Enhancement of Antagonistic Activities of *Pseudomonas Fluorescens* and *Bacillus Cereus* Against some Plant Diseases Strains

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ABSTRACT

The identification and the biological and molecular characterization of microbial biocontrol agents or microbial producers of bioactive compounds are of great interest in modern agriculture. Antagonistic strains against *Trichoderma* and *Fusarium* genus are able to produce various secondary metabolites, which can play a role in the mechanism of their biological activity. Particularly, they produce antifungal secondary metabolites, which by spreading through soil cracks could ensure the antagonists of an ecological advantage in the competition for the soil colonization against the pathogens.

Two strains *Pseudomonas fluorescens* and two strains of *Bacillus cereus* were isolated from soils of different areas of Saudia Arabia. Different antibiotic resistance patterns were isolated in each strain to select genetic marker for each strain. Antagonistic test of each strain against *Trichoderma* and *Fusarium* showed its role in the mechanism of their inhibition of two fungal activities in growth medium using inhibition zone diameter. Plasmid isolation and transformation between each two *Bacillus cereus* and also between each two *Pseudomonas flourosence* were carried out to determine correlation between biochemical functions with plasmids on antagonism against *Fusarium* and *Trichoderma*, some tested colonies on tested media indicated that some of them improve their inhibition zone t han their original wild type strains.

Key words: Bio-control agents, plasmid isolation, plant pathogen, *Fusarium, Trichoderma* and Transformation.

INTRODUCTION

Antagonistic microorganisms are able to produce various cell-wall degrading enzymes which may be involved in the cells lysis of phytopathogenic fungi. The use of antagonistic bacteria in biological control of plant pathogenic fungi may represent a promising alternative to the use of chemicals.

Diseases of roots, stems, aerial plant surfaces, flowers, and fruit are caused by a wide variety of pathogens. Because of this diversity, the antagonist species which negatively affect plant pathogens and the mechanisms by which they accomplish their beneficial action are also quite varied. Their biological and taxonomic diversity is covered in some details

in several texts and reviews, including Cook and Baker (1985), Fokkema and van den Heuvel (1986), Campbell (1989), and Adams (1990).

Successful biological control can be obtained through treated the plants by strains of *Pseudomonas* sp., *P. fluorescens* and *P. putida* (Lozano, 1986). *P. putida* can also be used for the control the root rot pathogen *Diplodia* spp. (Lozano, 1988 and Severns *et al.*, 2003).

Plasmids of bacteria are responsible for defining phenotypic traits. The plasmid profile of a cotton phylloplane bacterium belonging to genus *Pseudomonas* and antagonistic to the incidence of bacterial blight of cotton (Saha *et al.*, 2000).

Reguiation of antifungal metabolite production by biological control strains of *Pseudomonas* spp. is controlled by complex regulatory networks that respond to environmental and density-dependent signals and are coupled to the physiological status of the bacterium (Whistler *et al.*, 2000).

Accordingly, Biological control of soil borne diseases has been attracting situation. Many species of bacteria, fungi and plants produce enzymes that degrade chitin which is a major structural component of most fungal cell walls (Schinder, 1994). Various species of *Bacillus* have been proved secrete chitinase, including *Bacillus cereus* (Huang *et al.*, 2005). A number of methods have been used for typing *Bacillus* species e.g., stereotyping, bacteriophage typing, bacteriocin activities, antibiogram and biotyping, plasmid typing, analysis of fatty acid content, native – PAGE, small – subunit ribosomal RNA sequencing and genome analysis (Berber, 2004).

This research aimed to study isolating and identifying certain *Pseudomonas fluorescens* and *Bacillus cereus* and to study the efficiency of these strains in suppressing the growth of plant pathogenic fungus *Fusarium* and *Trichoderma*. Also study the effect of plasmid transfer within the same species to improve inhibition of the growth of two plant pathogens by transformed strains.

MATERIALS AND METHODS

Bacterial strains:

Two *Pseudomonas fluorescens* and two *Bacillus cereus* were isolated from soils of different areas of Saudia Arabia. Table (1).

Pathogen strains:

Samples of *Fusarium oxysporum* and *Trichoderma harzianum* were isolated from the infected plant roots according to Haseeb *et al.* (2005).

Isolation of indigenous *Pseudomonas flourosence* and *Bacillus cereus*.

A number of soil samples were collected from environmentally different fields in Saudia Arabia belonging to some areas, Table (1).

