

## IDENTIFICATION OF *RHIZOBIUM* ISOLATES POSSESSING ANTAGONISTIC ACTIVITY AGAINST *FUSARIUM OXYSPORUM* F.SP. *CICERIS*, THE CAUSAL AGENT OF FUSARIUM WILT OF CHICKPEA

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### SUMMARY

Using two cultivars (the susceptible ILC482 and the moderately resistant INRAT 87/1) of chickpea (*Cicer arietinum*), the antagonistic activities of 21 *Rhizobium* isolates were tested *in vitro* in dual culture, and *in vivo* under greenhouse and field conditions against *Fusarium oxysporum* f.sp. *ciceris* (Foc) race 0, the causal agent of Fusarium wilt of chickpea. In dual culture, 14 isolates inhibited the mycelial growth of the pathogen more than 30% and the most effective were Rh8, Rh11, Rh16 and PchSOM, which inhibited fungal growth more than 50%. Among the 14 *Rhizobium* isolates tested for volatiles, cyanide production and phosphate solubilisation, 8 significantly inhibited fungal growth by producing volatiles, 6 were positive for cyanide production and only three were able to solubilise phosphate. Isolate Rh8 produced the highest levels of volatiles, giving more than 10.7% fungal inhibition, and was the only one positive for both cyanide production and phosphate solubilisation. Greenhouse experiments on the same 14 isolates revealed the effectiveness of five: PchDMS, Pch 121, Rh5, Rh17 and Pch43. These reduced the percentage of wilted plants in both susceptible and moderately resistant cultivars. These percentages ranged from 12.5 to 54.6% in the susceptible cultivar ILC482 and from 8.3 to 29.1% in the moderately resistant cultivar INRAT 87/1. The best disease control was achieved by isolate PchDMS. Despite its effectiveness *in vitro*, isolate Rh8 was ineffective under greenhouse conditions. Field experiments showed that none of the 14 *Rhizobium* isolates significantly reduced the percentage of wilted plants of the susceptible cultivar ILC482, although with the moderately resistant cultivar INRAT 87/1 eight of the isolates significantly reduced wilt incidence. Inoculation of seeds with these isolates reduced the percentage of diseased plants from more than 48.6% in infected control plants to less than 20.3% in plants inoculated with the bacteria and infected with the pathogen.

The best protection against disease was obtained with isolates Pch43 and Rh4, which reduced the percentage of wilted plants to less than 8%. Besides their beneficial effects on disease control, our studies showed that rhizobia may improve plant growth and yield. These results indicate that *Rhizobium* isolates could be effective under commercial conditions in reducing the deleterious effects of Fusarium wilt.

*Keywords:* Biocontrol, chickpea, *Rhizobium*, Fusarium wilt.

### INTRODUCTION

Fusarium wilt caused by *Fusarium oxysporum* Schlechtend f.sp. *ciceris* (Padwick) Matuo & K Sato (Foc) is a major constraint to chickpea (*Cicer arietinum* L.) production throughout the world and particularly in the Indian subcontinent and the Mediterranean basin (Nene *et al.*, 1989). Yield losses caused by the disease amounted to 10% in India (Singh and Dahiya, 1973) and Spain (Trapero-Casas and Jiménez-Díaz, 1985) and up to 40% in Tunisia (Halila *et al.*, 1984; Halila and Harrabi, 1990).

*F. oxysporum* can survive in soil several years by means of chlamydospores (Haware *et al.*, 1996), which markedly reduce the potential of crop rotation as a disease management strategy. The most effective and practical method of control worldwide is to use fungicides (Gupta *et al.*, 1988) or resistant cultivars. However, the effectiveness of host resistance is curtailed by the occurrence of pathogenic races in Foc (Haware and Nene, 1982; Jiménez-Díaz *et al.*, 1989; Jiménez-Gasco *et al.*, 2004).

Efforts must be addressed toward developing new alternatives for more effective disease management. Biological control agents for plant diseases are currently being examined as alternatives to synthetic pesticides due to their perceived level of safety and minimal environmental impacts. Strains of several bacterial species such as *Bacillus*, *Pseudomonas* and recently the *Rhizobium* group were isolated and found to effectively control various soil-borne plant pathogenic fungi under greenhouse and field conditions. As compared to the other biocontrol agents, Rhizobia offer the great advantage of

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symbiotic nitrogen fixation by association with legumes (Peoples *et al.*, 1995).

