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## A Comparison Between Antibiotic-Resistant Mutants of Antagonistic Bacteria and Their Wild Types in Biological Control of Cotton Seedling Damping-Off Disease

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**Abstract:** In this research, antibiotic-resistant mutants of antagonistic bacteria to rifampicin (Rif) and nalidixic acid (Nal) were developed via spontaneous mutation and their effectiveness in controlling cotton seedling damping-off disease was compared with their wild types. Four wild types isolates belonged to *Pseudomonas fluorescens* and their mutants were tested against pre-and post emergence damping-off disease of cotton in greenhouse. The experiment was conducted in a completely randomized design with ten treatments and four replicates. A virulent isolate of *Rhizoctonia solani*, the causal agent of cotton seedling damping-off disease was used in the study and the effectiveness of bacterial isolates was evaluated based on the number of healthy seedlings 15, 30, 45 and 60 days after sowing. Results indicated that all Rif-Nal resistant mutants showed more effectiveness in disease control than their wild types 15 and 30 days after sowing. However, at days 45 and 60 after sowing, only three mutants were more effective than their wild types in controlling cotton seedling damping-off disease. Results of this study suggest that spontaneous mutation may affect and enhance the activity and performance of antagonistic bacteria.

**Key words:** Antagonistic bacteria, antibiotic-resistant mutants, biological control, cotton damping-off

### INTRODUCTION

Plant diseases create challenging problems in agriculture and pose real economic threats to both conventional and organic agriculture. Therefore, attempts to decrease plant diseases, which cause yield reduction in many crops, is very important and critical. Cotton is the most important fibre crop in the world including Iran (Heydari *et al.*, 2007). Cotton produces raw materials for the textile industry and also is a valuable food source due to the considerable oil and protein contents of seed. In year 2000, about 130 countries produced cotton and it is estimated that this crop was planted on 2.5% of the world's arable land area, making it one of the most important crops in terms of land use after food grains and soybeans (Weiss, 2000).

Like other crop plants, cotton is also susceptible to several plant pathogens including fungi. Seedling damping-off is considered as one of the most important diseases of cotton in Iran (Naraghi *et al.*, 2007). Several soil-borne fungi have been reported to be responsible for the occurrence of this disease. However, cultural and environmental factors that delay seed germination and seedling growth make the disease more severe. Cotton seedling damping-off occurs more frequently under cool,

wet conditions and seems to be more prevalent in sandy, low-organic-matter soils. Seedling diseases cause an estimated average annual yield loss of 5% and are usually the major disease problems in cotton production (Rothrock, 1996). Among agents causing seedling damping-off disease, *Rhizoctonia solani* is one of the most important fungi causing damping-off in cotton fields. Over the past 20 years, losses due to seedling diseases have averaged slightly over 3% in the United States. However, seedling diseases are a problem wherever cotton is grown (Gazaway, 1998).

Despite the effectiveness of fungicides, their widespread use has not eliminated cotton seedling damping-off caused by *R. solani* and other pathogens (Asaka and Shoda, 1993). Moreover, the widespread use of chemicals, mainly due to their potential harmful effects on non-target organisms, the development of resistant races of pathogens and the possible carcinogenicity of some chemicals have caused biological control with beneficial microorganisms to be considered as a significant alternative method to manage cotton seedling damping-off (Zaki *et al.*, 1998). A number of studies about using antagonistic bacteria and fungi for controlling seedling diseases have been carried out and a promising approach has been obtained (Brannen and Kenney,

1997; Howell *et al.*, 1997; Zaki *et al.*, 1998). *Rhizoctonia solani*-induced cotton seedling damping-off has also been suppressed by the antagonistic fungi including *Trichoderma* spp and *Gliocladium virens* in the field (Lewis and Papavizas, 1991), by *Pseudomonas fluorescens* (Howell and Stipanovic, 1979) and *Bacillus cereus* (Pleban *et al.*, 1995) in the greenhouse. *Burkholderia cepacia* (Mcloughlin *et al.*, 1992; Heydari and Misaghi, 1998), *Bacillus subtilis* (Asaka and Shoda, 1993), *Trichoderma* spp. (Cliquet and Scheffer, 1996) and also non-pathogenic binucleate *Rhizoctonia* (Villajuan *et al.*, 1996) have been reported to suppress *R. solani*-induced damping-off in other crops in the greenhouse.

Environmental stresses resulting from various factors such as antibiotics may affect antagonistic bacteria and result in genetic alteration such as mutation and these changes may affect the ability and performance of these bacteria (Ownley and Windham, 2003). One of these alterations is spontaneous mutation that its occurrence on antagonistic microorganisms in nature is possible.