Dilutions of soil samples were pasteurized on 80°C for 15 minutes; aliquots of 1 ml were then poured into plates of nutrient agar and incubated at 30°C for 48 hrs. Only developed colonies resemble *Bacillus sp.* morphologically were picked up, purified and identification. Different morphological and biochemical study were carried out including Gram staining, spore formation and position, swelling of sporangium, starch hydrolysis, glucose fermentation, nitrate reduction, catalase production and growth in 5% NaCl (Smith *et al.*, 1952) for *Bacillus scereus* and the methodology of Rachid and Ahmed (2005) for identification of isolated *Pseudomonas fluorescens*.

Table 1. Location and nomination of isolated strains

Strains	Nomination	Locations
Bacillus cereus	*Bacillus cereus* A	Jeddah
	Bacillus cereus B	Taif
P. fluorescens	*P. fluorescens* A	Al-Baha
	P. fluorescens B	Abha

Media and culture conditions:

Pseudomonas fluorescens and *Bacillus cereus* strains were grown at 27°C ±2°C in solid or liquid King's medium B (KB) broth (0.2 g peptone, 0.15 g K2HPO4, 0.15 g MgSO4.7H2O, 1.5 ml glycerol, 2 g agar and distilled water up to 100 ml) or Luria Bertani (LB) medium (1 g tryptone, 0.5 g yeast extract, 2 g agar, pH 7.0 and distilled water up to 100 ml). Bacteria were stored in 0.8% nutrient broth plus 0.5% yeast extract (NBY) broth

(Difco, Detroit, Mich.) plus 40% glycerol at −8°C. Starter cultures were grown in 10-ml dilute (1/10-strength) NBY broth in 20-ml screw top vials for 8 to 12 h at 27°C at 140 rpm, yielding approximately 10^9 CFU/ml. The fungal pathogen was grown in potato dextrose agar (PDA) medium (the filtrate of boiled 30 g diced potatoes, 2 g glucose, 2 g agar and distilled water up to100 ml). Fungal inhibition zone were determined after three days.

Bacterial antagonistic effect

Zone inhibition and growth reduction through dual culture were used to test the antibiosis of bacterial strains against the fungal pathogen *Fusarium oxysporum* and *Trichoderma harzianum*. A small plug of fungal inoculum (about 4 mm square) was placed in the center of peteri plates contained PDA agar with and without additional NaCl, bacterial cells from fresh cultures of the tested isolates were streaked near the edge of the plates. The plates were incubated at 28°C and inhibition zones around the streaks were measured after 5 days in which the reference plates were full completely by the growth of fungal pathogen on PDA agar.

Antibiotic resistance test

Resistance to antibiotics, chloramphinicol (Cm), streptomycin (Sm), tetracycline (Tc), rifampicin (Rif^r), and ampicillin (Amp^r) for Two *Pseudomonas fluorescens* and two *Bacillus cereus* strains were tested by streaking the isolates on LB plates containing one of these antibiotics. The final concentration were; 35 µg chloramphenicol / ml, 200µg streptomycin / ml, 100µg rifampicin/ml, 100µg ampicilin /ml, and 15µg tetracyclin / ml. Plates were incubated at 30°C for three days. Single and double spontaneous antibiotic resistance colonies were isolated as indicated in Table (3).

Isolation of plasmid DNA:

The plasmid DNA of the wild type strain (*Pseudomonas flourosence* and *Bacillus cereus*), and it's transformed colonies were isolated and identified by QIAGEN plasmid Kit 25 (Cat. No. 12123).

Transformation trials:

Transformation experiments were done as described by Ausubel (1995).

Gel electrophoresis:

Analysis of plasmid content was carried out using horizontal gel electrophoresis mini gel system (Molecular Bio. Products, inc., MBP™) at 60 V for 60 min. and then observing the pattern by UV transeluminator.

RESULTS

Isolation of *Bacillus* strains

Pseudomonas fluorescens and *Bacillus cereus* strains were isolated from soil samples collected from four Saudia Arabia areas (table 1). All *Bacillus* isolates were Gram positive; rod shaped, starch hydrolysis and could ferment glucose. More than 20 *Bacillu scereus* and *P. fluorescens* were isolated and subjected to the identification procedures of smith *et al.*, (1952) for *Bacillu scereus* and Rachid and Ahmed (2005) for *P. fluorescens*. Among identified *Bacillus cereus* and *P. fluorescens* strains, two strains of each were used for further studies.