Among the *Rhizobium* group, *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, and *Bradyrhizobium japonicum* have been used successfully against fungal pathogens belonging to the genera *Macrophomina*, *Rhizoctonia* and *Fusarium* (Ehteshamul-Haque and Ghaffar, 1993; Ozkoc and Deliveli, 2001). Rhizobia have several mechanisms of action that allow them to control pathogens. These mechanisms include competition for iron by production of siderophores (Carrillo and Del Rosario, 1992; Arora *et al.*, 2001), competition for nutrients (Essalmani and Lahlou, 2002), production of antibiotics (Chakraborty and Purkayastha, 1984; Ehteshamul-Haque and Ghaffar, 1993), promotion of plant growth, in terms of better shoot height, root length, dry weight and root nodulation (Siddiqui *et al.*, 2000; Siddiqui and Mahmoud, 2001), and induction of plant defence mechanisms (Abdelaziz *et al.*, 1996).

The aim of the present work was to characterize and select *Rhizobium* isolates with antagonistic activity against wilt caused by *F. oxysporum f.sp. ciceris*. Isolates were purified from chickpea nodules and their effects on fungal growth and disease development were assessed *in vitro*, in dual cultures, as well as *in vivo* under greenhouse and field conditions.

## MATERIALS AND METHODS

**Origin of microorganisms.** *Rhizobium* isolates were obtained from nodules of 50-day-old chickpea plants using the crushed-nodule method (Vincent, 1970). All isolates were purified and tested for their ability to form nodules on chickpea as previously described (Beck *et al.*, 1993). Twenty one isolates, among which 5 were provided by the Institut National de la Recherche Agronomique de Tunisie (INRAT), were grown and maintained on YEMA (Yeast Extract Mannitol Agar) medium (Vincent, 1970). The origin and the date of collection of each isolate is indicated in Table 1.

The isolate of *F. oxysporum f.sp. ciceris* race 0 (Foc) used was obtained by Halila and Strange (1996), from infected chickpea plants collected from the north of Tunisia, showing typical *Fusarium* wilt symptoms. This isolate induced leaf yellowing and stunting followed by plant death in susceptible chickpea cultivars. Monoclonial fungal cultures of the pathogen were stored in sterile sand tubes at 4°C. Active cultures were obtained from small aliquots of sand culture plated on potato dextrose agar (PDA). Fungal cultures were incubated at 25°C for 8 days with a 12 h photoperiod of fluorescent and near ultraviolet light.

**Chickpea cultivars.** Two chickpea (*Cicer arietinum* L.) cultivars ILC482 and INRAT87/1, respectively sus-

**Table 1.** Sources of *Rhizobium* isolates.

Isolates	Origin	Collection date
Rh1	Menzel Témim1 (Nabeul)	1992
Rh2	Menzel Témim2 (Nabeul)	1992
Rh3	Korba (Nabeul)	1992
Rh4	Belli (Nabeul)	1992
Rh5	Mida (Nabeul)	1995
Rh6	Menzel Bourguiba1 (Bizerte)	1992
Rh7	Menzel Bourguiba2 (Bizerte)	1992
Rh8	Ras jebel (Bizerte)	1992
Rh9	Ghezala (Bizerte)	1992
Rh10	Zaghouan (Zaghouan)	1992
Rh11	Testour1 (Béja)	1992
Rh12	Testour2 (Béja)	1992
Rh13	Ksar Mezouar1 (Béja)	1992
Rh14	Ksar Mezouar2 (Béja)	1992
Rh16	Mornaguia2 (Ariana)	1992
Rh17	Menzel Bourguiba (Bizerte)	1992
PchDMS	CIRAD (Montpellier)	1978
Pch43	ICARDA (Syria)	1988
Pch121	CIRAD (Montpellier)	-
Pch35T	INRA (Montpellier)	-
PchSOM	Maroc	1988

ceptible and moderately resistant to *Fusarium oxysporum f.sp. ciceris* (Foc) race 0, were used in this experiment.