Mutation is defined as any heritable change in genetic material and has been known as the only source of genetic variation (Blazquez, 2003). Some mutations arise as natural errors in DNA replication (or as a result of unknown chemical reactions); these are known as spontaneous mutations. Spontaneous mutations are derived from various sources, including errors made during replication of undamaged template DNA, mutagenic nucleotide substrates and endogenous DNA lesions. These sources vary in their frequencies and resultant mutations and are differently affected by the DNA sequence, DNA transactions and cellular metabolism (Drake, 1991; Maki, 2002). Mutations induced by chemical mutagens, radiation and transposons insertion are called induced mutations.

Since genetic phenomena including mutation may affect the characteristics and performances of microorganisms, therefore the objective of the present study was to investigate the impact of spontaneous mutation on the performance and activity of some antagonistic bacteria in biocontrol of cotton seedling damping-off disease.

## MATERIALS AND METHODS

**Selection and preparation of antagonistic bacteria:** Nine isolates of effective bacterial antagonists belonged to *Pseudomonas fluorescens* and *Bacillus* spp. were selected from our collection for this study. These bacteria have previously shown antagonistic activity against cotton seedling damping-off disease (Heydari *et al.*, 2005). Two hundreds  $\mu\text{L}$  of bacterial suspension were spread on

nutrient agar (NA) medium. The plates were then incubated at  $25^{\circ}\text{C}$  for 72 h. Bacterial suspensions were prepared from fresh culture at  $10^8$  cfu  $\text{mL}^{-1}$  using a spectrophotometer.

**Preparation of antibiotic solutions:** Two antibiotics, rifampicin and nalidixic acid, were used in this study. These antibiotics have commonly been used in previous studies (Mariano and McCarter, 1993; Heydari and Misaghi, 1998; Steddom and Menge, 2001). Antibiotic solutions were prepared at different concentrations including 10, 50 and  $100 \text{ mg mL}^{-1}$ .

**Development of antibiotic-resistant mutants:** In general, when bacteria are exposed to stress of antibiotics, some of them develop resistant mutants via spontaneous mutation. This characteristic of bacteria was used in this study and resistant mutants were developed by exposing them to different concentrations of antibiotics. Spontaneous mutants of all isolates that were resistant to both rifampicin (Rif) and nalidixic acid (Nal) were obtained by selecting bacterial colonies developed on King's B medium (KB) [15 g agar, 15 g proteose peptone No. 3, 1.5 g  $\text{K}_2\text{HPO}_4$ , 1.5 g  $\text{Mg SO}_4$ , 12.6 g glycerol, 1 L of distilled water (Heydari *et al.*, 1997) supplemented with 10, 50 and  $100 \text{ mg mL}^{-1}$  of each antibiotic] 3-10 days after culture plates were inoculated with each test bacterium. Spontaneous mutation occurred in bacteria under pressure of antibiotics and resistant mutants were grown on culture medium as small colonies. Suspensions were prepared from these mutant colonies in different concentrations of antibiotics. It should be stated that in some concentrations of antibiotics, no mutant colonies were developed. Four mutant isolates that had the highest number of colonies at different concentrations of antibiotics, were selected and were used in greenhouse experiment. Stability of Rif-Nal resistant mutants was confirmed by repeated culturing on KB and NA without the antibiotics.

**Selection of *Rhizoctonia solani* isolate:** One virulent isolate of *R. solani* (AG-4; Anastomosis Group 4) was selected from our collection, in which several isolates of *R. solani* had been isolated from the roots of cotton seedlings. It has shown the highest level of pathogenicity on cotton seedlings in our previous studies (Heydari *et al.*, 2005).

**Greenhouse experiment:** Wild type and mutant isolates of bacteria applied as seed treatment were tested for their biocontrol efficacy against cotton seedling damping-off disease in the greenhouse. Ten treatments [four wild type and four mutant isolates of bacteria, Cont<sup>+</sup> (without

fungus and bacteria) and Cont-(without bacteria but with fungus)] were used with four pots for each treatment. Each pot was filled with 2 kg of pasteurized soil (heated for 1 h at 115°C and 15 psi to destroy possible soil-borne pathogens). *R. solani* inoculum was prepared as described below:

*R. solani* (AG-4) was grown on Potato Dextrose Agar (PDA) for 6 days at 27°C. Twelve plugs (0.78 cm<sup>2</sup>) from the culture were sliced into 1- mm<sup>2</sup> sections and incorporated into the top 2 cm of the soil in each pot.

