BIOCONTROL OF CHICKPEA *FUSARIUM* WILT BY *BACILLUS* SPP. RHIZOBACTERIA

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Abstract: Among 131 rhizobacteria isolates, 29 potentially antagonistic strains were screened in *in vitro* assays. The five antagonistic *Bacillus* spp. Rb29, Rb6, Rb12, Rb4, and Rb15 showed the most inhibitory effect against FOC1 (from 25.63 to 71.11%), mycelial growth, and FOC2 (from 28.43 to 60.65%) *in vitro*. Results also revealed that production of volatile metabolite, components and inhibition of the test pathogen by volatile metabolites varied among different antagonistic rhizobacteria. Isolates Rb29, Rb6, Rb12, Rb4, and Rb15 produced more volatile metabolites which inhibited mycelial FOC growth by 40%. Chickpea *Fusarium* wilt severity caused by FOC1 was reduced from 60 to 99% in the susceptible cultivar ILC 482 treated with antagonistic *Bacillus* spp. (Rb29, Rb6, Rb12, Rb4, and Rb15) in pot assays and by 98, 81, 68, 64, 57.20%, respectively, in the field trials. As for their beneficial effects on disease control, the results revealed that *Bacillus* spp. may improve plant growth and disease control.

Key words: antagonistic strains, *Bacillus*, bacterized seed, *Cicer arietinum*, screening

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the world's fourth most important legume crop after Soybean, common bean, and peas. In developing countries, chickpea is a rich complement to the cereal diet since it has a high nutritive value. Mainly grown for its highly proteinated edible seeds, this crop can be used for both seed and forage production (Yadav *et al.* 2011). In several countries (Algeria, Morocco, Syria, Turkey, Pakistan, India, etc.), production of chickpea did not increase because of low productivity and unstable output (Labdi 1995). Causes of regression are agronomic, abiotic and biotic factors (Labdi 1995). In Algeria, where the market is very favorable for the Kabuli type chickpea, production is developing very slowly because of low yields and because of competition with other crops on limited land areas (Pluvinage 1990).

Chickpea *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato (FOC) is a destructive disease agent, and an important pathogen in Algerian economy (Labdi 1990).

The use of resistant cultivars is one of the most practical and cost efficient strategies for managing plant diseases. However, the efficiency of resistant cultivars in managing a disease can be seriously limited by pathogenic variability occurring in pathogen populations, including the existence of pathogenic races and pathotypes (Jiménez-Gasco *et al.* 2004). Increasing the use of chemical inputs causes several negative effects such as the development of pesticide resistance to applied agents. Chemical inputs also have an effect on non-targeted environmental impacts (Gerhardson 2002).

Lately, people are more and more concerned about fungicides and concerned because the limited use of cultural methods. Biological control has emerged as an important alternative in managing soil-borne plant diseases. Several rhizobacteria have been extensively used as biological agents to control many soil-borne plant pathogens, including FOC (Dileep Kumar 1999; Landa *et al.* 2004). Various biocontrol agents, including bacteria belonging to the genera *Bacillus*, *Pseudomonas*, and fungi such as nonpathogenic fusaria, have been used successfully. The results in reducing pathogenic fungal growth *in vitro* and disease development *in vivo* are significant. *Bacillus* spp. are gram-positive bacteria, frequently retrieved from the rhizosphere. This species has already been mentioned as a biocontrol agent in the work of many researchers (Landa *et al.* 2001; Johri *et al.* 2003). *Bacillus* spp. have also been shown to be potential candidates as biocontrol agents since they are abundant in soils and because they produce heat resistant spores apart from their active metabolites (Milner *et al.* 1996).

Our research was carried out as an alternative strategy to chemical control. We aimed to develop an effective biological control of FOC. For this reason, the most promising rhizobacteria antagonists towards FOC (mainly *Bacillus* spp.), were isolated and screened for *in vitro* trials. Our objectives were also to evaluate the potential efficacy of the antagonists for controlling chickpea *Fusarium* wilt in northwestern Algeria.

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MATERIALS AND METHODS

Isolation of rhizobacteria

Rhizobacteria isolates were isolated from rhizosphere soils of healthy chickpea plants in order to be used as natural biocontrol agents. The rhizosphere soil samples had been collected during the period from 2008 to 2010 from nine locations in Algeria (Table 1). The samples were placed in polyethylene bags, closed tightly, and stored in a refrigerator at 4°C until needed.

Isolation of rhizobacteria was performed using a soil dilution plating technique as described by Fang (1998). One gram of dried soil samples was suspended in 9 ml sterile distilled water, agitated for 1 min, and allowed to settle for 1 h. The suspension was subsequently diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. The 0.1 ml soil dilutions of 10^{-3} to 10^{-6} were separately streak-plated on nutrient agar (NA) medium, in triplicate. The plates were then incubated at 28°C for 24 h. From the countable plates, ten to fifteen representative colonies with different morphological appearances were selected and re-streaked on a new plate containing the same medium, to obtain pure colonies.