Bacterial antagonistic efficiency

The efficiency of *Bacillus cereus* and *P. fluorescens* strains to inhibit the growth of *Fusarium oxysporum* and *Trichoderma harzianum* were carried out using three different media. The results presented in Table (2) indicated that all strains indicate inhibition zones on King's medium, with different efficiencies. While these strains could not inhibit *Fusarium oxysporum* growth when Nutrient agar (NA) medium was used. Results also showed the antagonistic effect when $FeCl_3$ was added to King's medium. The *Bacillus cereus* strains used their antagonistic effect of *Fusarium* when adding $FeCl_3$. At the same time the antagonistic effect of *Bacillus cereus* strains was improved by adding $FeCl_3$. The effect of $FeCl_3$ was proved to be different in the two *B. cereus* strains used, *B. cereus* A showed better antagonistic efficiency. The best efficient strains for antagonistic effect was *B. cereus* A and *P. fluorescens* A. These two strains have been chosen for further study, Table (2).

Intrinsic antibiotics resistance of *Bacillus* isolates

Five antibiotics were used to test all obtained *Bacillus cereus* and *P. fluorescens* strains for their antibiotic resistance response, data are present in Table 3. The results showed that all *Bacillus cereus* A isolate were sensitive to rifampicin (Rif) and resistant to streptomycin (Sm), chloramphenicol (Cm), ampicillin (Ap) and tetracycline (Tc). At the same

time *Bacillus cereus* B strain proved to be resistant to rifampicin (Rif), chloromphinicol (Cm), ampicillin (Ap) and sensitive to (Tc) and streptomycine (St). *P. fluorescens* A were resistant to the three antibiotics, chloromphinicol (Cm), tetracycline (TC) and ampicillin (Ap) and sensitive to rifampicin (Rif) and streptomycin (Sm). While *P. fluorescens* B strain was sensitive to streptomycin (Sm) and chloramphenicol (Cm) and resistance to the other three antibiotics used. These results indicated that, the four isolates are genetically different in their patterns of antibiotics resistant.

Plasmid patterns of strains

The number and size of *Bacillus cereus* and *P. fluorescens* plasmids were isolated using QIAGEN plasmid Kit 25 (Cat. No.12123). The plasmid patterns of *Bacillus cereus* and *P. fluorescens* strains are present in Fig (1), Results showed different plasmids patterns among the four *Bacillus* and *pseudomonas* strains. The plasmid numbers were ranged from one to three plasmids, as three plasmids has been found in *Bacillus cereus* B strain, two plasmids were found in *Bacillus cereus* A and *P. fluorescens A* strain, one plasmid was found in *P. fluorescens* B strain.

Transformation of *B. cereus* A and *P. fluorescens A*

Transformation of competent cells of *B. cereus* A and *P. fluorescens A* was performed as described by Ausubel *et al.* (1995). *B. cereus* A and *P. fluorescens* A was chosen as recipient strain due to its sensitivity against the five tested antibiotics *B. cereus* A was sensitive to rifampicin (Rif). While, *P. fluorescens* A was sensitive to rifampicin (Rif) and streptomycine (St). The rest *Bacillus cereus* B and *P. fluorescens* B strains were used as donors of plasmids DNA. *B. cereus* A and *P. fluorescens* A competent cells were prepared as outlined by Ausubel *et al.* (1995).Plasmids of *Bacillus cereus B* donor strains were isolated by using the QIAGEN plasmid Kit 25 (Cat. No.12123). Fifteen µl of plasmid DNA (10ng) were added in a 15 ml test tube and kept on ice.

The competent cells were rapidly thawed and 100µl was quickly dispensed into the test tube. It was kept on ice for 30 min. and the mixture was then exposure to a heat shock at 42°C for 2 min. Adding 1 ml of LB broth and incubated at 37°C in a shaker incubator (200rpm) for 0.5 and 1 h. To isolate transformants, 100µl of appropriate dilution were spread on NA agar selective medium supplemented with appropriate concentrations of selection marker (Rif) and Tc).

Three transformants were selected from *Bacillus cereus* transformation procedure on the basis of antibiotic resistance pattern cells as shown in Table (3) that resist Rif and Tc antibiotics were selected as transformants and were designated as B.C1, B.C2 and B.C3. While, transformants P.F1, P.F2 and P.F3 from *P. fluorescens* transformation procedure. Cells that resist Rif and St antibiotics were selected as transformants. Controls run without plasmid DNA produced no colonies on selective medium, Table (4).

