were able to grow at temperatures from 4°C to 37°C, tolerate salt concentration (0.5 –2%) and produce siderophores and proteases. The results demonstrated the effectiveness of the rhizobia isolates and the referenced strains against the fungi isolates *in vitro* and *in vivo*. In dual culture, all rhizobia isolates inhibited the mycelial growth of the pathogens. The best disease control was obtained with isolate F3 from faba bean which inhibited the fungal growth with more than 70%. The highest inhibition growth was obtained against *Alternaria* spp.2*, Penicillium* spp.2*, Cladosporium* spp. and *Humicola* spp. with an inhibition rate ranging from 90 to 96%. In pot experiments, Rhizobia isolates from chickpea, lentil and faba bean reduced significantly all disease incidence with more than 75%; where the best fight was observed in lentil plants, while in faba bean no wilted plants were observed. Seeds inoculated with rhizobia and fungi in Petri dishes showed that isolates from faba bean and lentil were the most effective in reducing fungi disease incidence.
Conclusion: Rhizobia have a high potentiality to inhibit the growth of tested pathogens and could be fielded within an integrated disease management package.

Keywords: Biological control; leguminous; phytopathogenic fungi; Rhizobium; seeds treatment.

1. INTRODUCTION

Fungi are pre-eminent as plant pathogens. Roughly 70% of all the major crop diseases are caused by fungi (Oerke, 2006). Phytopathogenic fungi infect all aerial plant parts and destroy different agricultural crops. A large number of phytopathogenic fungal species have been described such as *Alternaria, Fusarium* and *Penicillium* [1].

These fungi became problematic because of the lack of effective control means, such as chemical products or some cultural practices (fumigation, seeds treatment, crop rotation,…) or the use of resistant plant varieties as insertion of genes or Genetically Modified Organisms… [2,3], while they were considered uneconomic, with harmful impact on environment. So it is important to find other control approaches which will make it possible to continue to fight against phytopathogens and decreasing the use of chemical products as it would be a cheaper method. Biological control of fungi by antagonistic agents constitutes an alternative method to control plant diseases. Microorganisms that can grow in the nodule of leguminous are ideal to be used as biocontrol agents, and can be protect host plants against pathogens attack [4,5]. In the last decade the *Rhizobium* group have shown to play a major role in plant pathogenic fungi biocontrol with a great potential [6]. The *Rhizobium* group, have been used successfully against several pathogens belonging to the genera *Macrophomina*, *Rhizoctonia*, *Pythium*, *Fusarium, Alternaria, Phytophtora, Ascochyta, Botrytis* and *Aspergillus* [7,8,9,10,11,12].

Microbial biocontrol agents may act against plant pathogens by three different ways: competition for space and/or nutrients [13], direct antagonism by antibiotics, enzymes and siderophores [7,14]; or induction of host plant defense mechanisms [15,16].

The objective of this study was to evaluate the potential of *Rhizobium* sp. strains isolated from leguminous species to control phytopatogenic fungi.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The thirteen rhizobia isolates used in this study were recovered from nodules of leguminous grown in soils from different sites in Algeria. Three referenced isolates, *Rhizobium Sullae* sp., *Rhizobium Sullae* RHF and *Rhizobium*

leguminosarum were isolated from *Hedyasarum* and chickpea, these strains were gifted by Professor Benguedouar (University of Constantine, ALGERIA). These can grow between 4 and 50°C and tolerate salt from 0.5 to 2%. One isolate F3 was isolated from faba bean field from M'sila, and nine strains were isolated from root nodules of Pea, lentil, faba bean and chickpea collected from experimental farm of Sétif 1 University (Algeria).

2.2 Isolation of Rhizobia

First ten *Rhizobium* were isolated and selected from faba bean, chickpea, pea and lentil roots. Nodules were detached from roots, washed in tap water to remove the adhering soil particles, and then nodules were dipped in alcohol followed by washing with 0.1% mercuric chloride ($HgCl_2$) solution for 30 s. Later the nodules were washed successively four times with sterilized distilled water and crushed with sterilized glass rod to obtain a milky suspension. An aliquot of this suspension was plated on Yeast Extract Mannitol Agar (YEMA) and incubated at 28°C for 72 h, then purified with several sub culturing on the same medium. The isolates were conserved and kept on YEMA medium containing 3 g of $CaCO_3$ and stored at 4°C as source cultures [17].

2.3 Identification of the *Rhizobium* Strains

2.3.1 Morphological and physiological characterization

Colony morphology was characterized on YEMA according to [18], on the base of to the color, shape and Gram staining. While physiological characterization was determined as follows; by studying the use of carbon sources, the effect of temperature and salinity, the resistance to antibiotics, the time of generation and the production of proteases and siderophores.

2.3.2 Temperature tolerance

The ability of bacterial strains to grow at high and low temperatures was monitored at 4, 37 and 50°C on YEMA plates as described by [19] and [20]. The control plate was incubated at 28°C.

2.3.3 Salt tolerance

For salt tolerance, the YEMA medium was supplemented with NaCl at 0.5, 1 and 2%, and growing isolates were observed after 72 hour at 28°C. While YEMA control contained 0.1% of NaCl [21].

2.3.4 Intrinsic antibiotic resistance

The intrinsic resistance was determined on YEMA containing the following antibiotics; ampicillin, penicillin G and amoxicillin. Each antibiotic was aseptically added to sterile YEMA medium at a concentration of 100 $\mu g.ml^{-1}$, inoculated with bacteria and incubated for 3 days at 28°C. Control plates contained no antibiotics [22].

