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EVALUATION OF PSEUDOMONAS AND BACILLUS BACTERIAL ANTAGONISTS FOR BIOLOGICAL CONTROL OF COTTON VERTICILLIUM WILT DISEASE

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Abstract: Verticillium wilt is considered the most important disease of cotton in the world, including Iran. Cultural practices and the use of resistant varieties are the most common strategies used to control Verticillium wilt of cotton. These strategies are not always available or effective. In recent years, biological control using fungal and bacterial antagonists, has been applied to control some cotton diseases including damping-off. In this study, we investigated the possibility of biological control of Verticillium wilt of cotton using bacterial antagonists. Suspension of eight bacterial strains of *Pseudomonas fluorescens* and *Bacillus* spp. isolated from different rhizospheric soils and plant roots in the Iranian cotton fields, were prepared with a concentration of 10^8 cfu/ml. Ten cotton seeds (cv Varamin) were then coated with each bacterial suspension and were planted in soil pre-inoculated with *Verticillium dahliae* microsclerotia. The efficacy of bacterial antagonists in reducing wilt disease was evaluated by determination of the disease index in different treatments. The results indicated that most isolates were effective in reducing disease (compared to the untreated control) 90 days after sowing. Isolates B5, B6, B2, B7, and B3 were the most effective, respectively, in reducing wilt index. In contrast, isolates B1, B4, and B8 did not significantly reduce the disease. In general, P. *fluorescens* isolates were more effective than Bacillus isolates. This study suggests that bacterial antagonists might be potential biological control agents of cotton.

Key words: *Bacillus subtilis, B. coagulans, B. polymyxa, Cotton, Pseudomonas fluorescens, Verticillium dahliae*

INTRODUCTION

Cotton is an important economic crop around the world including Iran. Cotton is cultivated in about 20 provinces of Iran (Zaki *et al*. 1998; Heydari *et al*. 2007; Naraghi *et al*. 2007). Harmful pests (insects, weeds, and plant pathogens) are among the most important yield-reducing agents in cotton fields (Heydari and Misaghi 1998; Naraghi *et al*. 2006; Heydari *et al*. 2007; Naraghi *et al*. 2007).

Verticillium wilt caused by *Verticillium dahliae* is considered the most important disease of cotton in the world and Iran (Nadakavukaren and Horner 1959; Ausher *et al*. 1975; Naraghi *et al*. 2006; Heydari *et al*. 2007; Naraghi *et al*. 2007). Cultural practices and the use of resistant varieties are the most common strategies to control Verticillium wilt on cotton. These strategies, however, are not always available or effective. Biological control using fungal and bacterial antagonists have been applied to control plant diseases in recent years (Weller 1988; Thomashow and Weller 1990; Pierson and Weller 1994; Amer and Utkhede 2000; Collins and Jacobson 2003; Heydari and Misaghi 2003; Bharathi *et al*. 2004; Manjula *et al*. 2004; Jataraf *et al*. 2005; Selim *et al*. 2005; Heydari *et al*. 2007; Naraghi *et al*. 2007; Jorjani *et al*. 2011).

Bacterial antagonists have been shown to be effective in biological control of several plant diseases. The species that have mostly been used include *Pseudomonas fluorescens, P. putida, Bacillus* spp., and *Burkholderia cepacia*.. For example, Jataraf *et al*. (2005) successfully controlled damping-off disease of tomato caused by *Pythium aphanidermatum* using an isolate of *Bacillus subtillis* in green house conditions (Jataraf *et al*. 2005). In another study, lettuce root rot disease was controlled using some bacterial antagonists (Amer and Utkhede 2000).

Manjula *et al*. (2004) used *B. subtilis* against citrus root rot disease. Their results showed that bacterial antagonists effectively controlled the disease. In addition to the above studies, biological control of sugar beet Cercospora leaf spot disease (Collins and Jacobson 2003), sugar beet damping-off disease (Jorjani *et al*. 2011), and wheat Take-all disease (Thomashow and Weller 1990; Pierson and Weller 1994) have also been achieved by application of *Bacillus* spp. and *P. fluorescens* bacterial antagonists.