**Effect of *Rhizobium* isolates in dual culture.** *In vitro* antagonism tests were performed on PDA in 9-cm Petri plates by applying a dual culture technique (Sadfi *et al.*, 2001). *Rhizobium* isolates were streaked across the centre of the plate, with a second streak made at right angles to the first. Four discs 5 mm in diameter cut from the edge of a 7 day-old culture of Foc were placed at each side of the antagonist. The distance between the two microorganisms was 2.5 cm. Plates were incubated at 25°C for one week. Percent growth inhibition of Foc (GI) after 7 days was calculated by the formula of Whipps (1987):  $(R1-R2)/R1 \times 100$ , where R1 is the fungal radial growth (measured in mm) in the direction opposite to the antagonist and R2 is the radial growth toward the antagonist. Growth inhibition (GI) was measured on a scale from 0 to 3 (Korsten *et al.*, 1995), where 0 = no growth inhibition, 1 = 1 to 25% growth inhibition, 2 = 26 to 50% growth inhibition and 3 = 51 to 75% growth inhibition.

**Volatile antifungal compounds, phosphate solubilization and cyanide production.** The production of volatile antifungal compounds by the 14 *Rhizobium* isolates with GI greater than 1 was assayed by a sealed plate method as described by Fiddman and Rossal (1993). From a 72 h Yeast Extract Mannitol Broth (YEM; Vincent, 1970) culture of rhizobia, 200 µl were spread on YEMA medium in a Petri dish. After incubation at 37°C for 24 h, a second Petri dish containing PDA, was inoculated with a 6-mm plug of the test fun-

gus in the centre of the plate, inverted and placed over the bacterial culture. The two plates were sealed together with Parafilm and further incubated at 25°C. This ensured that both organisms were growing in the same atmosphere though physically separated. As a control, a Petri dish containing agar medium without bacteria was placed over the PDA medium inoculated with the fungal pathogen. Fungal growth was measured as increases in radial growth of the test fungus over 24 h intervals for a period of 5 days. Each test was replicated 3 times.

Ability to solubilize phosphate was tested using the medium of Katznelson and Bose (1959). Dicalcium phosphate agar plates were inoculated with 24 h bacterial cultures and incubated at 28°C for 5 days. The colonies forming clarification halos were considered as phosphate solubilisers.

Cyanide production was detected as described by Bakker and Schippers (1987). Petri plates containing 10% Trypticase soy agar (TSA, Difco, Franklin Lakes, NJ USA) supplemented with 4.4 g of glycine per litre were inoculated with the bacteria and inverted after a piece of filter paper, impregnated with 0.5% picric acid and 2% sodium carbonate, had been placed in the lid of each Petri dish. The plates were incubated at 28°C for 3 to 5 days. A change in colour from yellow to orange-brown on the filter paper indicated cyanide production.

**Production of bacterial and fungal inoculum for greenhouse experiments.** Bacterial inoculum was prepared in 250-ml flasks containing YEM. After inoculation with bacteria, the flasks were incubated on a rotator shaker at 120 rpm and 28°C for 72 h. Before use, bacterial concentration was adjusted to  $10^8$  cells ml<sup>-1</sup> (OD<sub>620</sub> 0.8-0.9). Inoculum of Foc race 0 was obtained by transferring agar discs 4-mm in diameter cut from the edge of 7-day-old colonies growing on PDA, to 250-ml flasks (one disk/flask) containing 100 g of 9:1 sand maize medium (Haware and Nene, 1982). After incubation for two weeks at 25°C, the flasks were used to inoculate a mixture of soil, sand and peat (1/1/1), sterilized by autoclaving twice at 110°C for 1.5 h in batches of 20 kg. The soil and contents of flasks (20:1 w/w) were mixed and the inoculated soil distributed to 15-cm plastic pots.

**Effect of *Rhizobium* isolates on *Fusarium* wilt.** Chickpea seeds of the two cultivars ILC482 and IN-RAT87/1 were surface-sterilized by immersion in 2.5% sodium hypochlorite for 2-3 min, and washed thoroughly in 3 changes of sterile distilled water. The seeds were pre-germinated for three days in Petri dishes containing sterile distilled water and the seedlings were transplanted into plastic pots (200 cm<sup>3</sup>, 4 seedlings per pot, and 4 pots for each treatment) containing *Fusarium*-inoculated soil mixture (positive control). Negative controls

were grown in the uninoculated soil mixture. The pre-germinated seeds were inoculated with rhizobia by using a liquid suspension, at the rate of 2 ml per seedling at sowing time. The work was done in a greenhouse at 25±5°C and 60 to 90% relative humidity for a period of 12 weeks. Plants were watered as needed and fertilized weekly with 100 ml of Hoagland's nutrient solution. The experiment was done in duplicate.

