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Evaluation of biocontrol potential of *Pseudomonas* and *Bacillus* spp. against Fusarium wilt of chickpea

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Abstract

In this study, antagonistic effects of 6 isolates of *Pseudomonas* and 6 isolates of *Bacillus* genera isolated from rhizosphere of chickpea were evaluated against *Fusarium oxysporum* f. sp. *ciceris* as potential biocontrol agents *in vitro* and *in vivo*. Fungal inhibition tests were performed using plate assay. Each isolate were tested for the production of protease, siderophore, cyanide hydrogen, indole acetic acid, antifungal volatile and extracellular compound. Twelve isolates were selected according to their high antagonistic efficiency in *in vitro* which was shown as inhibition zones in the dual-culture assay. According to phenotypic properties, selected isolates were identified as *Bacillus subtilis* (B1, B6, B28, B40, B99, and B108), *Pseudomonas putida* (P9 and P10) and *P. aeuroginosa* (P11, P12, P66 and P112). The ability of bacterial isolates was varied in production of cyanide hydrogen, siderophore, protease and indole acetic acid (IAA). Biocontrol activity and plant growth promotion of bacterial strains were evaluated under greenhouse conditions, in which *P. aeuroginosa* (P10 and P12), *B. subtilis* (B1, B6, B28 and B99) and *P. aeuroginosa* (P12 and B28) provided better control ($P \leq 0.05$) than untreated control (15.8-44.8%) in seed treatment and soil-inoculation, respectively. The growth parameters (plant height, fresh and dry weight of plants) were significantly increased by B28, P12 and P112 isolates in both tests compared to the untreated control. Our results indicate that PGPR improve growth parameters in this plant and can help in the biocontrol of pathogen.

Keywords: Antagonistic bacteria, Biological control, Chickpea, Fusarium.
Abbreviations: d_day; h_hour; NA_Nutrient agar; KBA_King's medium B Agar; PDA_Potato dextrose agar; RH_Relative humidity; W_week; SKM_Skimmed milk agar; CRD_ completely randomized design; DMRT_ Duncan multiple-ranges test.

Introduction

Chickpea (*Cicer arieatinum* L.) is one of the most important crops growing in the Kurdistan province of Iran, but the yield and quality of chickpea are influenced by Fusarium wilt disease caused by the *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Sato & Matuo (Dueby et al., 2007). Fusarium wilt is one of the most important and destructive vascular disease of chickpea (Dileep kumar, 1999). Yield losses of chickpea due to fusarium wilt are estimated at 10% in India and Spain, 40% in Tunisia and 17% in Iran (Bouslama, 1980; Jamali et al., 2004). There are eight races of *F. oxysporum* f. sp. *ciceris* (0, 1A, 1B/C, 2, 3, 4, 5 and 6) which are identified by reaction on a set of differential chickpea cultivars (Jimenez-Gasco and Jimenez-Diaz, 2003; Haware and Nene, 1982). The most efficient method for the management of disease is using resistant cultivars (Jimenez-Gasco and Jimenez-Diaz, 2003; Navas-Cortés et al., 1998), although new races of the pathogen appear to overcome resistant genes. In addition, chemical control is not satisfactory, therefore biological control is an alternative to chemical control of the disease (Anjajah et al., 2003; Landa et al., 2004). Use of biological control agents, such as plant growth promoting rhizobacteria (PGPR), can be a suitable approach in control of disease (Schmidt et al., 2004). Plant growth promoting rhizobacteria (PGPR), such as *Pseudomo*nas and *Bacillus* strains, are the major root colonizers (Manikanda et al., 2010; Joseph et al., 2007), and can elicit plant defenses (Kloepper et al., 2004). Different mechanisms have been reported for their performance such as production of antibiotics, siderophore cyanide hydrogen, competition for nutrition and space, inducing resistance, inactivation of pathogen's enzymes and enhancement of root and plant development (Intana et al., 2008; Weller, 1988). *Pseudomonas* and *Bacillus* strains have great potential in control of fusarium wilt disease of chickpea (Anjajah et al., 2003; Hervas et al., 1997; Landa et al., 1997). The objective of this research was to evaluate antagonistic effects of some selected rhizobacteria on *Fusarium oxysporum* f.sp. *ciceris* under greenhouse conditions.

Results

Characterization of pathogen and Pathogenicity test

Eleven isolates of pathogen were obtained from infected chickpea plants. Among them six isolates were found to be significantly more pathogenic on chickpea c.v Kaka. These

were selected for *in vitro* and greenhouse studies (data not shown).