In vitro screening of rhizobacteria isolates for their antagonistic activity

Dual culture assay

In vitro inhibition of mycelial growth of FOC (FOC1 and FOC2) by the bacterial isolates were tested using the dual culture technique described by Landa *et al.* (1997) and Swain and Ray (2007), but with some modifications. Four agar discs from nutrient agar cultures of each rhizobacteria were placed equidistantly along the perimeter of Potato Dextrose Agar (PDA) plates and were incubated in the dark at 28°C. After 24 h, a 6 mm agar disc from fresh PDA cultures of seven-day-old FOC was placed in the center of the plate. Plates without rhizobacteria were used as the control and incubated at 25°C for six days. The inhibition zone, noted by the absence of any contact between the bacteria and FOC after incubation, was scored as described (Suarez-Estrella 2007). Each test was replicated three times. After 2 to 6 days of incubation, the interface region was observed under light microscope.

Distance culture assay

Rhizobacterial inhibition through production of the volatile antifungal substance (s) was measured following a modified method used by Fiddaman and Rossall (1995).

Table 1. Sampling locations and source of potentially antagonistic rhizobacteria

Number	Isolates	Department	Location	Year collected
1	Rb1	Mascara	Maoussa	2009
2	Rb4		Maoussa	2010
3	Rb6		Tighenif	2008
4	Rb12		El-Bordj	2008
5	Rb15		Maoussa	2010
6	Rb16		Maoussa	2009
7	Rb8	Sidi Bel-Abbes	Tassala	2008
8	Rb23			2009
9	Rb2	Tlemcen	Tlemcen	2008
10	Rb3			
11	Rb10			
12	Rb18			2009
13	Rb21			2010
14	Rb25			
15	Rb7	Tiaret	Si El Hawas	2008
16	Rb13		Tiaret	2008
17	Rb26		Tiaret	2009
18	Rb11	Mostaganem	Mostaganem	2008
19	Rb20			
20	Rb24			2010
21	Rb27			
22	Rb17	Relizene	Mendès	2008
23	Rb22	Chlef	Chlef	2008
24	Rb28			2009
25	Rb9	Guelma	Oued El Zanati	2008
26	Rb14		Guelma	2009
27	Rb19		Guelma	2010
28	Rb5	Constantine	Ain Abid	2008
29	Rb29			2009

Four agar discs from nutrient agar cultures of each rhizobacteria, were placed equidistantly along the perimeter of PDA plates. After incubation at 28°C for 24 h, a second Petri dish containing PDA with a 6 mm plug of the test fungus, was placed in the center of the plate, inverted and placed over the bacterial culture. The two plates were sealed together with Parafilm to prevent gas diffusion and then they were incubated at 25°C. This incubation ensured that both organisms were growing in the same conditions though they were physically separated. As a control, a Petri dish containing PDA medium without bacteria was placed over the PDA medium inoculated with the fungal pathogen (FOC1/FOC2). Any radial growth increase of the test fungus over 48h intervals, for a period of six days, was recorded. Each test was replicated three times.

Identification of efficient antagonistic rhizobacteria

The best-selected rhizobacterial isolates were tentatively identified as to the genus, according to the method described in Bergey's Manual of Determinative Bacteriology (Sneath 1986; Holt *et al.* 1994). Complementary biochemical traits were determined through API 20E tests (Földes *et al.* 2000).

Effect of *Bacillus* isolates on chickpea *Fusarium* wilt, in experiments conducted in pots and in the field

Chickpea cultivar

One FOC susceptible chickpea line (ILC 482) was used in this experiment. The seeds of ILC 482 were obtained from the Technical Institute of Field Crops in Saïda, Algeria.

Fungal inoculum preparation

Two isolates of FOC (FOC1 and FOC2), originated from Mascara (Algeria) were used in the present study.

After preparing monoconidial FOC isolates, the isolates were kept on a PDA medium and incubated at 25°C for 15 days. Spore suspensions were prepared by removing the spores from the sporulating edges of the culture with a sterile rod and adding 5 ml of sterile distilled water for better spore separation. Spore suspensions were sieved through two paper filters, and spore concentration was determined by the aid of a haemocytometer (10^6 conidia/ml) (El Aoufir 2001).

After observing visual symptoms of *Fusarium* wilt and death of young seedling, FOC1 was selected as the most virulent isolate, and was used (FOC1) as a target pathogen in this experiment.