At flowering (8 weeks), samples were harvested to assess the effect of the rhizobia on growth parameters. Shoots, roots and nodules of each plant were separated and dried at 70°C. The number of nodules as well as the dry weights of nodules, shoots and roots of each plant were recorded. Samples of dry shoots were then ground to a fine powder and subjected to Kjeldahl determination of nitrogen content. All values given are averages of three samples for each treatment.

**Disease assessment and data analysis.** Disease incidence was assessed at 12 weeks by counting the number of wilted plants. Foc was re-isolated from wilted plants by plating stem pieces from the crown region onto PDA. All experiments were replicated as completely randomised blocks and done in two replicates. Data were analysed by ANOVA followed by Duncan's multiple range test using Sigma stat statistical software (SPSS, Version 10).

**Field experiments.** Field experiments were done in a field in the Experimental Station of INRAT, in the Oued Beja aerea (north-west Tunisia) that was heavily and homogeneously infected with Foc race 0. The weather at this station is sub-humid, with average annual rainfall of 560 mm and temperature of 19°C.

The same two cultivars and 14 *Rhizobium* isolates tested in the greenhouse were studied under field conditions. Chickpea seeds were thoroughly soaked in the bacterial suspension ( $10^8$  cells ml<sup>-1</sup>) to ensure uniform coating of the surface. The field was subdivided into plots 2×2 m in size in each of which sixty seeds were sown. Each treatment had three replicates in randomised blocks. Non-bacterized seeds served as controls. Sowing was made during the first week of March 2003. Samples were harvested after 8 weeks to determine growth parameters. The total number of wilted plants was recorded at 12 weeks. Statistical analyses were performed as described for greenhouse experiments.

## RESULTS

***In vitro* experiments.** As shown in Table 2, among the 21 *Rhizobium* isolates tested in dual culture, 19 inhibited the growth of Foc race 0. Fourteen gave more than 30% inhibition and belonged to growth inhibition categories 2 and 3. Isolates Rh8, Rh16, PchSOM and

**Table 2.** Effect of *Rhizobium* isolates on *in vitro* growth of *Fusarium oxysporum* f.sp. *ciceris* race 0 and determination of the ability of the most effective bacterial isolates in dual culture to solubilise phosphate and to produce cyanide and volatiles.

<i>Rhizobium</i> isolates	% growth inhibition <sup>(+)</sup>	GI category <sup>(+)</sup>	% inhibition of fungal growth by volatiles	Phosphate solubilization	Cyanide production
Rh1	0	0	ND	ND	ND
Rh7	0	0	ND	ND	ND
Rh2	3.08	1	ND	ND	ND
Rh6	5.14*	1	ND	ND	ND
Rh10	20.39*	1	ND	ND	ND
Rh14	20.52*	1	ND	ND	ND
Pch35T	21.82*	1	ND	ND	ND
Rh9	32.42*	2	1.02	+	-
Rh3	32.42*	2	2.55	-	+
Rh13	32.98*	2	1.02	-	-
Rh17	33.33*	2	9.18*	-	-
Rh12	36.07*	2	0	-	-
Rh4	36.2*	2	4.59	-	+
Rh5	39.72*	2	7.65*	-	-
PchDMS	41.55*	2	0	-	+
Pch121	42.46*	2	8.16*	-	+
Pch43	47.03*	2	8.67*	+	-
Rh8	51.78*	3	10.71*	+	+
Rh16	56.16*	3	9.69*	-	-
PchSOM	59.81*	3	9.18*	-	+
Rh11	60.73*	3	8.16*	-	-

(+) = Percent growth inhibition compared to uninoculated control was determined after 7 days of incubation using Whipps' (1987) formula. Values assigned on a scale from 0 to 3 = were: 0 = no growth inhibition; 1 = 1 to 25%; 2 = 26 to 50%; 3 = 51 to 75%. Values followed by \* were significant ( $P=0.05$ ) by Duncan's multiple range test. ND = not determined.

Rh11 were the most effective *in vitro* and caused more than 50% growth inhibition.