Preliminary screening and characterization of biocontrol agents

Two hundred and thirty two bacteria were isolated from the rhizosphere and root of chickpea showing substantial inhibition zones against *Fusarium oxysporum* f. sp *ciceris* in *in vitro*. Twelve out of 232 bacterial strains showing high antifungal activity against pathogen were selected and used in the subsequent *in vitro* inhibition tests and greenhouse experiments (Fig 1 and Fig 2). Based on biochemical, physiological and morphological tests, selected isolates were identified as *B. subtilis* (B1, B6, B28, B40, B99, and B108), *P. putida* (P9 and P10) and *P. aeuroginosa* (P11, P12, P66 and P112) (Table 1 and 2).

In vitro antifungal activity

In dual culture test, *B. subtilis* strains were shown to have more growth inhibition of pathogen on the PDA medium than *Pseudomona*s isolates. *B. subtilis* B28 isolate showed the highest inhibition percentages (51.16%) than other isolates (Fig 2). Furthermore, the results indicated that *B. subtilis* and *Pseudomonas* spp isolates produced more extracellular metabolites and volatile compound, respectively (Table 3). *B. subtilis* B28 isolate produced 78.29% extracellular metabolites and *P. aeuroginosa* P12 produced 26.35% volatile compounds. These isolates more than other isolates had, higher inhibition against hyphal pathogen (Table 3).

Production of antifungal metabolites

Results of *in vitro* test using 12 bacterial strains against pathogen showed that different strains exhibited different combinations of antimicrobial metabolites such as protease, HCN, siderophore, IAA and cellulase. *Pseudomonas* isolates produced cyanide hydrogen. *P. aeuroginosa* P66 isolate indicated the most activity in production of cyanide hydrogen. The production of protease by *Bacillus* isolates was more evident than *Pseudomonas* isolates. Among them only *P. aeuroginosa* P11 and *P. aeuroginosa* P12 isolates demonstrated negative response. The data is summarized in Table 3. Production of indole acetic acid (IAA) by *Pseudomonas* isolates had more evident than *Bacillus* isolates in the presence or absence of trypthophan. *P. aeuroginosa* P112 showed higher IAA production (Fig 3).

Effects of bacterial antagonist on disease severity in greenhouse conditions

The first symptom of disease was appeared 21 days after inoculation. The effects of bacterial antagonist on the disease severity in both soil and seed treatments are shown in Table 4. *B. subtilis* B28 and *P. aueroginisa* P12 isolates showed more effects (44.18% and 41.86%) respectively, than other isolates (Table 4). These isolates have shown more effects in seed treatment (39.47 and 34.21%) (Table 4). The effects of all treatments on shoot length, root length, dry weight of shoots and roots of chickpea plants were determined 50 days after inoculation by the pathogen. All bacterial isolates had significant effects on plant growth compared with the non-treated control in both methods. *B. subtilis* B28 and *P. aueroginisa* P12 isolates significantly increased length of shoot and root of chickpea plants in soil and seed treatments. Moreover, these isolates increased dry weight of shoots and roots compared with the non-treated control in both methods (Table 5).