Bacterial inoculum preparation

Bacterial isolates were grown in nutrient broth on a rotary shaker at 28°C and 180 rpm for 24 h. the suspension was centrifuged in 50 ml capacity sterile plastic tubes at 5,000 rpm for 10 min. A new pellets-suspension was prepared in quarter-strength sterile distilled water to give a final concentration of 10^8 bacteria/ml (Idris *et al.* 2007).

Pot experiment

A pot experiment was conducted in 2009 and 2010 to evaluate the performance of bacteria as a bio-control agent against wilt. Four treatments and three replicates were done. We sowed each of three susceptible chickpea cultivar ILC 482 seeds in a 7 cm diameter sterilized surface (3% Sodium hypochlorite) plastic pot, filled 2/3 full with a sterilized soil mixture and peat (V/V). Sterilization was done at 120°C for 1 h three times in three days. Chickpea seeds were surface sterilized with 2% sodium hypochlorite for 3 min and rinsed three times with sterile distilled water and then dried. The experiment was replicated four times.

Preparation 1 including the non-inoculated control, was treated with sterile distilled water without bacterial and fungal inoculum (with no rhizobacteria and no pathogen). Preparation 2 was treated only with conidia of FOC1 (with pathogen and no rhizobacteria). Preparation 3 including seeds bacterized with *Bacillus* spp. was mixed with 10^6 conidia/ml of FOC1 (with rhizobacteria and pathogen). Preparation 4 containing seeds bacterized separately with the efficient rhizobacteria but with no conidia of the pathogen (rhizobacteria + no pathogen) was settled. Chickpea seeds were thoroughly soaked in a bacterial suspension containing 10^8 bacteria/ml to ensure a uniform coating of the surface.

Pots were kept under observation for wilt incidence for 40 days, and compared after sowing with the control pots.

Field experiment

As in the preceding experiment, the same cultivar was studied: five *Bacillus* isolates and four treatments. The field was subdivided into 2x2 m plots; in each plot 24 seeds were sown in three lines. Each treatment was replicated three times. The non-bacterized seeds were kept as the controls. Seeds were sown during the first week of March 2009.

When plants were ten-weeks-old, the total number of wilted plants was recorded. Evaluation of disease incidence was initiated when the first disease symptoms appeared on plants which had been only inoculated with FOC1. Subsequently, disease incidence was evaluated every three days for 20 to 22 days after inoculation.

Disease assessment

Disease reactions were assessed according to the severity of symptoms at 2 to 3 days intervals, using a 0 to 4 rating scale based on the percentage of foliage with yellowing or necrosis in acropetal progression (0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, and 4 = dead plant) (Landa *et al.* 1997). The percentage suppression of *Fusarium* wilt was calculated according to the disease severity index (Villajuan-Abgona *et al.* 1996). The FOC isolate, FOC1, was isolated again from wilted plants by plating stem pieces from the crown region onto PDA medium.

Statistical analysis

Data obtained from all *in vitro* experiments were subjected to analysis of variance (ANOVA) and significant means were separated through Newman-Keuls Multiple Range Test at $p < 0.05$ calculating also standard errors (SE). Each test was replicated three times.

RESULTS AND DISCUSSION

Isolating rhizobacteria and identifying efficient antagonistic isolates

Isolating rhizobacteria from different locations helps in the identification of organisms which have adapted to various environmental conditions. In the present study, a total of 131 rhizobacterial isolates was obtained from nine locations in Algeria: Mascara, Sidi Bel-Abbes, Tlemcen, Tiaret, Relizane, Mostaganem, Chlef, Constantine, and Guelma.

A primary selection was made from antagonism test plates where confluent bacterial growth from the chickpea rhizosphere inhibited fungal mycelia development. Pure rhizobacterial cultures isolated from those plates were tested for fungal antagonism.

With this procedure, 29 isolates inhibiting more than 20% of *F. oxysporum* f. sp. *ciceris* were obtained, and compared to fungi growing alone. Based on *in vitro* efficiency against FOC1 and FOC2, five rhizobacterial isolates (Rb29, Rb6, Rb12, Rb4, and Rb15) were selected. They were identified with the use of Bergey's Manual of Determinative Bacteriology, and subjected to morphological, physiological and biochemical properties of the *Bacillus* genus.

Colonies on Tryptic soya agar medium (TSA) from these isolates are wrinkled and white. These isolates are Gram positive, aerobic, rods forming endospores, hydrolyse starch, and motile. With these results, we confirmed that they all belong to the *Bacillus* genus.

In vitro screening of rhizobacteria isolates for antagonism against *F. oxysporum* f. sp. *ciceris*

Among 131 rhizobacteria tested, twenty-nine rhizobacteria isolated were screened by the dual culture method against two FOC isolates (FOC1 and FOC2). Antagonism was evident in Petri dishes through the different magnitudes of the FOC inhibition halo. All isolates significantly inhibited growth of FOC1 and FOC2 pathogens. All isolates also reduced FOC1 and FOC2 development where inhibition percentage varied from 25.63 to 71.11% and from 28.43 to 60.65%, respectively (Table 2).