As to the effect of *Rhizobium* volatiles on pathogen growth (Tab. 2), among the 14 isolates tested for volatiles activity, 12 were able to reduce the growth of the pathogen. The isolates Rh17, PchSOM, Rh16 and Rh8 seemed to be the most effective, giving more than 9% inhibition after 96 h of incubation.

Most *Rhizobium* isolates were unable to solubilise phosphate. Only Pch43, Rh9 and Rh8 produced a halo on dicalcium phosphate agar plates (Table 2). Six isolates, PchDMS, Rh4, PchSOM, Rh3, Pch121 and Rh8 were positive for cyanide production and Rh8, positive for phosphate solubilisation and cyanide production, gave the highest percentage of inhibition when tested for volatiles. It was classified among the most effective isolates in dual culture tests.

**Greenhouse experiments.** These tests showed that both the susceptible and moderately resistant cultivars reacted to *Foc* with a high incidence of wilt (Table 3). Nevertheless, 12 weeks after sowing, there was more disease (79% of plants wilted) in the susceptible cultivar ILC482 than in the moderately resistant cultivar INRAT87/1 (54%). Inoculation of pre-germinated seeds with isolates PchDMS, Pch121, Rh5, Rh17 and Pch43 significantly reduced the percentage of wilted plants in both the susceptible and the moderately re-

sistant cultivars.

This percentage ranged from 12.5 to 54.6% in ILC482 and from 8.3 to 29.2% in INRAT87/1. The best disease control was obtained with isolate PchDMS, which reduced wilt incidence to 12.5% and 8.5% respectively, in the two cultivars. *Rhizobium* isolates performed better in moderately resistant cultivar, where 9 isolates significantly reduced the percentage of wilt. Table 4 shows that at flowering, plants, whether or not inoculated with *Fusarium* (but in any case not deliberately inoculated with *Rhizobium*) showed no nodulation, in either cultivar. Inoculation with the different *Rhizobium* isolates led to nodule formation in both cultivars.

There was nevertheless more nodulation in the moderately resistant cultivar than in the susceptible cultivar. The two *Rhizobium* isolates PchSOM and Rh13 significantly increased the number as well as the dry weight of nodules in both cultivars in presence of *Foc*. Isolates PchDMS, Rh11 and Rh17 only did so in the moderately resistant cultivar. Although effective in controlling disease development isolate Pch43 did not increase the number of nodules, but significantly increased nodule weights in both cultivars (Table 4).

**Field trials.** There were heavy attacks on the control plants by the end of the experiment, with more than 48% and 66% of wilted plants, respectively, in the moderately resistant and the susceptible cultivar (Table

**Table 3.** Effect of *Rhizobium* isolates on wilt incidence in chickpea cultivars ILC482 (susceptible) and INRAT87/1 (moderately resistant) inoculated with *Fusarium oxysporum* f.sp. *ciceris* under greenhouse and field conditions at 12 weeks after sowing.

Treatment	Disease incidence (% of wilted plants)			
	Greenhouse		Field	
	ILC482	INRAT87/1	ILC482	INRAT87/1
Control	0	0	ND	ND
Foc (positive control)	79.2	54.2	66.2	48.7
PchDMS	12.5*	8.3*	33.9	41.6
PchSOM	70.8	20.8*	49.4	16.6*
Rh11	75.0	12.5*	54.0	29.7
Pch121	54.2*	29.1*	44.3	19.5*
Rh5	54.2*	20.8*	53.3	13.9*
Rh12	79.2	45.8	55.7	58.5
Rh8	83.3	54.1	57.8	20.2*
Rh4	95.8	45.8	49.4	7.7*
Rh9	87.5	50.0	39.1	16.6*
Rh16	70.8	54.2	46.3	11.1*
Rh13	70.8	12.5*	45.0	56.6
Rh17	54.6*	20.8*	50.9	49.8
Rh3	95.8	37.5*	45.9	43.9
Pch43	33.3*	12.5*	44.2	7.9*

Each value is a mean of 3 replicates. Values followed by \* were significant ( $P=0.05$ ), compared to the positive control, by Duncan's multiple range test. ND= not determined.

3). Inoculation of seeds with *Rhizobium* isolates generally reduced the percentage of wilted plants. However, this decrease was not significant in the susceptible cultivar. Interestingly, 8 of the *Rhizobium* isolates very effectively controlled the disease in the moderately resistant cultivar, reducing wilt incidence by more than 60%. The best disease control was achieved by isolates Rh4, Rh16 and Pch43, which reduced wilt by more than 77% compared to the control (Table 3).