Discussion

Fusarium oxysporum f. sp. ciceris is economically significant disease on chickpea. Due to the soil-borne nature of the disease use of chemical methods for the control of disease is rarely successful. Inconsistencies in biocontrol under varying environmental conditions have been a common limitation of soil borne pathogens. The present research was conducted to evaluate the efficacy of *Pseudomonas* spp. and *B. subtilis* strains against the pathogen. The use of rhizobacteria for plant disease control is more effective when rhizobacteria is isolated from rhizosphere of the same host plant (Weller, 1988). In this study, all bacterial isolates were selected from rhizosphere of healthy chickpeas in fields. Some bacterial isolates showed high inhibition activity on pathogen, whereas others showed only mild or no activity at all. About 5% (12 of 232) of isolates showed antagonistic activity against *Fusarium oxysporum* f. sp. *ciceri*s in *in vitro*. Most isolates tested exhibited no or weak antagonistic activity against the pathogen on PDA plates assay. Similar results have previously been reported (Eedogan and Benlioglu, 2010; Zheng et al., 2011; Khot et al., 1996; Eleftherio et al., 2004). Results indicated that various individual PGPR strains applied as soil drench or seed treatment in suppressing of pathogen (Shouan Zhang et al., 2010; Das et al., 2008).
In vitro results illustrated that all *B. subtilis* isolates (except B108) produced more extracellular metabolites than *P*seudomonads isolates and inhibited pathogen growth, whereas ability to produce volatile compounds was low by these isolates in comparison with *Pseudomonads* isolates (Table 3). These results are in agreement with the research previously carried out by Romanenko et al (2000) and Kamal et al (2009). Results of volatile compounds and cyanide hydrogen production were similar in both tests Pseudomonas isolates had better performance than *Bacillus* isolates (Table 3). This result demonstrated that cyanide hydrogen was one of the most important volatile compounds of *Pseudomonas* spp. Cyanide hydrogen is upsetter of perspiration and chelating agent of metals and has been reported as effective in control of cucumber wilt caused by *Pythium ultimum* (Keel et al., 1996). Protease which has interference in wall degrading of fungal pathogen (Ahmadzadeh and Sharifi-Tehrani, 2009) is produced by all isolates, especially *Bacillus* spp. (Table 3). Production of siderophore by *Pseudomonas* isolates were not shown in 1000 µm $FeCl_3$ concentration, which demonstrated competition between *Geotrichum candidatum* and Pseudomonas isolates for obtaining iron. Maximum production of siderophore proved in 25 µl $FeCL_3$ concentration. The ability of antagonistic bacteria to produce siderophore was widely distributed between pseudomonas isolates. Gupta et al (2008) reported that the maximum production of siderophore occurred in 10 µl $FeCL_3$ concentration. Previous reports indicated the induction of siderophore by *P. aeroginosa* on KB medium (Baudoin et al., 1998; Kloepper et al., 1980b). The production of indole acetic acid was shown by all isolates in the presence or absence of tryptophan in Pseudomonas isolates. *P. aeuroginosa* P112 isolate had the most activity in IAA production (Figure 3). Xie et al (1996) indicated that compared to other strains, *Pseudomonas* strains had higher levels of IAA production. Our results have shown that by adding tryptophan, production of indole acetic acid increased and this was similar to the results obtained by Ali et al (2009). It is possible to suggest that tryptophan acted as a

Table 1. Biochemical, physiological and morphological properties of Pseudomonas isolates.

	Pseudomonas isolates					
Tests	P9	P10	P11	P12	P66	P112
Gram reaction	-	-	-	-	-	-
Aerobic growth	+	+	+	+	+	+
Anaerobic growth	-	-	-	-	-	-
Fluorescent pigment	+	+	+	+	+	+
Growth at 41C°	+	-	-	+	+	+
Growth at 4 C°	-	+	+	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+
Starch hydrolysis	+	-	+	-	-	-
Levan production	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Arabinose	-	+	+	-	-	-
Sorbitol	-	V	+	-	-	-
Galactose	-	+	+	-	-	-
Inositol	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-

Positive reaction: +; Negative reaction: - ; Variation reaction: V

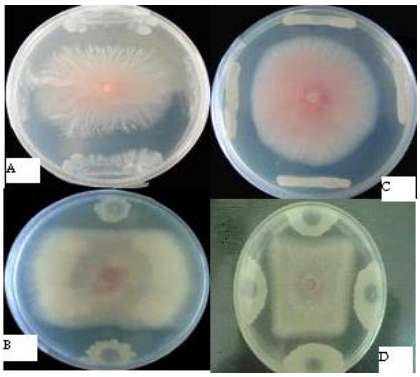

Fig 1. The effect of bacterial isolates on *Fusarium oxysporum* f. sp. *ciceris* on PDA media by dual -culture assay (A and B, *Pseudomonas*; C and D, *Bacillus*).

precursor and therefore led to presence of this material in rhizosphere by plant exudates (Kamilova et al., 2006). Authoritative rhizobacteria can convert tryptophan to indole acetic acid resulting in increasing plant growth. According to results presented at this study suppression of disease was better in soil treatment as shown in greenhouse experiments. Similar results reported by Jamli et al (2004). This may be related to the rate and extent of colonization of roots by isolates in soil treatment, since soil treatment pots were completely saturated by bacterial isolates. In both methods, *P. aueroginisa* P12 and *B. subtilis* B28 isolates were the most effective in reduction of disease under greenhouse conditions (Table 4). In our research, unlike disease incidence, disease severity decreased significantly by rhizobacteria. It seems that performance of rhizobacteria was effective in disease development. This delay may be due to either preliminary induced systemic resistance (Vanloon et al., 1998) or preliminary colonization which suppress pathogen onset directly via different mechanisms such as production of antibiotics and volatile compound competition for space or nutrition (Mafia et al., 2009). A significant correlation was found between pathogenesity test (*in vitro*) and reduction disease severity (*in vivo*) particularly in the dual culture test (Table 6). Therefore, it is concluded that a complex mechanisms were involved in biocontrol process. Growth parameters such as shoot length, root length, dry shoot weight and dry root weight were significantly higher in both seed and soil treatment methods compared to untreated control. *P. aueroginisa* isolates had the most effects on all growth factors in soil and seed treatments (Table 5). Previous work showed that many microorganisms such as bacteria can had positive influence on plant growth and plant health as PGPR (Zakira Naureen, 2009; Erdogan and Benlioglu, 2010; Kloepper et al., 1980a; Dileep Kumar, 1999). The role of *Pseudomonas* spp. in promoting root and shoot growth of different crops has been demonstrated (Kamal et al., 2008). The use of rhizobacteria against fusarium wilt of chickpea plant caused by the *Fusarium oxysporum* f. sp *ciceris* not only suppressed the disease incidence and decreased the number of seedlings with wilting symptoms, but also increased growth parameters of the plant.