2.3.5 Carbon substrate

Three carbon sources used by bacteria were tested on YEMA, where mannitol has been replaced by glucose, galactose or saccharose. The growth was observed after 3 days of incubation at 28°C [23].

2.3.6 Generation time

Generation time was determined by inoculating rhizobial isolate in 50 ml sterilized YEM broth and incubated at 28°C for 24 h with stirring at150 rpm. The growth in term of turbidity was measured by taking the absorbance of the culture sample at 610 nm every 2 h, and generation time was calculated using the following formula:

Generation time = (T2–T1)/ 3.3 (log10 OD2- log10 OD1)

Where (T2-T1) is the difference of two time intervals at any two point in log phase in growth curve; (log10 OD2- log10 OD1) is the difference between the log10 value of OD2 at time T2 and OD1 at time T1 [24].

2.3.7 Production of proteases

Production of proteases was tested by inoculation of *Rhizobium* on milk agar medium and incubated at 28°C for 72h. The presence of a halo zone around the colony revealed positive protease. Protease production reflects the antagonistic activity of *Rhizobium* [25].

2.3.8 Production of siderophores

Siderophore production was highlighted using 50 ml of solution of Chrome Azurol Sulfate CAS [26] in 2.5 ml Ferric perchlorate reagent, poured onto rhizobia plates, orange color would appear

if the test is positive or blue if the test is negative [27].

2.4 Fungal Isolation

Fungal strains were isolated from infected plants showing diseases symptoms: wheat, olive, almond, eucalyptus, faba bean, quince, raspberry, grape, ivy and violet flowers collected from Sétif (east) and Béjaia (north-east of Algeria). Tissue sections were surface-disinfected by immersion in 5% sodium hypochlorite solution for 30s then rinsed twice in sterile distilled water, dried on sterile filter paper and placed onto potato dextrose agar (PDA) in 9-cm Petri dishes. Dishes were incubated at 28°C for 7 days. Fungal cultures of the pathogens were purified and stored in tubes containing PDA at 4°C. The isolates were identified morphologically from microscopic and macroscopic characters according to [28].

2.5 In vitro Antagonistic Activities against Phytopathogens

The antagonistic effect of the selected strains in addition to the referenced strains towards fungi was estimated *in vitro* by confrontation, and *in vivo* in pots experiments and on seeds.

In vitro inhibition of fungal mycelia growth by the bacterial isolates was tested using the dual culture technique described by [29] and [30]. Four discs from 72h culture of rhizobia were equidistantly placed on the margins of PDA plates and incubated at 28°C for 24 h, and then an agar disc of fungal mycelia from 7 days old cultures was placed at the center of the PDA plate and incubated at 28°C for seven days. Plates without bacteria containing only the fungus served as control.

Percent fungal growth inhibition was calculated using the formula of [31]:

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

Where r is the radius of the fungal colony opposite the bacterial colony and R is the maximum radius of the fungal colony in the control.

2.6 In vivo Antagonism of *Rhizobium* towards Fungal Isolates

All bacterial isolates which induced more than 50% inhibition of mycelia growth *in vitro* assay were selected for the *in vivo* evaluation. The experiments *in vivo* were performed as follows.

Chickpea, pea, lentil and faba bean seeds surface were sterilized with 5% sodium hypochlorite for 1 min and rinsed five times with sterile distilled water, and soaked in distilled water for 1 h. Then seeds were inoculated with bacteria (10^8ufc/ml, soaked in YEM liquid medium containing strains) and transplanted in plastic pots containing sterilized soil for 21 days.

After 21 days, plants were inoculated with pathogens by depositing some drops (3 to 4) of spore suspension ($4x10^6$spore/ml) overall surface of three different leaves of each plant. Two batches of seeds were used. The first batch received rhizobia suspension (10^8ufc/ml) and plants were inoculated with fungal spores ($4x10^6$spore/ml), the second batch plants were inoculated with fungal spores only and watered as needed.

One to two weeks after leaves inoculation, virulence of the pathogen was evaluated as previously described by [32] from 0 to 4 where: 0 healthy plant (0%), 1 slight yellowing (25%), 2 high yellowing (50%), 3 wilting (75%) and 4 death (100%).

2.7 In vitro Seedling Germination Tests

An *in vitro* seedling assay was performed as previously described by [33] and [34], to test the ability of the *Rhizobium* sp. isolates to suppress the effect of fungal isolates on seedling germination. Seeds were sterilized as described earlier and then they were soaked in the10^8ufc/ml bacterial suspension. A total of 4 seeds were aseptically placed on plates with filter paper impregnated with sterile distilled water and incubated at 28°C for one day. One day after, seeds were inoculated with some drops of spore suspension ($4x10^6$ spores.ml-1), after 7days of incubation at 28°C the degree of root necrosis was scored as previously described by [35] from 0 to 4 where:

0 healthy seedlings (0%), 1 necrosis of the extremity (25%), 2 root cutting (50%), 3 reduction of cotyledon (75%), 4 severe necrosis (100%).

All the tests in this work were in triplicate.

2.8 Statistical Analysis

Data were analyzed by the one way analysis of variance (ANOVA) for comparing means of 3 or

more treatments/doses, to estimate the effects of rhizobia on fungi inhibition and to avoid the error. The test with $P<.05$ was considered as statistically significant. This was followed by Fisher's test when the number of treatments was under than 5 and over 2 (leaf and seed treatments).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Physiological characteristics

3.1.1.1 Morphological Characteristics of rhizobia

The isolates are Gram-negative and rod shaped. Colonies are circular translucent yellow to creamy, raised with smooth edges, slimy, and did not absorb red color when cultured in YEMA containing Congo red.