According to the results of the above-mentioned studies, different mechanisms and modes of action have

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been involved in the antagonistic ability of the applied bacteria. These mechanisms include antibiosis (antibiotic production), siderophore production, inducing systemic resistance (ISR), lytic enzyme secretion, and hormone production (Thomashow and Weller 1990; Pierson and Weller 1994; Amer and Utkhede 2000; Manjula *et al*. 2000; Collins and Jacobson 2003; Jataraf *et al*. 2004; Jorjani *et al*. 2011).

The objectives of the present study were to find an effective and ecologically-friendly strategy for management of Verticillium wilt in cotton using bacteria antagonistic to *V. dahliae*.

MATERIALS AND METHODS

Materials

Chemicals, microbial growth media, and the ingredients used were of laboratory chemical-reagent grade and were purchased from Tehran's chemical market, Iran. Culture media used in this study included Nutrient Agar (NA), King's B (KB), Potato Dextrose Agar (PDA) and Czapeck-dextrose broth (Difco, Detroit, MI). Cotton seeds (cv. Varamin) were obtained from the Iranian Cotton Research Institute.

Microbial cultures

In vitro screening had previously been done on the *P. fluorescens* and *Bacillus* spp. isolates used in the present study. They were obtained from the Microbial Culture Collection, Beneficial Microorganisms Research Laboratory, Iranian Research Institute of Plant Protection, Tehran, Iran. Bacterial cultures were routinely maintained on Kings B medium (KB) and were sub-cultured twice a month.

Preparation of bacterial suspensions

Bacterial cells were harvested after five days of growth in NA medium, centrifuged at 6,000 rpm for 15 min and re-suspended in phosphate buffer (0.01 M, pH 7.0). The concentration of the bacterial suspension was adjusted to approximately 10^8 cfu/ml using a spectrophotometer. The bacterial strains were kept at –80°C in 44% glycerol. Cells from stocks were first grown on KB medium to verify their purity. The inoculum was produced by transferring one loop-full from the culture to 100 ml of KB broth in a 250 ml Erlenmeyer flask and incubating at room temperature (25±2°C) on a shaker at 150 rpm for 48 h.

Preparation of *V. dahliae* inoculum for the greenhouse experiment

In this experiment, one isolate of *V. dahliae* was used (Naraghi *et al*. 2007). Preparation of the *V. dahliae* microsclerotia suspension was carried out according to the procedure described by Naraghi *et al*. (2007) as follows:

The fungus was maintained in 250 ml flasks containing 100 ml of Czapeck dextrose broth (Difco, Detroit,MI). Flasks were shaken continuously at 160 rpm for 17 days under ambient conditions (21–22°C). To collect microsclerotia, flasks were removed from the shaker and allowed to stand for 30 min so that microsclerotia settled on the bottom of the flask. The top layers containing fungal hyphae and conidia, were removed by decanting. Microsclerotia were re-suspended in 100 ml of sterile distilled water (SDW) and allowed to settle for 30 min. Additional floating hyphae and conidia were decanted. The re-suspending and settling procedure were repeated three times. Microsclerotia were then re-suspended in 100 ml of SDW and macerated twice for 1 min each time in an omnimixer (Sorvall, Newtown, CN) at full speed. After maceration, microsclerotia were suspended in SDW, allowed to settle for 30 min and the top layer decanted off and discarded. This procedure was repeated three times.

The slurry containing the microsclerotia was spread uniformly in a thin layer in a 9 cm diameter glass petri plate using one plate for each original flask. The slurry was dried in a laminar air flow hood for 1–2 h. Plates were then covered and transferred to a 30°C incubator for 30 h to destroy the remaining hyphae and conidia. Microsclerotia from each plate were re-suspended in 100 ml of SDW and macerated twice for 1 min in an omnimixer at full speed. Suspension related to each plate was allowed to settle for 30 min. The top layer was discarded and the bottom layer was suspended in 1 ml of SDW in a 1.6-cm-wide x 15-cm-heigh test tube.

Pot soil inoculation was carried out as follows:

Two hundreds microsclerotia were used for each gram of pot soil. One ml of suspension containing microsclerotia was poured in to a 2 cm-diameter petri plate and the number of microsclerotia was determined using a stereo-dissecting microscope.

Evaluation of the antagonistic effects of bacterial antagonists as seed treatment on pathogenecity of *V. dahliae* in the greenhouse

A completely randomized experiment with 10 treatments and four replications was designed and carried out in the green house. Each replication consisted of a 20-cm-diameter and 40-cm-height plastic pot containing three kg pasteurized field soil collected from the Golestan province cotton fields. Five seeds of the cotton cultivar Varamin were planted in each pot. Treatments included:

1. Soil and seed without inoculum (the negative control).
2. Soil inoculated with *V. dahliae* and untreated seeds (the positive control).
3. to 10. Soil inoculated with *V. dahliae* and seeds treated with suspension of each bacterial antagonist (B1-B8) (Table 1).