Isolates Rb29, Rb6, Rb12, Rb4, and Rb15 were the most efficient *in vitro* and caused growth inhibition of FOC1 and FOC2 above 50%. The highest inhibitory effect was noted in Rb29 with a 71.11% rate. On the other hand, the lowest inhibitory effect towards FOC1 was noted in Rb27/Rb3 with a 25.63/26.65% rate. The lowest inhibitory effect towards FOC2 was in Rb27 and Rb3 with a 28.43/28.62% rate. The control plates without rhizobacteria, were completely covered by pathogen mycelia showing no fungus growth inhibition. The mean mycelium growth inhibition of the most effective bacterial isolates revealed that inhibition was highly significant ($p < 0.05$). According to these results, Rb29, Rb6, Rb12, Rb4, and Rb15 were used in the further experimentation.

Table 2. Screening for antagonistic rhizobacteria against *F. oxysporum* f. sp. *ciceris* plant pathogen of chickpea

Rhizobacterial isolats	% mycelial inhibition* of FOC in					
	a dual culture assay			a distance culture assay		
	FOC1[a]	FOC2[a]	mean[b]±SE	FOC1[a]	FOC2[a]	mean[b]±SE
Rb1	43.33	34.26	38.80 f ±4.54	15.66	15.24	15.45 x ±0.21
Rb2	37.77	36.45	37.11 f ±0.66	25.41	25.57	25.49 n ±0.08
Rb3	26.66	28.61	27.64 h ±0.98	28.56	28.67	28.62 h ±0.06
Rb4	57.50	55.71	56.60 c ±0.89	33.33	37.20	35.27 e ±1.94
Rb5	38.98	39.02	39.00 f ±0.02	21.66	22.84	22.25 r ±0.59
Rb6	64.16	54.71	59.44 b ±4.73	44.68	45.74	45.21 a ±0.53
Rb7	44.43	35.86	40.15 f ±4.28	19.99	20.33	20.16 t ±0.17
Rb8	45.55	40.56	43.05 f ±2.50	26.66	26.74	26.70 k ±0.04
Rb9	41.42	36.80	39.11 f ±2.31	28.89	29.60	29.24 g ±0.36
Rb10	35.55	31.83	33.69 g ±1.86	26.66	26.87	26.77 j ±0.11
Rb11	53.33	41.85	47.59 e ±5.74	16.67	17.10	16.88 w ±0.22
Rb12	58.53	50.54	54.54 d ±4.00	40.00	42.90	41.45 c ±1.45
Rb13	46.55	40.20	43.38 f ±3.18	20.34	20.66	20.50 s ±0.16
Rb14	43.33	42.71	43.02 f ±0.31	28.61	28.60	28.60 h ±0.00
Rb15	56.72	52.56	54.64 d ±2.08	43.00	43.33	43.17 b ±0.16
Rb16	35.66	37.17	36.42 f ±0.75	19.56	20.23	19.89 u ±0.33
Rb17	48.88	35.77	42.33 f ±6.56	21.66	22.85	22.26 r ±0.59
Rb18	45.55	35.72	40.64 f ±4.92	22.33	22.84	22.59 r ±0.26
Rb19	47.50	38.57	43.03 f ±4.46	21.88	22.85	22.37 r ±0.48
Rb20	45.44	38.53	41.99 f ±3.46	25.88	26.65	26.27 l ±0.39
Rb21	45.77	32.85	39.31 f ±6.46	18.33	19.04	18.69 v ±0.36
Rb22	36.66	31.56	34.11 fg ±2.55	30.48	30.50	30.49 f ±0.01
Rb23	30.00	31.71	30.86 g ±0.85	22.44	23.82	23.13 q ±0.69
Rb24	33.33	30.71	32.02 g ±1.31	24.94	25.71	25.32 o ±0.38
Rb25	46.66	39.60	43.13 f ±3.53	23.33	23.90	23.61 p ±0.28
Rb26	52.22	42.56	47.39 e ±4.83	24.67	26.59	25.63 m ±0.96
Rb27	25.63	28.42	27.03 i ±1.40	14.11	14.28	14.19 y ±0.08
Rb28	50.00	42.84	46.42 e ±3.58	26.66	27.50	27.08 i ±0.42
Rb29	71.11	60.65	65.88 a ±5.23	36.66	40.00	38.33 d ±1.67

*inhibition percentage of *F. oxysporum* f. sp. *ciceris* radial growth 6 days after incubation

[a]the values are the means of three replications

[b]means followed by a different letter are significantly different ($p \leq 0.05$)

No physical contact between any of the tested antagonistic rhizobacteria and FOC1 or FOC2 was noticed. Moreover, an inhibitory halo suggesting the presence of fungistatic metabolites secreted by rhizobacteria was seen. Change in FOC colony mycelia color was also observed.