Table 5 shows that by the end of the experiment, *Fusarium*-infected control plants were all nodulated. Inoculation with the different *Rhizobium* isolates generally did not increase the number or dry weight of nodules in either cultivar since rhizobia were effectively acquired from the field soil. As in the greenhouse experiments, field experiments showed that the number and the weight of nodules were generally greater in the moderately resistant cultivar as compared to than in the susceptible cultivar. With the other growth parameters results varied according to the bacterial isolate and the chickpea cultivar used (Table 5).

Nevertheless, the two isolates PchSOM and Pch43 seemed to be the most effective rhizobia, conferring significant increases in root and shoot weights as well as nitrogen content in both moderately and fully susceptible cultivars.

## DISCUSSION

Reduction of fungal growth *in vitro* by certain of the rhizobia and formation of inhibition zones were pre-

sumably due to the metabolites released by the bacteria into the culture medium. These metabolites may include antibiotics and/or cell-wall degrading enzymes. Different studies have implicated antifungal secondary metabolites produced by *Rhizobium* spp. in the control of plant diseases caused by pathogenic fungi (Ehteshamul-Haque and Ghaffar, 1993; Perdomo *et al.*, 1995; Siddiqui *et al.*, 2000).

We tested rhizobia for volatiles, cyanide production, and phosphate solubilisation because previous studies suggested that bacteria possessing these traits can increase plant growth (Sperber 1958; Bakker and Schippers, 1987; Glick *et al.*, 1995). Indeed, some P-solubilising organisms have been reported as plant growth promoters, but rigorous proof is lacking (De Freitas *et al.*, 1997; Whitelaw *et al.*, 1997). In the case of cyanide production, inoculation of wheat by recombinant cyanide-producing strains of *Pseudomonas putida* resulted in suppression of *Septoria tritici* blotch and leaf rust (Flaishman *et al.*, 1996).

Volatiles may also contribute to inhibition of fungal pathogen growth. Indeed, several studies have shown the importance of volatiles in the biocontrol of different plant diseases (Gagné *et al.*, 1991). Our experiments showed that three isolates (Pch43, Rh9 and Rh8) were phosphate solubilisers, six produced cyanide (PchDMS, Rh3, PchSOM, Rh4, Pch121, and Rh8) and eight (Rh5, Pch121, Pch43, Rh17, PchSOM, Rh16P, Rh8 and Rh11) significantly inhibited pathogen growth by producing volatiles.

Greenhouse experiments showed that inoculation of chickpeas with Foc reduced plant growth and caused

**Table 4.** Effect of *Rhizobium* isolates on growth parameters and shoot nitrogen content (% N) in the two chickpea cultivars ILC482 and INRAT87/1 inoculated with *Fusarium oxysporum f.sp. ciceris* under greenhouse conditions 8 weeks after sowing.

Treatment	Nodule number		Nodule dry weight (10 <sup>-2</sup> g)		Root dry weight (g)		Shoot dry weight (g)		% N	
	ILC	INRAT	ILC	INRAT	ILC	INRAT	ILC	INRAT	ILC	INRAT
Control	0	0	0	0	0.144	0.385*	0.833	1.727	4.2*	4.6*
Foc	0	0	0	0	0.085	0.131	0.815	1.131	3.2	3.2
PchDMS	6.7	12.3*	4.800*	9.870*	0.251*	0.301*	1.757*	1.430	4.5*	5.0*
PchSOM	13.3*	12.7*	3.870*	7.100*	0.193	0.271	0.981	1.420	4.3*	5.3*
Rh11	1.0	13.3*	0.250	15.000*	0.157	0.290	0.810	1.415	2.9	3.3
Pch121	2.7	3.3	0.533	2.330	0.143	0.263	0.938	1.388	2.3	3.2
Rh5	3.0	1.0	2.230*	0.267	0.195	0.184	1.708*	1.101	3.0	3.5
Rh12	1.7	4.3	0.467	6.030*	0.166	0.264	1.544*	1.298	4.2*	6.3*
Rh8	1.0	1.0	0.233	0.330	0.106	0.209	0.962	1.317	3.1	4.3*
Rh4	1.0	1.0	0.233	0.267	0.089	0.135	1.239	1.140	4.5*	5.0*
Rh9	1.0	2.7	0.300	0.333	0.175	0.230	1.184	1.573	4.8*	5.1*
Rh16	0.3	1.3	0.267	0.400	0.098	0.221	0.864	1.205	5.8*	6.2*
Rh13	9.0*	5.0*	3.370*	6.830*	0.146	0.245	0.804	1.245	4.0	6.5*
Rh17	4.0	12.7*	6.200*	7.530*	0.127	0.305*	0.663	1.137	4.6*	6.8*
Rh3	3.3	2.3	0.330	2.170	0.156	0.276	0.943	1.698	4.0	5.6*
Pch43	2.7	2.7	2.230*	6.800*	0.259*	0.308*	2.257*	1.971*	4.3*	5.0*