Materials and methods

Microorganism and culture conditions

Fusarium oxysporum f. sp. *ciceris* were isolated from infected chickpea plants growing in Kurdistan province. The infected chickpea stems were cut into small pieces of 1-1.5 cm, surfaces were sterilized with 1% sodium hypochlorite for 1.5 min, washed in sterile distilled water twice and cultured on PDA medium. Bacterial antagonists were isolated from

Table 2. Biochemical, physiological and morphological properties of Bacillus isolates.

Tests	Bacillus isolates					
	B1	B6	B28	B40	B99	B108
Gram reaction	+	+	+	+	+	+
Aerobic growth	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Spore position	C*	C	C	C	C	C
Growth at 45 ^{C}	+	+	+	+	+	+
Oxidase	+	+	+	+	-	-
Catalase	+	+	+	+	+	+
Utilization of citrate	+	+	-	+	+	+
Starch hydrolysis	+	+	+	+	+	+
Growth at 7% NaCl	+	V	+	+	+	V
Anaerobic growth in glucose broth	-	-	-	-	-	-
Growth in pH 5.7	+	+	+	+	+	+
Arabinose	+	+	v	+	+	+
Mannitol	+	+	+	+	+	+
Xylose	+	+	+	+	+	+

Positive reaction: + Negative reaction: - Variation reaction: V

*C, Central (Position of bacterial spore)

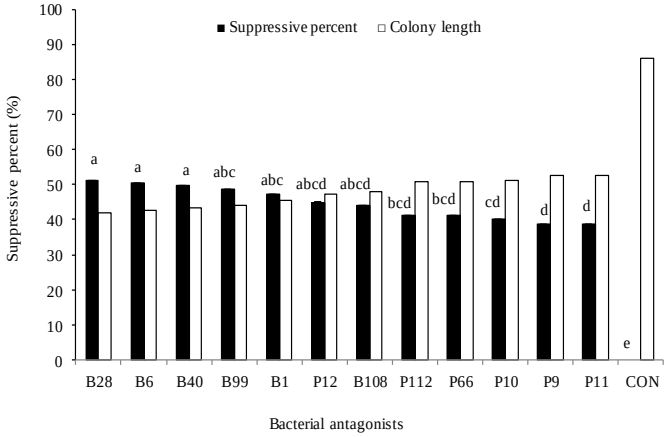

Fig 2. The effect of bacterial antagonists on the hyphal growth of *Fusarium oxysporum* f. sp. *ciceris* in dual culture test. Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to DMRT

rhizosphere of chickpea plants collected from various locations in Kurdistan province (Sanandaj, Marivan, Kamyaran and Dehgolan) from July to September 2008. Approximately one g of root tissue was excised from each sample, surface sterilized using in NaClo (0.5% available chlorine) for 2 min, then rinsed in sterile distilled water. Soil samples were shaken in 9 ml sterile water for 3 min at 160 rpm. Serial dilution was made from 10^{-3} to 10^{-7}. Then 0.2 ml of each dilution was spread onto NA medium and Pseudomonas F Agar medium. Plates were incubated at 27-29^{o}C for 48 h. Bacterial cells were harvested and suspended in 10 mM $MgSO_4$ to final concentration of 10^8 cells/ml.