3.1.1.2 Determination of growth temperature

Maximum growth of all tested strains was ranked between 4° and 37°C, the percentage of isolates that grew decreased to reach 30% at 50°C. Although 70% of the isolates that tolerated 4 to 50°C were isolated from faba bean, while other strains (from pea, lentil and chickpea) were able to grow between 4 and 37°C.

3.1.1.3 Determination of carbon sources

Most of the isolated rhizobia strains were able to use the tested carbon substrates. All tested strains grew on glucose, galactose and saccharose.

3.1.1.4 Determination of salt tolerance

Chickpea, pea, faba bean and lentil rhizobia exhibited a wide diversity in their salt tolerance and all isolates grew on the medium containing 0.1% of NaCl. All the tested rhizobia continue to grow between 0.5 to 2% of NaCl.

3.1.1.5 Determination of antibiotic resistance

The evaluation of resistance of rhizobia to antibiotics showed that most of the isolates exhibited high resistance to penicillin G and amoxicillin. In the presence of ampicillin 70% of isolates were resistant. Eighty eight to 92% of rhizobia from faba bean, pea, lentil and chickpea were resistant to the tested antibiotics. Almost 67% of chickpea and faba bean strains were resistant to ampicillin.

3.1.1.6 Time generation, production of proteases and siderophores

The isolates have doubled their population between 1.9 and 6.4 h (Table1). On the basis of their generation times, 92% of the isolates were considered as fast growers (GT< 6 h), and 08% as slow growers (GT > 6 h).

Several isolates show a translucide haloes on milk agar corresponding to proteolytic activity (Table 1). Otherwise all of the isolates showed orange haloes indicating siderophores production.

3.1.2 Fungi characteristics

The cultural and microscopic characteristics (Fig. 1) of fungal strains indicate that the isolated pathogenic fungi belong to different genera: two species of *Alternaria* spp., two species of *Fusarium* spp., two species of *Penicillium* spp., one species of *Cladosporium* spp., one *Humicola* spp. and one *Aspergillus* spp.

3.1.3 In vitro antifungal activity of rhizobia

Different results and behavior were obtained according to each antagonist-pathogen combination (Table 2). The highest inhibition growth was 90 to 96% obtained with faba bean isolates against *Alternaria* spp. 2, *Penicillium* spp.2, *Cladosporium* spp. and *Humicola* spp., whereas no inhibition growth (0%) was observed

Table 1. Generation time and production of protease and siderophores by strains of Rhizobia

Strain	A6	Sam12	F	F_3	F_2m	Fmd	P	P3	Pd	Pcx	Pcd	Pc_3	L
Gt(h)	2.22	3.46	2.78	2. 39	4	2.9	5.18	5.3	3.1	1.98	6.41	2.8	5.2
Protease	+	+	-	+	+	+	+	+	-	-	+	-	+
Siderophore	+	+	+	+	+	+	+	+	+	+	+	+	+

(+): production, (-): no production, Gt(h): generation time in hour; A6, F, Sam12 are referenced strains from respectively Hedyasarum and chickpea; F_3 ,F_2m and Fmd: strains from faba bean ; P ,P_3 and Pd: strains from pea; Pcx ,Pcd and Pc_3: strains from chickpea; L: strain from lentil.

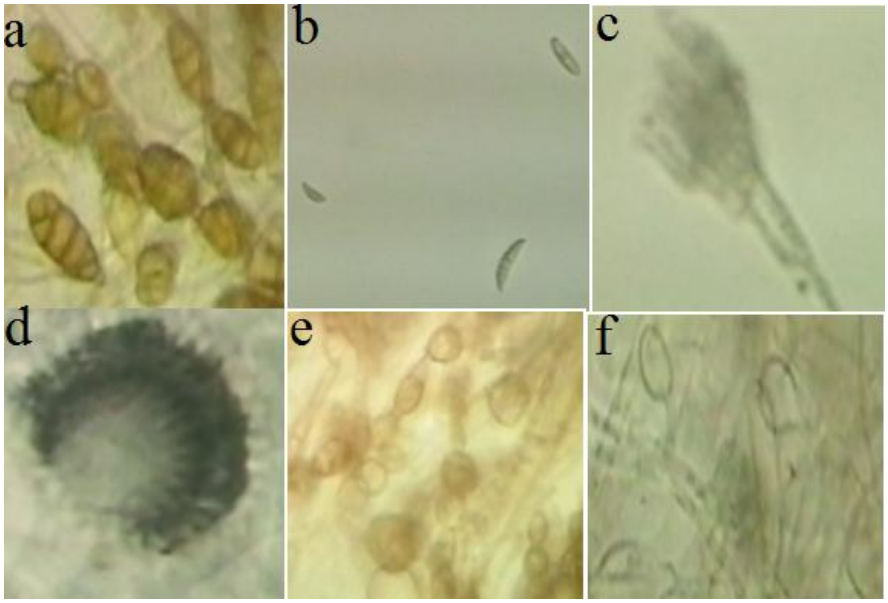

Fig. 1. Microscopic characteristics of fungi isolates
a. Alternaria spp., b. Fusarium spp., c. Penicillium spp., d. Aspergillus spp., e. Cladosporium spp., f. Humicola spp.

against *Penicillium* spp.2 with one isolate from pea (Pd) and two isolates from chickpea(Pcx and Pc3).

The F_3 strain from faba bean showed a significant reduction in growth of all fungal strains by an average percentage of 70%, however inhibitory effect of the pea strain Pd was only 42%.