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

severe wilting in the susceptible as well as in the moderately resistant cultivar. Interestingly, application of *Rhizobium* isolates significantly reduced the wilting index and increased plant growth. These rhizobia also increased nitrogen content and dry weight of nodules, roots and shoots. Several other workers have noticed the beneficial effects of rhizobia on plant growth and reduction of diseases incidence (Smiley *et al.*, 1986; Hussain and Ghaffar, 1990). Siddiqui and Singh (2004) reported better plant growth, higher transpiration, better root nodulation, and lower wilting index in chickpea plants infected with Foc and inoculated with rhizobia.

Our experiments revealed the effectiveness of the bacterial isolates PchDMS, Pch121, Rh5, Rh17 and Pch43. These significantly reduced the percentage of wilted plants in both the susceptible and moderately resistant cultivars. Based on *in vitro* dual culture experiments, all these isolates were classified in category GI2. Despite their high effectiveness *in vitro* (GI3), isolates Rh8, Rh16, PchSOM and Rh11 were either completely (on both cultivars) or partially (on the susceptible cultivar) ineffective under greenhouse conditions. These results are in concordance with the idea that antagonistic microorganisms performing best *in vitro* are not necessarily the most effective *in vivo* in presence of the host plant, and vice versa (Chérif *et al.*, 2002). This is particularly exemplified by *Rhizobium* isolate Rh8, which caused more than 50% fungal growth inhibition in dual culture, produced the highest levels of volatiles and was the only isolate positive for both cyanide production and phosphate solubilisation, but showed no effective-

ness *in vivo* under greenhouse conditions. Similar results were obtained by Sadfi *et al.* (2001), who showed that two *Bacillus* isolates effectively inhibited the growth of *Fusarium roseum* var. *sambucinum* *in vitro* but failed to reduce dry rot development *in vivo* on potato tubers.

By contrast, the isolates PchDMS and Pch43 performed better *in vivo* than *in vitro*, resulting in giving the best levels of disease control particularly under greenhouse conditions. These isolates were also effective in promoting chickpea growth, leading to generally better shoot and root dry weights compared to the control. These benefits may be attributed to better disease control in presence of the bacteria and/or to better nutrition, due especially to higher nodulation and phosphorus uptake (Alagawadi and Gaur, 1988). These two isolates did not in general significantly improve chickpea nodulation, indicating that the effectiveness of these bacteria in promoting plant growth could not be based only on their nitrogen fixation potential. Different studies have indicated that phosphate solubilisation may play a key role in plant growth promotion (Chabot *et al.*, 1996). For instance, the results of Halder *et al.* (1990) showed that strains of *Rhizobium leguminosarum* biovar *viceae* and *Mesorhizobium sp.* nodulating chickpea were the most effective solubilisers of phosphate. Nevertheless our studies showed that while isolate Pch43 is a phosphate solubiliser, isolate PchDMS was not. This suggests that protection against fungal attack may be highly significant in improving the growth observed in chickpeas treated with effective rhizobia. This is also supported by the fact that in the greenhouse iso-

**Table 5.** Effect of *Rhizobium* isolates on growth parameters and shoot nitrogen content (% N) in the two chickpea cultivars ILC482 and INRAT87/1infected with *Fusarium oxysporum* f.sp. *ciceris* under field conditions 8 weeks after sowing.