***Preliminary screen*ing**

Two hundred and thirty two bacterial isolates were tested for their ability to produce antifungal substances against *Fusarium oxysporum* f. sp. *ciceris* using a dual-culture *in vitro* assay on PDA plates. Five µl of each bacterial suspension (10^8 cfu/ml) was placed on the plate. After 48 h incubation at 28^{o}C for 48 h, a single 6 mm diameter mycelial disc was placed at the center of plates. Then, plates were incubated at 27-29 ^{o}C in darkness and after 7 days the growth diameter of the pathogen (distance between the point of placement of fungal disk and actively growing edges of the fungus) was measured. The percentage of growth inhibition was calculated according to Erdogan and Benlioglu (2010) method. This experiment was conducted twice. Bacteria with inhibitory potential were selected for further experiments.

Identification of bacterial antagonist

Initially, the selected isolates were identified based on gram positive, spore forming, fluorescent pigment production, aerobic or anaerobic growth. To identify *Pseudomonas* isolates, oxidase, catalase, arginine dihydrolase, starch hydrolysis, gelatin hydrolysis, levan production, nitrate reduction, arabinose, sorbitol, galactose, inositol, trehalose, growth at 41˚C, growth at 4˚C tests were further performed. To identify *Bacillus* isolates, motility, oxidase, catalase,

Table 3. *In vitro* activity of antagonists against *Fusarium oxysporum* f. sp. *ciceris* and production of antimicrobial metabolites.

Antagonisms	DC(%)	EM(%)	VC(%)	PT	CH	ST	IAA
P. putida P9	38.7^{d}	38.7^{d}	23.2^{ab}	+	+	+	+
P. putida P10	40.3^{cd}	37.1^{d}	22.5^{ab}	+	++	+	+
P. aueroginosa P11	38.7^{d}	37.1^{d}	22.5^{ab}	-	+	+	+
P. aueroginosa P12	44.9^{abcd}	43.4^{d}	26.3^{a}	-	+	+	+
P. aueroginosa P66	41.1^{bcd}	24.0^{e}	24.8^{ab}	+	++++	-	+
P. aueroginosa P112	41.1^{bcd}	19.4^{e}	7.0^{d}	+	+++	+	+
B. subtilis B1	47.3^{abc}	52.8^{c}	20.1^{bc}	+	-	-	+
B. subtilis B6	50.4^{a}	67.4^{b}	17.8^{c}	+	-	-	+
B. subtilis B28	51.2^{a}	78.3^{a}	22.5^{ab}	+	-	-	+
B. subtilis B40	49.7^{a}	69.8^{b}	6.2^{d}	+	-	-	+
B. subtilis B99	48.8^{ab}	75.9^{a}	5.4^{d}	+	-	-	+
B. subtilis B108	44.2^{abcd}	13.2^{f}	3.1^{e}	+	-	-	+
Non infected Control	0^{e}	0^{g}	0^{f}	-	-	-	-

Data are means of four replicates. Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to DMRT. DC = Percent growth inhibition in dual culture method, EM= Percent growth inhibition in extracellular metabolites method, VC= Percent growth inhibition in volatile compound method, PT= protease test, CH= cyanide hydrogen, ST= Siderophore test, IAA= indole acetic acid.

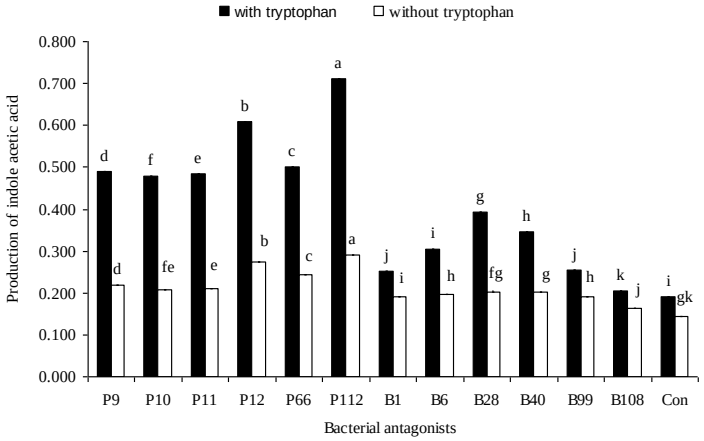

Fig 3. The amount of indole acetic acid production by *Pseudomonas* and *Bacillus* isolates with or without tryptophan. Data are means of three replicates. Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to DMRT

utilization of citrate, starch hydrolysis, growth at 7% NaCl, growth at 45˚C, anaerobic growth in glucose broth, growth in pH 5/7, arabinose, mannitol and xylose utilization were assessed (Shaad, 1988).