In the other hand, the best average reduction percentages of fungal growth by all rhizobia were found against *Alternaria* spp. 2 and *Humicola* spp. (77%). The lowest percentages were found against *Fusarium* spp.2 and *Penicillium*, 41-44% respectively. The referenced strains A6 and F isolated from *Hedyasarum* inhibited respectively *Penicillium* spp.2*, Humicola* spp. and *Fusarium* spp.1 from 52% to 95%.The average percentage of all mycelial growth inhibition induced by the rhizobial isolates in dual culture test was 60%.

3.1.4 *In vivo* antagonism of *rhizobium* towards *fungi*

Almost 75% of chickpea plants inoculated by chickpea rhizobia and faba bean plants inoculated by faba bean rhizobia looked healthy showing no symptoms. Almost 20% of plant showed yellowing color in chickpea caused by *Aspergillus* spp. and *Cladosporium* spp.*,* also 25% with black color in faba bean caused by *Fusarium* spp.1*, Penicillium* spp.2 and *Humicola* spp., while 5% of wilting by *Alternaria* spp.2 and *Fusarium* spp.1 appeared in chickpea but no wilt in faba bean (Figs. 2 and 3).

In our study, almost 35 % of pea rhizobia protected completely the host plants. While 45% of plants looked yellow, caused by *Alternaria* spp.1*, Fusarium* spp.1 and *Humicola* spp.*,* and 20 % of wilting caused by *Alternaria* spp.2 and *Humicola* spp. (Fig. 4). However, the referenced strains A6 and F isolated from *Hedyasarum* showed the best results in chickpea, faba bean and lentil but fail to protect pea plants (Fig. 5).

3.1.5 *In vitro* antagonism of *Rhizobium* towards fungi inoculated on seeds

Rhizobia isolates had variable and significant effect on germination of chickpea, pea, faba bean and lentil seeds inoculated with spore suspension. Interestingly, application of *Rhizobium* sp. isolates significantly reduced the wilting. Disease in inoculated seeds with *Fusarium* spp.1*, Cladosporium* spp. and *Humicola* spp. was significantly reduced and the inhibition rate varied from 16 to 90% (Figs. 6 to 9) with the best suppression of fungi observed in faba bean (90%) and lentil (81%).

Table 2. Inhibition percentage of phytopatogenic fungi by rhizobia

Fungi \ Bacteria	A6	Sam12	F	F_3	F_2m	Fmd	P	P_3	Pd	Pcx	Pcd	Pc_3	L	APIB
Alternaria spp.1	63	72	61	65	55	65	66	65	69	62	74	70	58	65
Alternaria spp.2	75	87	72	95	90	82	77	77	77	75	70	50	85	77
Fusarium spp. 1	62	67	85	72	62	67	62	75	62	65	50	87	80	68
Fusarium spp. 2	50	50	52	35	50	52	57	25	97	32	50	37	15	41
Penicillium spp.1	22	55	37	44	44	77	20	40	33	55	44	37	55	43
Penicillium spp.2	95	24	52	60	20	44	84	96	0	0	72	0	32	44
Cladosprium spp.	16	70	29	91	91	16	37	25	33	58	70	91	83	54
Humicola spp.	85	75	75	95	92	90	92	82	0	77	80	75	92	77
Aspergillus spp.	67	75	59	78	72	75	60	40	70	70	74	81	69	68
APIF	59	63	63	70	64	63	61	58	42	54	64	58	63	60

APIF: Average percentage of inhibition of each fungi; APIB: Average percentage of inhibition of each bacteria; F_3,F_2m and Fmd: strains from faba bean; P,P_3 and Pd: strains from pea; Pcx, Pcd and Pc_3 ,Sam12 : strains from chickpea; L: strain from lentil; A6 and F: strains from Hedyasarum and they are referenced strains with Sam12.

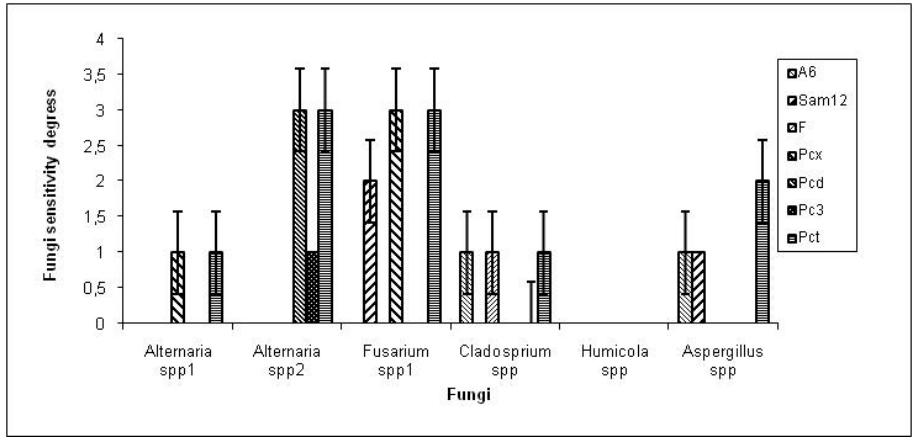

Fig. 2. Effect of rhizobia on chickpea wilt incidence

0: healthy plant, 1: slight yellowing, 2: high yellowing, 3: wilting; Pcx, Pcd and Pc_3: strains from chickpea; A6, F and Sam12: referenced strains respectively from Hedyasarum, and chickpea; Pct: control plant of chickpea.