Bacteria were introduced into the rhizosphere by placing them on cotton seeds. Ten mL of an aqueous suspension containing 10<sup>8</sup> colony forming units (cuf) mL<sup>-1</sup> of each test Rif-Nal-resistant bacterium and their wild type from a 72-h-old NA culture, were mixed with 2 mL of a 2% aqueous suspension of methyl cellulose. Eighty seeds were evenly coated by rolling them in the mixture for 30 min and coated seeds were dried at 25°C for approximately 24 h. Twenty seeds (Varamine variety) were sown in each pot and pots were placed in the greenhouse at 18 to 20°C under 16-h-light/8-h-dark conditions. Pots were arranged in a completely randomized design and were watered from the top with 300 mL of water. This amount of water was sufficient to wet the entire soil column in the pot without any drainage. The incidence of cotton seedling damping-off was assessed by counting the number of emerged seedlings in each pot (plant stand) 15, 30, 45 and 60 days after sowing.

**Statistical analysis:** The number of healthy seedlings from the greenhouse experiment were analyzed using MSTATC statistical software. Data were tested by Analysis of Variance (ANOVA) and means were compared using Duncan's multiple range test.

**RESULTS**

Development of antibiotic-resistant mutants. The results of developing bacterial mutants resistant to Rif-Nal antibiotics are shown in Table 1. No mutant colonies were observed for two isolates (CKK-2, CGM-4) at any concentration of the antibiotics. However, two other isolates (SCH-11, Q<sub>18</sub>), developed mutant colonies at the all concentrations of antibiotics while other bacterial isolates, developed resistant mutants only at one concentration (Table 1).

Among the developing mutants, four mutants that had the highest number of colonies at different concentrations of antibiotics were selected and used in the greenhouse experiments (Table 2).

Efficacy of mutants vs. wild type isolates in suppressing cotton seedling damping-off disease in the

Table 1: Mutants developed from nine bacterial isolates at different concentrations of the combined rifampicin-nalidixic acid antibiotics

Bacterial isolate <sup>e</sup>	Concentration of Rif-Nal antibiotics (mg L <sup>-1</sup> )		
	10	50	100
CKK-2	-	-	-
CKK-3	+	-	-
CKK-9	-	+	-
CGM-3	-	-	+
CGM-4	-	-	-
Sch-3	+	-	-
Sch-11	+	+	+
Q <sub>18</sub>	+	+ <sup>†</sup>	+
Q <sub>27</sub>	-	- <sup>‡</sup>	+

<sup>e</sup>CKK-9 = *B. polymyxa*, CGM-4 and SCH-3 = *B. subtilis*, other isolates belonged to *Pseudomonas fluorescens*, <sup>†</sup>positive = Development of antibiotic-resistant mutants, <sup>‡</sup>minus = No development of antibiotic-resistant mutants

Table 2: The effective concentration of antibiotics that induced the highest number of mutant colonies of selected bacterial mutants

Selected mutant isolates	Effective concentration of antibiotics (mg L <sup>-1</sup> )
CKK-3	10
Sch-11	50
Q <sub>18</sub>	100
Q <sub>27</sub>	100

Table 3: Efficacy of mutants vs. wild type bacterial isolates in suppressing cotton seedling damping-off disease in the greenhouse 15, 30, 45 and 60 days after sowing

Treatment <sup>e</sup>	Number of healthy seedlings <sup>f</sup> after (days)			
	15	30	45	60
Cont <sup>e</sup>	19.50a <sup>‡</sup>	19.50a	19.00a	19.00a
Q <sub>18</sub> (Mu)	18.75a	15.25b	14.25b	14.25b
Q <sub>18</sub> (Wt)	13.00b	12.75c	12.50b	12.50b
Sch-11(Mu)	18.50a	18.50a	18.50a	18.50a
Sch-11(Wt)	9.00c	9.00d	8.25c	8.25c
CKK-3(Mu)	18.00a	17.75a	17.75a	17.50a
CKK-3(Wt)	10.25c	10.00d	9.75c	9.75c
Q <sub>27</sub> (Mu)	17.75a	18.25a	18.00a	17.75a
Q <sub>27</sub> (Wt)	8.25c	8.00d	8.00c	8.00c
Cont-	4.50d	4.500e	4.25d	4.25d

<sup>e</sup>Cont<sup>e</sup> = positive control (pathogen-free, bacteria-free control), Mu = mutant, Wt = wild type, Cont- = negative control (treated with pathogen but not with the bacteria), <sup>f</sup>Values represent the average of four replicates, <sup>‡</sup>Means within each column followed by the same letter are not, significantly different according to least significant difference test (p = 0.05)

greenhouse. All Rif-Nal resistant mutants were more suppressive to the damping-off disease than their wild types up to 30 days after sowing. However, only three mutants continued to be more effective than their wild types in biocontrolling seedling damping-off up to 60 days after sowing. The least suppressive mutant was the mutant of Q<sub>18</sub> isolate. After 60 days, there were no significant differences among three mutant isolates and the positive control (pathogen-free, bacteria-free control). The least number of seedlings emerged (= highest level of disease incidence) was recorded in the negative control (treated with pathogen but not the bacteria), which had significantly higher level of post-emergence damping-off than all treatments that received bacteria (wild type or mutant isolates) (Table 3).