Table 2. Zone inhibition of isolated *Bacillus cereus* and *P. fluorescens* and their transformants

Pf A	Pf B	Pf1	Pf1	Pf1	B.CA	B.CB	B.S1	B.S2	B.S31
++	+	+++	+++	+++	+	++	+++	+++	+++

- : No inhibition zone
+: The number of + (% 1-4) corresponding to degree of inhibition

Table 3. Antibiotics resistance of *Bacillus cereus* and *P. fluorescens* and their transformants.

Bacterial isolates	Rif	Sm	Cm	Ap	Tc
P. fluorescens A	-	-	+	+	+
P. fluorescens B	+	-	-	+	+
P. fluorescens (Pf1)	+	-	+	+	+
P. fluorescens (Pf2)	+	-	+	+	+
P. fluorescens (Pf3)	+	-	+	+	+
Bacillus cereus A	-	+	+	+	+
Bacillus cereus B	+	-	+	+	-
Bacillus cereus (B.S1)	+	+	+	+	+
Bacillus cereus (B.S2)	+	+	+	+	+
Bacillus cereus (B.S3)	+	+	+	+	+

+ = Resistant
- = sensitive

Table 4. Doner and recipient transformation procedures and their transformants.

Recipient strain	Donors strains	Transformants
Bacillus cereus A	*B. cereus* B	B.C1, B.C2 and B.C3
P. fluorescens A	*P. fluorescens B*	P.F1, P.F2 and P.F3

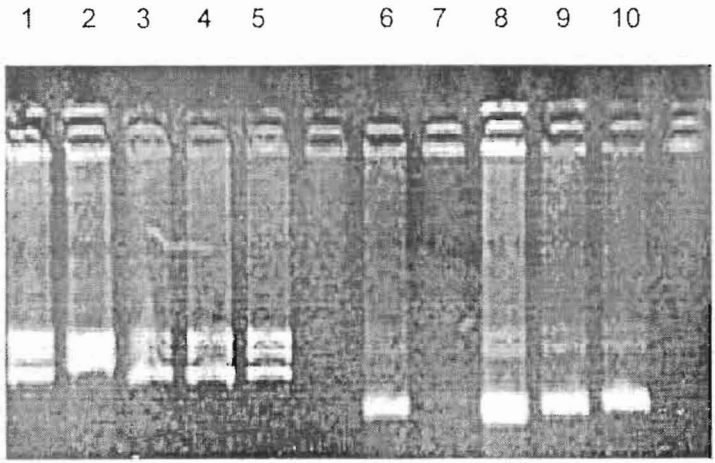

Fig. 1. Plasmid profile of *P. fluorescens* and *Bacillus cereus* strains and their transformants.
Bacillus cereus A (Lane 1), *Bacillus cereus* B (Lane 2), *Bacillus cereus* B.S1 (Lane 3), *Bacillus cereus* B.S2 (Lane 4), *Bacillus cereus* B.S3 (Lane 5), *P. fluorescens* (Pf1) (Lane 6), *P. fluorescens* B (Lane 7), *P. fluorescens* A (Lane 8), *P. fluorescens* Pf2 (Lane 9) and *P. fluorescens* Pf3 (Lane 10).

DISCUSSION

Aiming to develop effective biocontrol agents against *Fusarium oxysporum* and *Trichoderma harzianum*, four bacterial strains were isolated and identified from local soils in Saudia Arabia. Two of these strains were belong to *Bacillus cereus* (A and B), the other two were belong to *P fluorescens* (A and B). Isolates of *Bacillus cereus* and *P. fluorescens* have shown to possess in vitro inhibitory activity against tested *Fusarium oxysporum* and *Trichoderma harzianum*. Many strains of *Bacillus cereus* and *P fluorescens* proved to be potential biocontrol agents against fungal pathogens The principal mechanism of this antifungal action involves the production of antibiotics (Fravel. 1988). However, it is likely that several mechanisms act in concern to active control, including the production of volatiles, which have a significant effect on soil microbiology (Linderman and Gilbert, 1957). *B subtilis* strains also produce volatiles that antagonize a range of organisms including the soil born plant pathogens *Rhizoctoniz* and *Pythium* (Wright and Thomson,1958; Fiddman and Rossal, 1993). The suppression of wilt disease by *Fusarium* and yield increases in cotton have been reported (Kenny *et al*., 1992), while inoculation with *Bacillus* spp. strains increase the number of healthy cotton plants by 13.3% versus a standard chemical seed treatment (Brannen 1995). The antifungal activity of *B subtilis* is achieved via the production of iturins, which possess broad spectrum of antibiotic activity (Klich *et al*., 1991). *Bacillus* spp. have been also reported to suppress diseases caused by pythium *solani* and *Fusaium* spp (Willer,1988: Kenny *et al* , 1992; Branen, 1995).