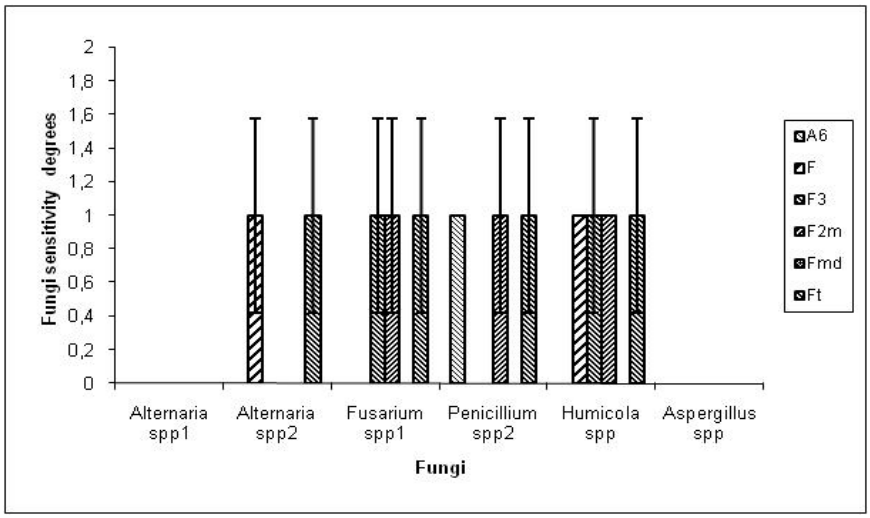

Fig. 3. Effect of rhizobia on fababean wilt incidence

0: healthy plant, 1: slight yellowing, 2: high yellowing,3: wilting; F_3, F_2m and Fmd : strains from faba bean; A6 and F: referenced strains respectively from Hedyasarum; Ft: control plant of faba bean.

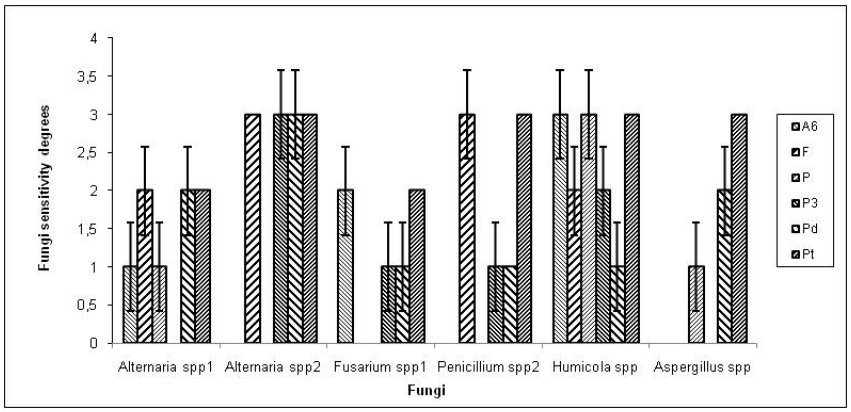

Fig. 4. Effet of rhizobia on pea wilt incidence

0 healthy plant,1 slight yellowing, 2 high yellowing,3 wilting; P, P3 and Pd: strains from pea ; A6 and F: referenced strains from Hedyasarum, Pt: control plant of pea.

3.2 Discussion

Plant pathogens especially fungi are the most important agents causing serious losses world-wide to agricultural products. Biological control is the most promising and viable method using microbial agents to suppress diseases [36]. Rhizospheric microorganisms are perfect for use as biocontrol agents; since the rhizosphere adduces the front-line defense for roots against pathogenic fungi.

The present study was conducted to isolate efficient fungal inhibitory rhizobia from leguminous. Rhizobia have special physiological characteristics. [37] Mentioned that *Rhizobium* strains failed to absorb Congo red stain. Polysaccharide production is another characteristic of rhizobia which is implicated in important processes as infection and nodule formation [38]. The isolated rhizobia were viscous, because of exo-polysaccharides production. There would be formation of long viscous filaments [39,40]. Polysaccharides are involved in the protection of rhizobia against deleterious biotic and abiotic stress factors in the soil [38] and in the protection of the nitrogenase against oxygen diffusion through the nodule cells [41].

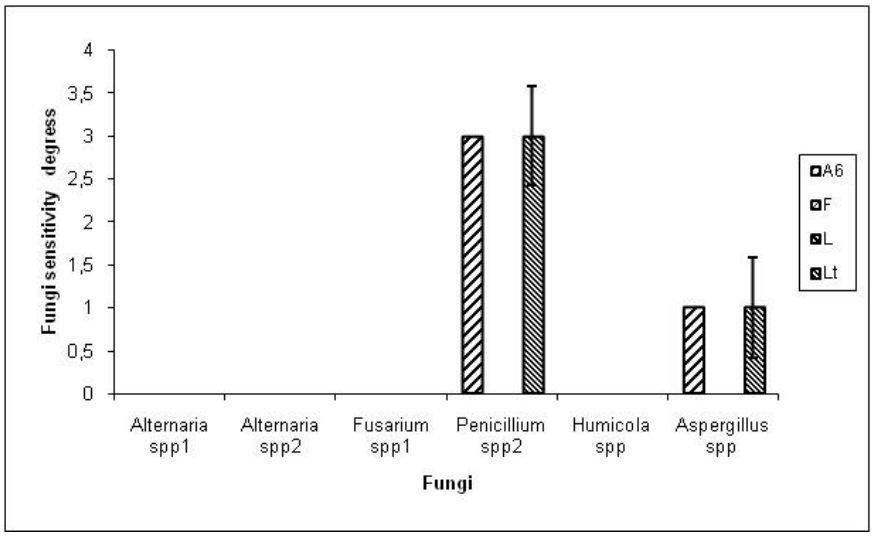

Fig. 5. Effet of rhizobia on lentil wilt incidence

0: healthy plant, 1: slight yellowing, 2: high yellowing, 3: wilting; A6 and F: referenced strains from Hedyasarum; L: strain from lentil; Lt: control plant of lentil.