Protease production

Bacterial isolates were tested for production of protease by growing them on skim milk agar (SKM) (Chantawannakul et al., 2002). An ability to clear the skim milk suspension in the agar was taken as evidence for the secretion of protease. Non- bacteria inoculated plates were used as the control.

Hydrogen cyanide production

Production of Hydrogen cyanide was determined on nutrient agar medium. 100 µl of bacterial culture (48 h) were streaked on the surface of medium, then sterilized filter papers were soaked in 2.0% Na_2CO_3 in 5.0% (w/v) picric acid placed in the upper lid of the petri dish. The petri dishes were sealed with parafilm and incubated at 30°C for 4 days. A change in the color of the filter paper from yellow to reddish brown was accepted as an index for cyanogenic activity. Non - inoculated plates with bacteria was used as control (Alstrom, 1987).

Siderophore production

This experiment was done based on Weller and Cooks (1983) method. Firstly, KBA media were prepared and 0, 25, 50, 100, 1000, µM of $FeCl_3$ were added. 100 ml of bacterial isolates were placed on the surface of medium and the plates were incubated at 28˚C for 5 days. Then a suspension of *Geotrichum candidatum* in sterile water was prepared at a concentration of 1×10^{6} cfu/ml, sprayed on the surface of media and incubated at 28˚C for 72 h. Inhibition growth of *G. candidatum* around bacterial colonies was considered as an effect of siderophore production by bacterial isolates.

Indole acetic acid production (IAA)

The production of IAA was determined as described by Bric et al (1991). Bacterial strains were inoculated into nutrient broth (peptone, 5g; yeast extract, 1.5g; beef extract, 1.5g; and

Table 4. The effects of bacterial isolates on disease index of Fusarium wilt of chickpea in soil and seed treatment under greenhouse conditions

Antagonisms	soil treatment			seed treatment		
	DS	DRP (%)	DI (%)	DS	DRP (%)	DI (%)
P. putida P9	2.9^{cd}	18.6	100	2.8^{ab}	10.5	100
P. putida P10	2.8^{c}	20.9	100	2.6^{ab}	15.8	100
P. aueroginosa P11	3.0^{cd}	16.3	100	3.2^{b}	0	100
P. aueroginosa P12	2.1^{ab}	41.9	91.7	2.1^{a}	34.2	91.7
P. aueroginosa P66	2.9^{cd}	18.6	100	2.4^{ab}	23.7	100
P.aueroginosa P112	2.9^{cd}	18.6	100	2.7^{ab}	13.1	100
B. subtilis B1	2.7^{bc}	23.2	100	2.3^{ab}	28.9	100
B. subtilis B6	2.7^{bc}	25.6	100	2.2^{ab}	28.9	100
B. subtilis B28	2.0^{a}	44.2	83.3	1.9^{a}	39.5	91.7
B. subtilis B40	2.9^{cd}	18.6	100	2.5^{ab}	21.1	100
B. subtilis B99	2.7^{bc}	23.2	100	2.4^{ab}	23.7	100
B.subtitlis B108	3.0^{cd}	16.3	100	3.1^{b}	2.6	100
Infected control	3.6^{d}	0	100	3.2^{b}	0	100

Data are means of four replicates. Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to DMRT. DC= disease severity (Campbell and Madden 1990), DRP= Disease reduction percentage (Campbell and Madden 1990), DI= disease incidence (Yun Cao et al. 2001).

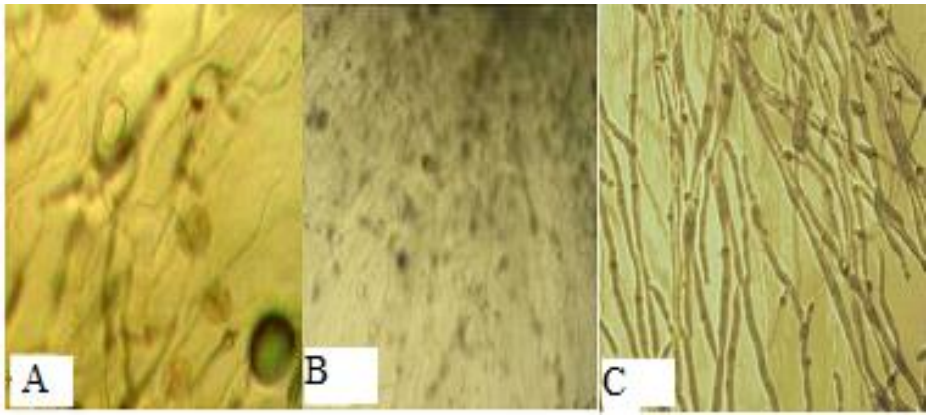

Fig 4. Changes of pathogen's body in interaction with antagonist in dual culture test. A and B, Infected treatment; C, Non infected.