A microscope was used to make observations, thus we think that with mycelium collected from the interface region having the best tested rhizobacteria, the isolates Rb29, Rb6, Rb12, Rb4, and Rb15 caused a modification in the mycelium appearance. These modifications were: mycelia color changing from white to red, reddish brown or darker brown. With these isolates, a coagulation of fungal cytoplasm that can be observed up to the hypha was detected, resulting in the presence of small vesicles and the appearance of big vacuoles. In this case, the destructive effect of FOC by rhizobacteria was high, resulting in serious damage of the hyphae, associated with a series of degradation events.

The mycoparasitic potential of *Bacillus* spp. is well documented (Johri *et al.* 2003; Saharan and Nehra 2011). Thus, this phenomenon has often been used as a means for *in vitro* screening of biocontrol agents (Elad *et al.* 1980). Similar conclusions have been reported by El Hassni *et al.* (2007) and Idris *et al.* (2007). They reported a modification of the fungal mycelium appearance, due to antifungal secondary metabolite production. Generally, biocontrol capacity through antagonistic bacteria involves either competition (Elad and Chet 1987) or bacterial metabolite production, such as siderophores, hydrogen cyanide, antibiotics or extracellular enzymes for antagonism towards plant pathogens (Kamilova *et al.* 2005; Sang *et al.* 2006). It has been reported that *Bacillus* spp. contains various biocontrol characteristics including secondary metabolites, the colonizing potential, and the production of competitors (Yoshida *et al.* 2001; Schmidt *et al.* 2004).

Rhizobacteria isolates volatile metabolite effects on *F. oxysporum* f. sp. *ciceris in vitro*

Higher volatile metabolite concentrations were produced by isolates Rb29, Rb6, Rb12, Rb4, and Rb15: they inhibited FOC1 and FOC2 at a rate varying from 33.33 to 44.66% and 37.2 to 45.75%, respectively (Table 2). While other isolates moderately inhibited its growth from 19.56 to 30.5%. The lowest volatile metabolite activity was observed in Rb27, Rb1, Rb11, and Rb21: they inhibited target pathogen FOC1 from less than 14.11 to 18.33%, and they inhibited FOC2 from 14.28 to 19.05%. The antagonistic potential was also noted to vary through volatile metabolites, and direct parasitism on the pathogen among different isolates of an antagonist rhizobacteria isolate. In addition, a change in mycelia color which was a different than the mycelium color of the control, close to the FOC colony was observed. We noted a stronger antibiosis mechanism of antagonistic rhizobacteria and a higher pathogen inhibition through volatile metabolites. Volatile toxic substances produced by antagonists spread easily and inhibit pathogen growth *in vitro*.

Five isolates highly efficient towards two isolates of *F. oxysporum* f. sp. *ciceris*, were finally selected based on the performance of *in vitro* isolates selected for their efficiency on individual pathogens. These selected efficient strains were used for further studies.

The production of antifungal compounds and siderophores is a primary mechanism in suppressing disease by *Bacillus* spp. and fluorescent *Pseudomonas* spp. (Edwards *et al.* 1994).

Peptide antibiotics and several other compounds which are toxic to plant pathogens have been recovered from several *Bacillus* strains (Yu *et al.* 2002).

Bacillus spp. efficiency on chickpea *Fusarium* wilt disease in pot and field experiments

Pots assay

These tests showed that susceptible cultivar reacts to FOC1 with a high incidence of *Fusarium* wilt. Nevertheless, 6 weeks after sowing, there was 100% more disease on wilted plants. Bacterized seeds with isolates Rb29, Rb6, Rb12, Rb4, and Rb15 significantly reduced the percentage of wilted plants, from 99–60%. All chickpea seedlings inoculated by a conidial suspension of FOC1 had a 3.8 mean score rating of infection, and showed foliage with yellowing or necrosis in acropetal progression. This is characteristically distinctive of *Fusarium* wilt with 100% wilt incidence. Compared to plants from seeds treated with the *Bacillus* isolates towards FOC1, we noted less yellowish foliage and low disease severity. We demonstrated that *Bacillus* isolate treatments enhance chickpea growth in pots under controlled conditions, even if growth promotion varies with the treatment.