## DISCUSSION

The important point about using antagonistic microorganisms in biological control of plant diseases is the effect of environmental stresses on the activities of these agents. These factors may affect the microorganisms of the soil and different parts of plants and cause changes which can influence the ability and performance of microbial communities (Ownley and Windham, 2003). Spontaneous mutation is one of the changes that its occurrence on organisms including antagonistic bacteria in nature is possible. In this study the effect of this phenomenon on some antagonistic bacteria was studied.

Results of the present study indicate that spontaneous mutation may enhance the antagonistic activity of the bacteria against cotton seedling damping-off disease. In other words, while bacteria are under stresses of antibiotics, they may get modified to be able to survive. Some bacteria are naturally resistant to antibiotics however some of them acquire and develop resistance via spontaneous mutation or in other species of bacteria during horizontal gene transfer (Blázquez, 2003).

In this study, antagonistic isolates including *P. fluorescens* and *Bacillus* spp. were tested. These bacteria are effective antagonists against root and crown diseases of plants and are considered to be soil inhabitants. When these bacteria are added to the soil, they are well adapted in soil and produce several metabolites including antibiotics and siderophores (Ganeshan and Kumar, 2006). Phenazin-1-carboxylic acid is one of such antibiotics produced by these bacteria (Thomashow and Weller, 1990). The anti-fungal metabolite 2,4-diacetyl phloroglucinol play a major role in the biocontrol capabilities of *P. fluorescens* (Delany *et al.*, 2000).

Previous studies have indicated that the selection of antibiotic-resistant mutant bacteria is proposed to occur in a drug concentration range which is called Mutant selection window (Zhao and Drlica, 2002). On the other hands, bacteria have different range of tolerance to antibiotics that are likely to be related to their genetic and physical properties. Some bacteria may survive in high concentration of antibiotics and produce mutants, while others may not survive in low concentration of antibiotics. These differences among bacterial isolates were observed in this study.

Antagonistic activity of isolates (mutants and wild types) used as seed coating against cotton seedling damping-off disease was studied in the greenhouse. Bacterization delivers antagonists of soil-borne pathogens to the target as close as possible. Seed

treatments place the antagonists in the infection court (the seed coat surface) at planting, before the seed can be attacked by the pathogen (Brannen and Kenney, 1997).

Results indicated that 3 mutant bacteria were more effective than their wild types in controlling cotton seedling damping-off disease. This suggests that spontaneous mutation could be an effective technique in enhancing the antagonistic activity of bacteria. Enhancing the activity of bacterial antagonisms may be attributed to the effect of spontaneous mutation on antagonistic mechanisms such as antifungal metabolites production, establishment, colonization and genetic characteristics.

Results of the greenhouse experiment showed significant differences among the number of healthy seedlings between all wild type and mutant isolates in the primary stage of seedlings growth (up to 30 days after sowing). However, 60 days after sowing, all of the mutants except one isolate were more effective than their wild types in biocontrol of cotton damping-off. This may be related to genetic or phenotypic differences among isolates and their survivability in the rhizosphere.

In general, the results of this study suggest that genetic alteration such as spontaneous mutation may cause some changes in the antagonistic efficiency and mechanisms of microorganisms. These changes observed in this study via comparison between the antibiotic-resistant mutants and their wild types in biocontrol of cotton seedling damping-off disease. It is important that in real plant environment (fields) many soil factors such as residues of herbicides, soil pH, soil moisture, etc. may affect the performance of biocontrol activity of microorganisms (Heydari *et al.*, 1997).

Another point related to this study is the applicability of using bacterial mutants as markers in the field to study interactions between environmental factors and these bacteria. These antibiotic-resistant mutants may also be used to study and determine the population changes and survival of antagonistic bacteria used for controlling soil-borne pathogens under field condition. For example, by using antibiotic-resistant mutants, the survival of *Pseudomonas viridiflava* on tomato leaves and some species of weeds (Mariano and McCarter, 1993), the effect of soil chemical pesticides on population of some antagonistic bacteria under field condition (Heydari *et al.*, 1997), the effect of soil environmental factors on population of bacteria (Heydari and Misaghi, 1998) and the population of *P. putida* to control root rot disease of citrus (Steddom and Menge, 2001) have been investigated.

Finally, the results of this study may have practical application in disease management strategies formulation. A better understanding of interactions between

environmental factors and antagonistic microorganisms as well as the changes resulting from these interactions may provide us useful information for increasing and enhancing the efficiency of the biocontrol agents used for suppressing plant diseases in the field.

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