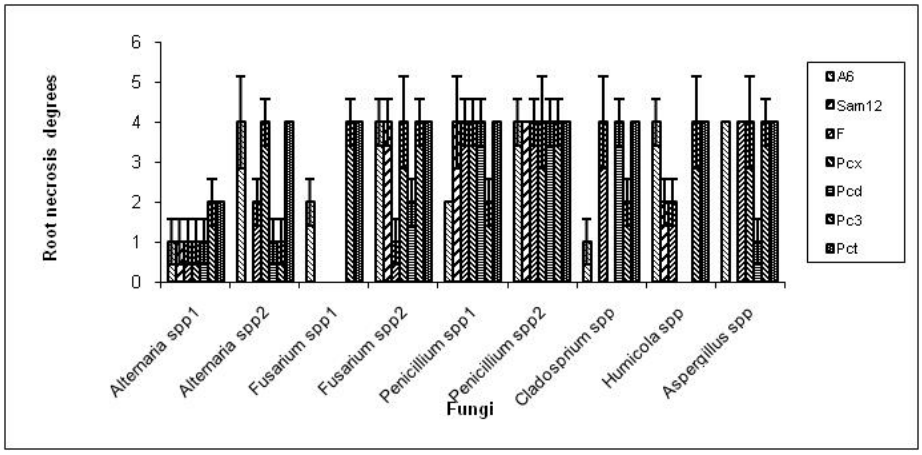

Fig. 6.**Effect of *Rhizobium* inoculation on fungi suppression of chickpea seeds**

0: healthy seedlings, 1: necrosis of extremity, 2: root cutting,3: reduction of cotyledon, 4 :severe necrosis; A6, F and Sam12: referenced strains respectively from Hedyasarum and chickpea; Pcx,Pcd and Pc $_3$: strains from chickpea; Pct: control of chickpea.

[19] found that generation times (GT) in rhizobia of chickpea was ranged from 50 min till 9 hours, while GT for isolates obtained from chickpea in our study were ranked from 1.98 to 6.41h. [42] reported a GT between 1 and 8 h in *Mesorhizobium.* [43] showed that GT of *Sinorhizobium* is ranked between 3 and 5 h, that why some of our isolates(Sam12,F_2m,Pd) may belong to *Mesorhizobium* or *Sinorhizobium* according to their GT. Whereas, [37] showed that GT of rhizobia strains isolated from *Acacia* legume tree growing in South Riyadh, is ranged between 2.07 and 3.85 h. The GT for the tested strains (A6 and F) from *Hedyasarum* a legume tree growing in Algeria were 2.22 and 2.78 h respectively. Moreover, GT for our isolates from faba bean were similar to those of [44], where they detected in faba bean isolates a GT of 1.9 to 4.3 h. Also [24] found that root nodulating bacteria isolated from pea plant had an average GT between 3.0 to 3.6 h; while our strains have a GT between 3.1 to 5.3. High amount of EPS production is mostly by the fast growing strains and lowest amount produced by the slow growing strains. However, intermediate values were also obtained with few fast and slow growing strains [41].

Several studies showed that *Rhizobium* can grow at 45°C and more [45,46,47,48]. However [37,44] noted that rhizobia isolates grew at temperatures from 15 to 35°C,while [49] showed that some rhizobia were also able to grow at 4°C (55%) and at 55°C (22%), few isolates of rhizobia grew at temperatures of 5 and10°C [44,50]. Earlier [51] suggested that rhizobial strains isolated from hot areas might be able to survive at high temperatures better than the strains isolated from cooler regions. The regions of isolation of our rhizobia are hot (Temperatures for Sétif and M'sila are ranked from 38 to 44°C) that why our isolates tolerate high temperatures.

Biocontrol agents are more efficient in the inhibition of the mycelial growth from the same agro-ecological origin [52]. Earlier [4] rules that rhizobacteria is most effective in the control of plant diseases affecting the host plant of these.

The inhibition rate of the tested rhizobia in our study was more than 80% against *Fusarium* spp.1 and 90% against *Alternaria* spp.2 (Table 2). *R. leguminosarum* have a lethal effect on the conidies of *Fusarium oxysporum* [13]. [6] Showed tip deformation, abnormal intercalary swelling, cytoplasm degeneration and hyphae lysis in pathogenic fungi such as *Sclerotinia sclerotiorum*, *Macrophomina phaseolina Rhizoctonia solani* and *Fusarium oxysporum*, during interaction with rhizobia. In another way, [53] showed that among the 21 isolates of *Rhizobium* examined from chickpea, 19 prevented the growth of *Fusarium oxysporum*, fourteen gave inhibition more than30% and four more than 50% inhibition of growth *in vitro*. [54] found that rhizobia inhibit *Fusarium* in wheat with more than 60%, also [9] reported an inhibition of 36% of *Fusarium* sp. and 54% of *Alternaria alternata*.