Field assay

We first observed that seed germination is much better when seeds were only inoculated with FOC1. Mashooda Begum *et al.* (2003) reported the same result. They showed that colonization of the bacterial strains *B. pumilus* (SE-34), *B. pasteurii* (T4), *B. subtilis* (IN 937-6), and *B. subtilis* (GB-03) reduced seed mycoflora incidence. This indirectly enhances seed germination percentage and the seedling strength index. In addition, we observed that the typical yellowing form of symptoms start on the bottom shoots and gradually go up until there is severe leaf sclerosis and flaccidity. Wilt appears after 25 days in all seedlings that were inoculated only with FOC1. We found a 3.7 average wilt severity and a 97.93% wilt incidence. We note that *Bacillus* isolates: reduced disease severity caused by FOC1 (Fig. 1), that disease incidence decreased, and that chickpea seedlings were significantly protected against *Fusarium* wilt. There were differences within the isolates. We found that *Bacillus* spp. (Rb29) had a stronger ability to control FOC1 compared to the ability of Rb15. We also found that disease appearance drops with isolates Rb29, Rb6, Rb12, Rb4, and Rb15 at 98, 81, 68, 64, and 57.20%, respectively. The decrease in disease is significant in bacterized treatment. We noticed a big difference. Plants treated with isolates Rb29 get significantly stronger if compared to plants resulting from seeds inoculated only with FOC1 conidia. In this case, treatment with *Bacillus* spp. Rb29 enhances the biomass of plants and increases the total number of leaves**.** Our investigations suggest, that if we compare plants infected only with FOC1, a modified rooting system may
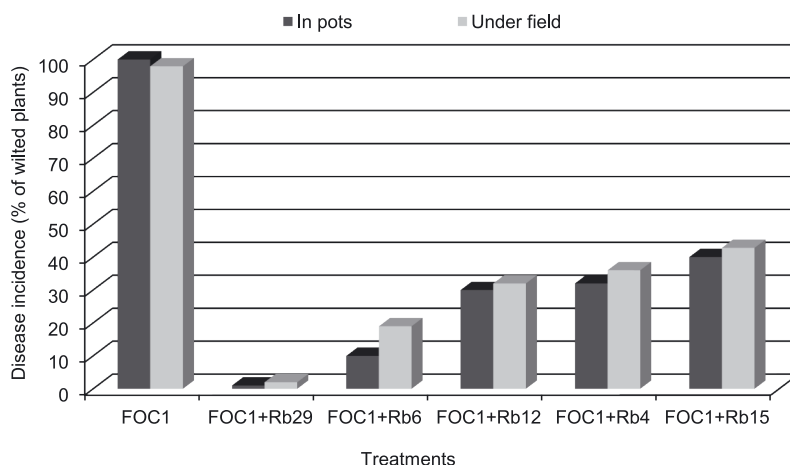
Fig. 1. Reduction of Fusarium wilt (%), in pots and in the field conditions, and a comparison between inoculated plants by FOC1 alone and inoculated plants treated by *Bacillus* spp.

be related to the increased growth response caused by *Bacillus* isolates. Plants infected only with FOC1, displayed stunted and reduced root systems.

Bacillus spp. from spores, are resistant to unfavorable conditions and can thus be adapted to the field. According to Gardener (2004), diverse populations of aerobic endospore-forming bacteria appear in agricultural fields, this may directly and indirectly contribute to crop productivity. Multiple *Bacillus* spp. and *Paenibacillus* spp. can promote crop health in varied ways. In addition, through the work of Reva *et al.* (2004), and Demoz and Korsten (2006), we know that some *Bacillus* spp. are good root colonizers and can effectively protect infection regardless of soilborne or airborne pathogens.

Choudhary and Johri (2009) demonstrated that the number of *Bacillus* strain activities suppress pathogens or otherwise promote plant growth. Improvements in plant health and productivity are mediated through three different ecological mechanisms: (i) pathogen antagonism, (ii) host nutrition and growth promotion, and (iii) plant host defense stimulation. Rhizobacteria are ideal for use as biocontrol agents. Rhizobacteria inhabit the rhizosphere that provides the front line defense for roots against attack by pathogens. Pathogens find antagonism from rhizobacteria before and during primary root infection. Rhizobacteria are reported to provide protection against several plant pathogens. Generally, rhizobacteria traits associated with plant pathogens biocontrol include: antibiotic synthesis (Haas and Defago 2005), production of low molecular weight metabolites such as hydrogen cyanide with antifungal activity (Dowling and O'Gara, 1994), production of enzymes including chitinase, b-1-3-glucanase, protease, and lipase. These enzymes can lyse some fungal cells (Chet and Inbar 1994). According to O'Sullivan and O'Gara (1992) and Loper and Henkels (1997), producing oxidative stress enzymes such as catalases, superoxide dismutases, peroxidase, and polyphenol oxidases is effective in scavenging active oxygen species, out-competing phytopathogens for nutrients and occupying niches on the root surface.