NaCl, 5g; each per liter) with or without tryptophan (500 g/ml) and incubated at 30°C for 5 days. A 5-ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 2 ml supernatant was transferred to a fresh tube to which 100 µl of 10 mM orthophosphoric acid and 4 ml of reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) were added. The mixture was incubated at room temperature for 25 min, and the absorbance of pink color developed was read at 530 nm using a spectrophotometer.

Production of volatile antibiotics

Firstly, 200 µl of bacterial suspension (1×10^7 cfu/ml) from each isolate were sprayed on the surface of a petri plate containing nutrient agar medium and incubated at 27-30°C for two days. In another petri plate containing PDA medium, a 5 mm disk of a 7-days-old pure culture of *Fusarium oxysporum* f. sp. *ciceris* was placed at the centre. Then both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension. Plates were sealed with parafilm. In the control plates, bacterial suspension was replaced with sterile water. Plates were incubated at 27-29°C for 48 h and the percentage of inhibition zone was calculated for each isolates (Fiddaman and Rossall, 1993). For each treatment, there were four replicates and the experiment was repeated twice.

Determination of extracellular compound

A 0.2 µm cellophane membrane was placed on PDA plates and 200 µl of antagonistic bacterial suspension (1×10^7 cfu/ml) were inoculated in the center of plates. Plates were incubated at 27-29°C for 48 h, then the membrane with the grown bacterial strains was removed and the plate was inoculated in the middle with a five mm disk of a pure culture of *Fusarium oxysporum* f. sp. *ciceris*. Plates were incubated at 27-30°C for 7 days and the radius growth of the pathogen was measured. Sterile double-distilled water replaced the bacterial suspension in control plates. There were four replicates for each treatment and the experiment was repeated twice (Zakira Naureen et al., 2009; Kraus and Loper, 1990).

Investigation of bacterial antagonistic activity in microscopy scale

Samples were taken from the edge of hyphal growth of the *Fusarium oxysporum* f. sp *ciceris* and treated with bacterial isolates in dual culture. Shape and color of mycelia in

Table 5. The effects of bacterial isolates on growth parameters of plants in soil and seed treatments under greenhouse conditions

antagonists	Soil treatment				Seed treatment			
	LS (cm)	LR (cm)	DWS (gr)	DWR (gr)	LS (cm)	LR (cm)	DWS (gr)	DWR (gr)
P. putida P9	32.66^{bcd}	15.12^{abc}	0.65^{bcd}	0.17^{bcd}	30.83^{bc}	14.66^{bc}	0.64^{abc}	0.16^{bc}
P. putida P10	33.79^{abc}	16.0^{abc}	0.77^{ab}	0.19^{abc}	35.33^{ab}	16.33^{ab}	0.62^{abc}	0.16^{bc}
P. aueroginosa P11	31.66^{d}	13.91^{c}	0.59^{cd}	0.151^{d}	30.33^{bc}	14.44^{bc}	0.51^{c}	0.11^{b}
P. aueroginosa P12	35.16^{a}	17.16^{a}	0.81^{a}	0.21^{a}	37.22^{a}	18.22^{a}	0.86^{a}	0.24^{a}
P. aueroginosa P66	32.45^{cd}	15.83^{abc}	0.76^{ab}	0.17^{abcd}	32^{abc}	16.44^{ab}	0.69^{abc}	0.16^{bc}
P. aueroginosa P112	34.58^{ab}	16.33^{ab}	0.77^{ab}	0.21^{ab}	34.66^{ab}	17.33^{ab}	0.77^{ab}	0.19^{ab}
B. subtilis B1	33.54^{abcd}	15.79^{abc}	0.67^{abcd}	0.18^{abcd}	33.11^{ab}	16.27^{ab}	0.65^{abc}	0.16^{ab}
B. subtilis B6	33.83^{abc}	16.41^{ab}	0.75^{abc}	0.19^{abc}	33.77^{ab}	15.83^{ab}	0.69^{abc}	0.19^{ab}
B. subtilis B28	35.0^{a}	17.04^{a}	0.81^{a}	0.21^{a}	35.44^{ab}	18.22^{a}	0.86^{a}	0.24^{a}
B. subtilis B40	32.33^{cd}	14.5^{bc}	0.64^{bcd}	0.16^{cd}	32.5^{abc}	14.5^{bc}	0.56^{bc}	0.12^{c}
B. subtilis B99	32.41^{cd}	15.29^{abc}	0.69^{abcd}	0.19^{abcd}	32.44^{abc}	15.44^{ab}	0.56^{bc}	0.15^{bc}
B. subtilis B108	31.54^{d}	13.87^{c}	0.57^{d}	0.16^{cd}	27.88^{c}	12.33^{c}	0.52^{c}	0.11^{c}
Non infected control	32.25^{cd}	14.16^{bc}	0.64^{bcd}	0.17^{bcd}	31.44^{abc}	15.66^{ab}	0.57^{bc}	0.15^{bc}