The basic mechanisms behind such a protection may be due in part to the produced metabolites cited previously (Table 1). Rhizobia may act by some way of competition for nutrients by displacing the pathogens [7] as iron by production of siderophores [6,8,41] due to lack of iron required for "sclerotia" germination and hyphal growth [55] secretion of antibiotics [6,8] HCN [6] [55] antifungal [56] direct parasitism by proteases acting as cell wall degrading enzymes [8]; or influence of the plant defense mechanism by stimulating the production of phytoalexins [6] or phenolic compounds by plants. These latter are the determinants of ISR in Rice induced by *Rhizobium* against pathogenic attack of *Rhioctonia solani* [56]. ISR was also obtained by co-inoculation of *Pseudomonas fluorescens* and *Rhizobium leguminosarum* in faba bean (*Vicia faba* L.) against faba bean yellow mosaic virus [57]. Otherwise, induced systemic resistance was attributed to LPS produced by *Rhizobium elti* in potato plants infected by the Cyst Nematode *Globodera pallida* [58]. Different studies underline the potential of the bacterial antagonists by the suppression of plant disease and inducing mechanisms of plants defense [9,59,60,61,62].

Rhizobium leguminosarum from bean was the most effective isolate on three fungal genera with percent inhibitions ranged between 14.65 and 16.03% for *Rhizoctonia solani* isolates, 14.62 to 30.35% for *Pythium ultimum* isolates and 14.58 to 29.75% for *Fusarium oxsporum* isolates [8]. [7] showed that two rhizobia isolates were able to inhibit a widely occurring plant pathogen; *Macrophomina phaseolina* that causes charcoal rot in groundnut by siderophores production. Also *Rhizobium meliloti* inhibited the growth of *Macrophomina phaseolina, Rhizoctonia solani* and *Fusarium solani in vitro* [63]. Other experiments showed that strains of rhizobia were able to prevent growth of *Fusarium oxysporium* and *Rhizoctonia solani* [64].

Rhizobium strains of chickpea tested *in vitro* prevent *Rhizoctonia solani* growth and reduce their development from 50% to 70%, with observed inhibiting halo that suggest the presence of fungistatic metabolites secreted and change of mycelial color between the end and the center of the colony of *Rhizoctonia solani*. While microscopic observations revealed the deformation of their apex [65]. Similar results were observed in our study with strain F_2m from faba bean against *Cladosporium* spp. [10] showed that *Rhizobium leguminosarum* had antagonistic effect on *Fusarium oxysporum* and *Phytophthora megasperma* by 66.3 and 62.1%, respectively. However an isolate of *Rhizobium leguminosarum* in our study inhibited *Fusarium* spp. by 50 to 67%. In the same way *Rhizobium* isolates inhibited the strongly growth of *Ascochyta pisi* moderately *Botrytis cinerea* and only slightly *Fusarium graminearum* [11] and also the growth of *Aspergillus niger* and *Fusarium oxysporum* [12].

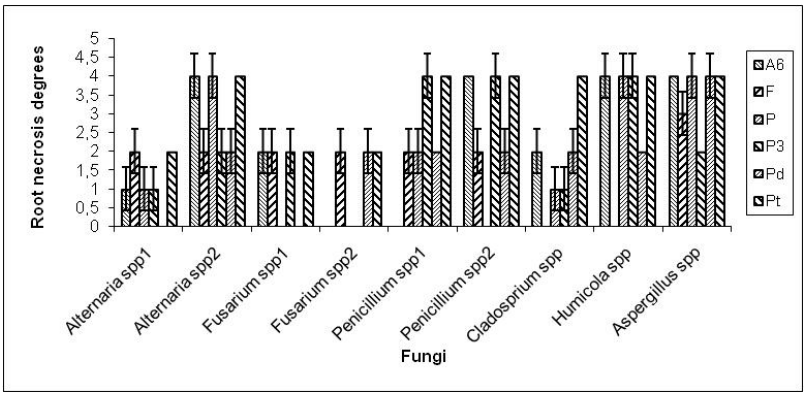

Fig. 7. Effect of *Rhizobium* inoculation on fungi suppression of pea seeds

0: healthy seedlings, 1: necrosis of extremity, 2: root cutting, 3: reduction of cotyledon, 4: severe necrosis; A6 and F: referenced strains from Hedyasarum; P,P_3 and Pd: strains from pea; Pt: control of pea.

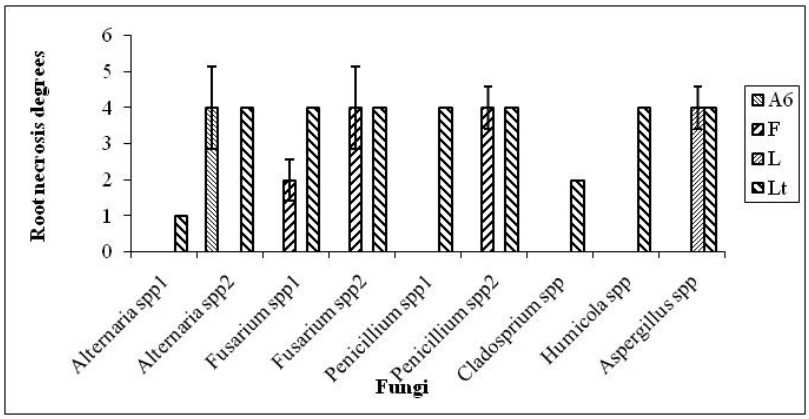

Fig. 8. Effect of *Rhizobium* inoculation on fungi suppression of lentil seeds

0: healthy seedlings, 1: necrosis of extremity, 2: root cutting,3: reduction of cotyledon, 4 :severe necrosis; L: strain from lentil; A6 and F: referenced strains from Hedyasarum, Lt: control of lentil.