Rhizosphere competition with biocontrol agents is potentially important for controlling plant diseases. This study led to the selection of potential biocontrol agents against chickpea *Fusarium* wilt caused by *F. oxysporum* f. sp. *ciceris*, and demonstrated that local isolates of *Bacillus* spp. have a prospective use as biological control agents to protect chickpea plants. Characterizing efficient biocontrol agents against soilborne diseases is important for carrying out a successful action in complex environmental conditions and dynamic rhizosphere.

REFERENCES

Chet I., Inbar J. 1994. Biological control of fungal pathogens. Appl. Biochem. Biotechnol. 48 (1): 37–43.

Choudhary D.K., Johri B.N. 2009. Interactions of *Bacillus* spp. and plants – with special reference to induced systemic resistance (ISR). Microbiol. Res. 164 (5): 493–513.

Demoz B.T., Korsten L. 2006. *Bacillus subtilis* attachment, colonization, and survival on avocado flowers and its mode of action on stem-end rot pathogens. Biol. Control 37 (1): 68–74.

Dileep Kumar B.S. 1999. Fusarial wilt suppression and crop improvement through two rhizobacterial strains in chickpea growing in soils infested with *Fusarium oxysporum* f. sp. *ciceris*. Biol. Fert. Soils 29 (1): 87–91.

Dowling D.N., O'Gara F. 1994. Metabolites of Pseudomonas involved in the biocontrol of plant disease. Trends Biotechnol. 12 (Suppl. 4): 133–141.

Edwards S.G., McKay T., Seddon B. 1994. Interaction of *Bacillus* species with phytopathogenic fungi. Methods of analysis and manipulation for biocontrol purposes. p. 101–118. In: "Ecology of Plant Pathogens" (J.P. Blakeman, B. Williamson, eds). Wallingford, UK, CAB International, 384 pp.

El Aoufir A. 2001. Étude du Flétrissement Vasculaire du Pois Chiche (*Cicer arietinum*) Causé par le *Fusarium oxysporum* f. sp. *ciceri*. Evaluation de la Fiabilité de L'analyse Isoenzymatique et de la Compatibilité Végétative pour la Caractérisation des Races Physiologiques. Thèse de doctorat, Université Laval, Canada, 161 pp.

El Hassni M., El Hadrami A., Daayf F., Chérif M., Ait Barka E., El Hadrami I. 2007. Biological control of bayoud disease in date palm: selection of microorganisms inhibiting the causal agent and inducing defense reactions. Environ. Exp. Bot. 59 (2): 224–234.

Elad Y., Chet I., Katan J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology 70 (2): 119–121.

Elad Y., Chet I. 1987. Possible role of competition for nutrients in biocontrol of Pythium damping-off by bacteria. Phytopathology 77: 190–195.

Fang Z.D. 1998. Plant Pathology Technology. 3rd ed. Beijing: Chinese Agriculture Press, 243 pp. (in Chinese).

Fiddaman P.J., Rossall S. 1995. Selection of bacterial antagonists for the biological control of *Rhizocotonia solani* in oilseed rape (*Brassica napus*). Plant Pathol. 44 (4): 695–703.

Földes T., Bànhegyi I., Herpai Z., Varga L., Szigeti J. 2000. Isolation of *Bacillus* strains from the rhizosphere of cereals and *in vitro* screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage micro-organisms. J. Appl. Microbiol. 89 (5): 840–846.

Gardener B.B.M. 2004. Ecology of *Bacillus* and *Paenibacillus* spp. Agricultural Systems Symposium the Nature and Application of Biocontrol Microbes: *Bacillus* spp. Phytopathology 94 (11): 1252–1258.

Gerhardson B. 2002. Biological substitutes for pesticides. Trends Biotechnol. 20 (8): 338–343.

Haas D., Defago G. 2005. Biological control of soil borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3 (4): 307–319.

Haware M.P. 1990. Fusarium wilt and other important diseases of chickpea in the Mediterranean area. Options Méditerranéennes, Série Séminaires 9: 61–64.

Holt J.G., Kreig N.R., Sneath P.H.A., Staley J.T., Williams S.T. 1994. Bergey's Manual of Determinative Bacteriology. 9th ed. Williams & Wilkins, Baltimore, USA, 816 pp.

Idris H.A., Labuschagnea N., Korsten L. 2007. Screening rhizobacteria for biological control of Fusarium root and crown rot of sorghum in Ethiopia. Biol. Control. 40 (1): 97–106.

Jiménez-Gasco M.M., Navas-Cortés J.A., Jiménez-Díaz R.M. 2004. The *Fusarium oxysporum* f. sp. *ciceris/Cicer arietinum* pathosystem: a case study of the evolution of plant-pathogenic fungi into races and pathotypes. Int. Microbiol. 7 (2): 95–104.