It was found that each of isolates of *Bacillus cereus* and *P fluorescens* has genetically different patterns of inhibition effect against *Fusarium oxysporum* and *Trichoderma harzianum* based on the used media In other words the pattern of inhibition might be affected by composition of media used. These results are in agreement with the results of Liao (1989), which would be due to siderophore production or for antibiotic production by isolated strains.

Most of the plasmids described in bacillus and pseudomonas strains are cryptic plasmids (Tanaka and Koshikawa 1977; Tanaka *et al*., 1977; Uozumi *et al* 1980) Few exceptions are existed as that determine bacteriocine production and tetracycline resistance, respectively in *B. cereus* (Bernhard *et al* 1978). Large variety of specific biochemical functions such as fertility resistance to antifungal drugs, production of

bacteriocine has been attributed to these genetic elements (Bernardo *et al.*, 1978).

Plasmid isolation revealed that most strains of *Bacillus cereus* and *P. fluorescens* harbour plasmids that are different in number and molecular weight, indicating that these strains are indeed independent isolates. The two *Bacillus cereus* a and B.

The susceptibility of bacilli and *Psoudomonas* to deferent antibiotics has been studied also previously; it should be possible to identify species on the bases identified in the present study and were of susceptibility tests (Burke and McDonald. 1983; and Kvllner.1978)

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الملخص العربي

تحفيز أنشطة التضاد لكل من بكتريا بسيدومونس فلوريسنس وكذا بكتريا باسيللس سيريس ضد بعض السلالات الممرضة للنبات

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يعتبر التوصيف البيولوجي والجزيئي للوسائط البكتيريا التي لها دور في التحكم الحيوي وكذلك النواتج الميكروبية ذات النشاط الحيوي من أهم الأنشطة في الزراعة الحديثة. والسلالات البكتريا التي تضاد كل من جنسي الفيوزاريم والترايكودرما لها القدرة علي إنتاج مواد أيضيه ثانوية والتي من الممكن أن تلعب دوراً في ميكانيكية أنشطتها الحيوية ، خاصة وأنها تنتج مواد ايضيه ثانوية مضادة للفطريات والتي عن طريق نشر هذه المواد من خلال تشققات التربة من الممكن ان تؤمن إنتاج مواد مضادة للفطريات مفضلة بيئيا ولقد تم عزل سلالتين من نوع بسيدومونس فلوريسنس وسلالتين من نوع باسيللس سيريس وذلك من أربع مناطق مختلفة من المملكة العربية السعودية ولقد اختبرت هذه السلالات لمقاومتها ضد المضادات الحيوية حيث ادي ذلك الي عزل سلالات مقاومة حيث استخدمت هذه المقاومة كمعلم وراثي لكل سلاله .

ولقد اختبر ايضا التضاد لهذين النوعين ضد كل من جنسي الترايكودرما والفيوزاريم ولقد اظهر هذا الاختبار ان هذه السلالات البكتيرية لها المقدرة علي تثبيط الأنشطة الحيوية لكلا الفطرين ، وذلك عن طريق تحديد مساحة منطقة تثبيط النمو في البيئة . وبالإضافة الي ذلك فقد تم عزل البلازميدات وإحداث التحول الوراثي بين سلالتي الباسيللس سيريس وايضا سلالتي بسيدومونس فلوريسنس وذلك لتحديد درجة التلازم بين الوظيفة البيوكماوية والبلازميدات لتضاد كل سلالتي البكتيريا ضد الفطريات ولقد اختبرت مستعمرات من هذه البكتريا المحولة علي بيئة اختباريه والتي أوضحت ان بعضا منها اثبت مقدرة علي تحسين القدرة علي تثبيط النمو مقارنة بالأصل البري .