Treatment	Nodule number		Nodule dry weight (10 <sup>2</sup> g)		Root dry weight (g)		Shoot dry weight (g)		% N	
	ILC	INRAT	ILC	INRAT	ILC	INRAT	ILC	INRAT	ILC	INRAT
Foc	17.4	28.4	3.43	4.02	0.117	0.150	0.624	0.671	11.7	17.3
PchDMS	24.4	20.4	5.15	2.01	0.157*	0.157	1.177*	0.836*	20.1*	19.6
PchSOM	15.8	36.3	3.05	7.26*	0.145	0.203*	1.131*	1.270*	26.6*	24.5*
Rh11	13.8	27.4	1.84	4.73	0.118	0.171	0.837	0.951	13.8	10.3
Pch121	21.1	24.3	4.67	4.77	0.132	0.197	1.261*	0.944	18.7*	19.9
Rh5	14.1	18.1	2.85	1.43	0.128	0.191	1.184*	0.792	21.9*	23.8*
Rh12	21.6	30.9	7.50	6.70*	0.139	0.201*	0.994	1.160*	14.5	11.8
Rh8	19.9	36.3	4.50	4.00	0.141	0.181	1.014	0.884	12.6	24.0*
Rh4	18.4	25.3	3.20	6.20*	0.120	0.175	0.886	1.032*	22.4*	26.5*
Rh9	20.0	28.7	2.78	3.92	0.123	0.185	1.024	0.971*	20.5*	16.7
Rh16	14.6	17.4	2.43	2.11	0.156*	0.165	0.718	0.686	11.9	17.0
Rh13	18.8	15.0	1.96	2.31	0.116	0.184	0.646	0.839	14.9	17.7
Rh17	20.0	16.3	3.68	4.23	0.119	0.181	0.925	0.838	13.3	13.9
Rh3	21.9	26.1	2.60	4.07	0.125	0.175	0.838	1.049*	15.4	17.0
Pch43	19.7	19.0	5.04	6.03*	0.158*	0.202*	1.083*	1.112*	18.4*	24.6*

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

lates PchSOM and Pch13 significantly increased nodulation in both cultivars, but did not decrease disease incidence or improve plant growth.

Although the basic mechanisms behind such protection are not clearly defined, the possibility that competition, antibiosis, direct parasitism and induced resistance by the antagonistic bacteria, may operate synergistically after inoculation with effective *Rhizobium* isolates cannot be ruled out. In the present study we showed that: i) despite the presence of the pathogen at high concentrations in greenhouse and field experiments, the bacteria were able to colonize plant roots and to form nodules; ii) most *Rhizobium* isolates were able to form inhibition zones *in vitro*, suggesting the production of antibiotics, and some isolates also produced volatiles and cyanide; iii) different isolates performed poorly or moderately *in vitro*, such as PchDMS, Rh5 and Pch43, but effectively protected the plant *in vivo*; iv) whether under greenhouse or field conditions, *Rhizobium* isolates gave better results in the moderately resistant cultivar than in the susceptible cultivar, indicating that induction of plant defence mechanisms could not be excluded.

Van Peer *et al.* (1991) showed that strain CS417 of *Pseudomonas* sp. suppressed *Fusarium* wilt in the moderately resistant carnation cv Pallas more efficiently than in the susceptible cv Lena. Similar results were obtained by our group when *Bacillus* isolates were tested against *Ascochyta* blight and *Fusarium* wilt of chickpea (unpublished data). A growing body of evidence from different studies underlines the potential of bacterial antagonists in promoting plant disease suppression by inducing plant defence mechanisms (Kloepper *et al.*, 1992; Onge-

na *et al.*, 1999; Chérif *et al.*, 2003). We also recently showed that our isolates PchDMS and Pch43 can induce peroxidase and polyphenoloxidase activities as well as the accumulation of phenolic compounds in chickpea roots pre-treated with the bacteria and challenged with Foc (Arfaoui *et al.*, 2005).

Our field studies showed that in presence of the moderately resistant cultivar, 8 *Rhizobium* isolates could significantly control disease development despite heavy colonisation by the pathogen of the sick plot used. The results could well be better under natural conditions of low pathogen pressure in the field, and our *Rhizobium* isolates might be effective even in susceptible cultivars.

Our results therefore suggest that the selected rhizobia could be used effectively as biocontrol agents of chickpea wilt. They could be fielded within an integrated disease management package also including moderately resistant cultivars, limited fungicide application and effective cultural practices.

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