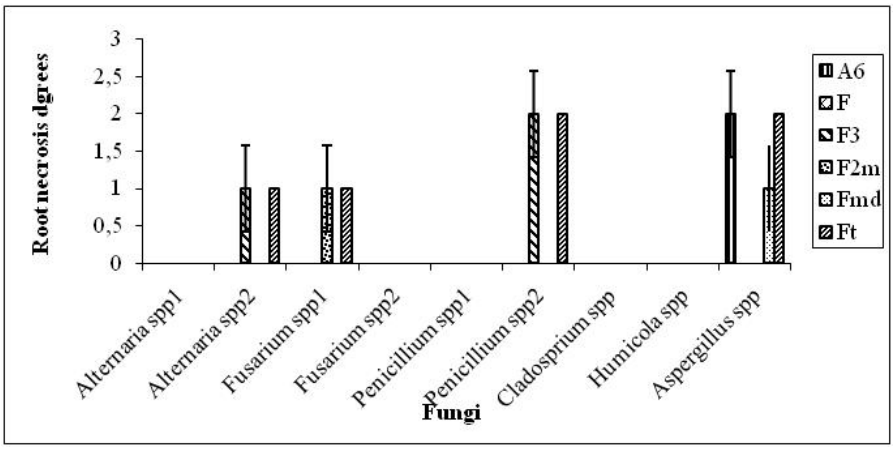

Fig. 9. Effect of *Rhizobium* inoculation on fungi suppression of faba bean seeds

0 : healthy seedling, 1 : necrosis of extremity, 2: root cutting,3 :reduction of cotyledon ,4: severe necrosis; A6 and F: referenced strains from Hedyasarum; F_3, F_2m and Fmd : strains from faba bean; Ft: control of faba bean.

Several studies noted benefits of the rhizobia on the growth of plants and reduction of diseases incidence [66,67]. [68] reported that treatment of broad bean seeds with *Rhizobium leguminosarum* resulted in significant reduction in damping-off caused by different fungal pathogens such as *Fusarium oxysporum, Fusarium solani, Macrophomina phaseolina, Rhizoctonia solani* and *Sclerotium rolfsii. Rhizobium* species significantly reduced fusarial foot [69] and root rot in chickpea [69,70,71] in common bean [70,71]; wilt in chickpea and bush bean [53,72].

Also, the results of [60] indicate that five trains of rhizobia inhibited *Rhizoctonia solani* in chickpea whose looked healthy with no symptoms and the inhibition of the fungi growth by some isolates of *Rhizobium* varied from 60 to 87%. In addition, *Rhizobium leguminosarum* reduced disease severity of *Phytophtora megasperma*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotium rolfsii* by 52.9, 50, 67.9 and 52%, respectively [10]. Also *Fusarium solani* and *Macrophomina phaseolina* and their interaction with rhizobia, in pots, improved seed germination percentages and reduced the root rot disease index significantly [73]. The best disease control almost 90% was obtained with isolate from lentil (L) on lentil plants (Fig. 5). [74] have shown that the wilting index was 2 when lentil plants pathogen-inoculated were treated with *Rhizobium* sp.

F3 inhibited in dual culture *Alternaria* spp. 2 and *Humicola* spp. growth with more than 90%, and was also effective *in vivo*. Pc3 showed similar results *in vivo* against *Cladosporium* spp. However the isolate P from pea was mostly performing *in vitro* than *in vivo* (Table 2 and Fig 4*)*. In contrast to the isolate Pd which was better *in vivo* than *in vitro*. Experiments of [53] indicated that four isolates of rhizobia were effective *in vitro* but were completely or partially ineffective *in vivo*. On the other hand, they observed that two of the isolates carried out better *in vivo* than *in vitro*. Our results would be in agreement with the idea that antagonistic micro-organisms carrying out are best *in vitro* but not *in vivo* and vice versa [75].

The treatment of seeds by rhizobia and fungal pathogen *in vitro* is not studied despite of their importance. In our work seeds necrosis varied from 10 to 45% according to the tested pathogen *Fusarium* spp. 2*, Penicillium* spp.1, *Penicillium* spp. 2 and *Aspergillus* spp. [65] found that the isolates of rhizobia have a variable and a significant effect on the germination of chickpea seeds inoculated with *Rhizoctonia solani*. The percentage of germination varied from 40 to 90%. These rhizobia improve seeds germination of chickpea and reduce necrotic root with 80%. However, few studies have been carried out on the effect of rhizobial strains and phytopatogenic fungi on seeds germination *in vitro*.

In addition to the nitrogen supply Rhizobia promote growth of the plant as a symbiotic partner in several ways, such as mobilization of nutrient, enhancement in stress resistance, solubilization of phosphates, production of phytohormones and siderophores. Siderophores act as iron source for the plant under iron depleted conditions [41] Siderophore production for rhizosphere colonization has also been recorded as one of the important mechanism by certain PGPRs (*Bradyrhizobium japonicum, Rhizobium leguminosarum* and *Sinorhizobium meliloti*) [76,77] to promote plant growth activity. Some of them have been earlier reported as bio-fertilzers as: *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* [78].

Increased population of *Mesorhizobium loti* MP6 in the rhizosphere reflects its potentiality to use root exudates as energy source which facilitate its rhizosphere colonization. The antagonistic nature and relative adherence give MP6 the protection during fungal infection that helps in establishing and resisting against preexisting deleterious microorganisms occupying the microbial niche in the rhizosphere [55]. On the other hand, *Rhizobium* protect non-host/non-nodulating plants (rice) against pathogenic fungi (*R. solani*), and increase their productivity [56].

4. CONCLUSION

In accordance with the obtained results from all the conducted tests, we concluded that *Rhizobium* isolated from the east of Algeria provides a high level of protection against the studied pathogenic fungi belonging to different genera, without specificity. Furthermore *Rhizobium* would be a good element to reduce infestation and the use of these strains will help farmers at combating diseases in field and to prevent fungal attack.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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