Johri B.N., Sharma A., Virdi J.S. 2003. Rhizobacterial diversity in India and its influence on soil and plant health. Adv. Biochem. Eng./Biotechnol. 84: 49–89.

Kamilova F., Validov S., Azarova T., Mulders I., Lugtenberg B. 2005. Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environ. Microbiol. 7 (11): 1809–1817.

Labdi M. 1990. Chickpea in Algeria. Options Méditerranéennes, Série Séminaires 9: 137–140.

Labdi M. 1995. Etude de la résistance à l'anthracnose (*Ascochyta rabiei*) chez le pois chiche (*Cicer arietinum* L.). Thèse de Doctorat, ENSA de Montpellier, France, 143 pp.

Pluvinage J. 1990. Chickpea in the Mediterranean production systems: two contrasting examples of possible developments in Algeria and France. Options Méditerranéennes, Série Séminaires 9: 133–136.

Landa B.B., Hervas A., Bethiol W., Jimenez-Diaz R.M. 1997. Antagonistic activity of bacteria from the chickpea rhizosphere against *Fusarium oxysporum* f. sp. *ciceris*. Phytoparasitica 25 (4): 305–318.

Landa B.B., Navas-Cortés J.A., Hervás A., Jiménez-Díaz R.M. 2001. Influence of temperature and inoculum density of *Fusarium oxysporum* f. sp. *ciceris* on suppression of Fusarium wilt of chickpea by rhizosphere bacteria. Phytopathology 91 (8): 807–816.

Landa B.B., Navas-Cortés J.A., Jiménez-Díaz R.M. 2004. Influence of temperature on plant–rhizobacteria interactions related to biocontrol potential for suppression of Fusarium wilt of chickpea. Plant. Pathol. 53: 341–352.

Loper J.E., Henkels M.D. 1997. Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. Appl. Environ. Microbiol. 63 (1): 99–105.

Mashooda B., RavisankarRai V., Lokesh S. 2003. Effect of plant growth promoting rhizobacteria on seed borne fungal pathogens in okra. Indian Phytopathol. 56 (2): 156–158.

Milner J.L., Silo-Suh L., Lee J.C., He H., Clardy J., Handelsman J. 1996. Production of kanosamine by *Bacillus cereus* UW85. Appl. Environ. Microbiol. 62 (8): 3061–3065.

O'Sullivan D.J., O'Gara F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. 56: 662–676.

Reva O.N., Dixelius C., Meijer J., Priest F.G. 2004. Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. FEMS Microbiol. Ecol. 48 (2): 249–259.

Saharan B.S., Nehra V. 2011. Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. 2011, p. 21.

Sang M.K., Chiang M.H., Yi E.S., Park K.W., Kim K.D. 2006. Biocontrol of Korean ginseng root rot caused by *Phytophthora cactorum* using antagonistic bacterial strains ISE13 and KJ1R5. Plant. Pathol. J. 22 (1): 103–106.

Schmidt C.S., Agostini F., Leifert C., Killham K., Mullins C.E. 2004. Influence of soil temperature and matric potential on sugar beet seedling colonization and suppression of Pythium damping-off by the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. Phytopathology 94 (4): 351–363.

Sneath P.H.A. 1986. Endospore forming Gram-positive rods and cocci. p. 1104–1137. In: "Bergey's Manual of systematic Bacteriology" (N.R. Krieg, J.G. Holt, eds.). Vol 2, Williams and Wilken, Baltimore, MD, 2898 pp.

Suarez-Estrella F., Vargas-Garcia C., Lopez M.J., Capel C., Moreno J. 2007. Antagonistic activity of bacteria and fungi from horticultural compost against *Fusarium oxysporum* f. sp. *melonis*. Crop. Prot. 26 (1): 46–53.

Swain M.R., Ray R.C. 2007. Biocontrol and other beneficial activities of *Bacillus subtilisisolated* from cow dung microflora. Microbiol. Res. 164 (2): 121–130.

Villajuan-Abgona R., Kagayama K. Hyakumachi M. 1996. Biocontrol of *Rhizoctonia* damping-off of cucumber by non pathogenic binucleate *Rhizoctonia*. Eur. J. Plant Pathol. 102 (1996): 227–235.

Yadav J., Verma J.P., Tiwari K.N. 2011. Plant growth promoting activities of gungi and their effect on chickpea plant growth. Asian J. Biol. Sci. 4 (3): 291–299.

Yoshida S., Hiradate S., Tsukamoto T., Hatakeda K., Shirata A. 2001. Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology 91 (2): 181–187.

Yu G.Y., Sinclair J.B., Hartman G.L., Bertagnolli B.L. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol. Biochem. 34 (7): 955–963.