Data are means of four replicates

Means in the column followed by different letter's indicate significant differences among treatments at $P \leq 0.05$ according to DMRT

LS= length of shoot, LR= length of root, DWS= dry weight of shoot, DWR= dry weight of root

Table 6. Correlation between pathology tests *in vitro* and the rate of reduced disease severity *in vivo*.

Correlation	Dual culture	Extracellular metabolites	Volatile compound
RDS in soil treatment	0.65^{*}	0.52	0.60^{*}
RDS in seed treatment	0.62^{*}	0.71^{**}	0.46

** Correlation is significant in level of $P \leq 0.01$. * Correlation is significant in level of $P \leq 0.05$.

infected treatments were evaluated and compared to that of control under a microscope at magnification of × 100.

Greenhouse experiments

Pathogenicity test

Fusarium oxysporum f. sp *ciceris* isolates recovered from infected plants were tested for their pathogenicity on chickpea plants, c.v Kaka. Seeds were sown in sterilized soil infested with 10^6 cfu/g soil of pathogen into 10-cm-diameter pots. Control pots were not inoculated with pathogen. The wilting incidence was assessed up to five weeks after sowing.

Greenhouse screening of bacterial strains against disease

A total of 12 isolates of Pseudomonas (P9, P10, P11, P12, P66, P112) and *Bacillus* (B1, B6, B28, B40, B99, B108) were selected for greenhouse assays.

Efficacy of seed treatment for chickpea Fusarium wilt control

Chickpea seeds were soaked for 30 min in a bacterial-methylcellulose suspension (10^8 cfu/seed) and dried under a laminar flow hood. Control treatments consisted of non-treated dry seeds which were coated with 1% methylcellulose. Then the seeds were sown in sterile soil infested with the pathogen at a rate of 10^6 cfu/g soil into 10-cm-diameter pots. In each pot, three seeds were sown and the treatments were replicated five times in a randomized complete design (Dileep Kumar, 1999).

Efficacy of soil drenching for chickpea Fusarium wilt control

In this experiment both the pathogen and the antagonist were added to soil of pots at a concentration of 10^6 spores/g soil and 10^8 cfu/g soil, respectively. Then three seeds were sown in each pots. All pots were maintained in a glasshouse at 22 to 28°C, 60-70% RH, 16 h light and 8 h darkness. Plants were watered twice a week and once a week added the fertilizer solution (NPK) (Jamali et al., 2004).

Diseases severity and disease incidence assessments

Disease severity was assessed for each plant on a 0 to 4 rating scale according to the percentage of chlorosis, necrosis and wilt, as follows: 0= healthy, 1=1-33%, 2=33%-66%, 3= 66%-100%, 4= dead plant (Campbell and Madden, 1990). Disease incidence assessments were calculated using method reported by Yun Cao et al (2011) with the following formula:

Percent disease incidence = [(number of infected plants)/total number of plants] × 100.

Disease reduction percentage (DRP) was calculated by the method described by Yun Cao et al (2011) using the following formula:

$DRP = 1- D_T/D_C \times 100$ (D_T = Disease incidence percentages in treatment; D_C = Disease incidence percentages in control).

At the end of the experiments, shoot length, root length, dry weight of shoot and root were determined for each plant.

Statistical analysis

Experiments were designed as a completely randomized design (CRD) with four replications, and all analyses were conducted using the SAS software (SAS institute, Inc., 2003). The means were compared by Duncan multiple-ranges test (DMRT) at $P \leq 0.05$.

Conclusion

Using a resistante cultivar is the best method in controlling disease. However, due to the possible appearance of new races in pathogen and breaking of resistance in host defence, this method is not sufficient for control of disease. Therefore,

we suggested that application of rhizobacteria in combination with resistant cultivars can obtain beneficial results and provide significant protection against the *Fusarium oxysporum* f. sp. *ciceris*, through chickpea root colonization.

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