As table 1 indicates, eight bacterial isolates were used in this study from which four (B1-B4) belonged to different species of *Bacillus* spp. Among them, two isolates identified as *B. subtilis* (B1 and B2) and two other isolates (B3 and B4) belonged to *B. coagulans* and *B. plymyxa*, respectively. Other isolates (B5–B8) belonged to *P. fluorescens.* All antagonists (B1–B8) shown in table 1 were isolated from the roots of cotton plants in two different cotton growing area of the Golestan province in Iran (Gorji Mahalleh and Karkandeh).

The Disease Infection Index was determined 90 days after planting according to the procedure described by Iakutkin and Popov by evaluating the percent of wilt symp-

Table 1. Characteristics of bacterial antagonistic isolates evaluated against Verticillium wilt of cotton under greenhouse conditions in Iran

No.	Isolate code	Isolate identification	Isolation host	Isolation location
1	B1	*Bacillus subtilis*	cotton	Gorji Mahalleh – Golestan Province
2	B2	*B. subtilis*	cotton	Karkandeh – Golestan Province
3	B3	*B. coagulans*	cotton	Karkandeh – Golestan Province
4	B4	*B. polymyxa*	cotton	Gorji Mahalleh – Golestan Province
5	B5	*Pseudomonas fluorescens*	cotton	Karkandeh – Golestan Province
6	B6	*P. fluorescens*	cotton	Karkandeh – Golestan Province
7	B7	*P. fluorescens*	cotton	Karkandeh – Golestan Province
8	B8	*P. fluorescens*	cotton	Karkandeh – Golestan Province

toms on the leaves of cotton plants in different treatments (Naraghi *et al.* 2007). Collected data were subjected to analysis of variance (ANOVA) and the mean comparison was performed using Co-Stat statistical software (CO-HORT, CA, USA).

RESULTS

Results of Verticillium wilt index on cotton leaves 90 days after planting, are presented in table 2. The Verticillium wilt index and percent of disease varied among treatments, with five out of eight bacterial antagonists (B2, B3, B5, B6, and B7) reducing wilt index and disease percent significantly (compared to the untreated control). Isolate B5 was the most effective with a 60% disease reduction compared to the untreated control (Table 2). Isolates B6, B2, B7, and B3 were also significantly effective in reducing disease index and incidence (Table 2). Although the remaining three isolates (B1, B4, and B8) reduced the disease index, the reduction was not statistically significant compared to the untreated control (Table 2).

DISCUSSION

The use of beneficial micro-organisms for the management of plant diseases have gained the attention of agricultural scientists in recent years. Bacterial antagonists are capable of employing several different antagonistic mechanisms, such as: antibiosis, siderophore production, various enzyme secretion, hormone production, and inducing systemic resistance in host plants (Thomashow and Weller 1990; Pierson and Weller 1994; Amer and Utkhede 2000; Manjula *et al*. 2000; Collins and Jacobson 2003; Jataraf *et al*. 2004; Jorjani *et al*. 2011). Due to these modes of action, they have been accepted as strong potential candidates for biological control of plant pathogens, particularly soil-borne ones, such as *V. dahliae* – the cause of Verticillium wilt in cotton, for which a chemical control method is not available.

Results of the present study indicate the possibility of managing cotton Verticillium wilt disease efficiently by seed treatment with antagonistic bacteria. According to the results, most of the bacterial isolates showed effectiveness in controlling and reducing Verticillium wilt disease. The inhibitory effects of several bacterial antagonistic iso-

Table 2. Effect of bacterial antagonists on Verticillium wilt disease on cotton plants, 90 days after planting, under greenhouse conditions in Iran

No.	Treatment code	Treatment description	Disease index	Disease percent
1	Control-	soil without fungal inoculum + untreated seeds	*0 f	0
2	Control+	soil with fungal inoculum + untreated seeds	3.14 a	79
3	B1	soil with fungal inoculum + seeds treated with B1 bacterium	2.97 a	74
4	B2	soil with fungal inoculum + seeds treated with B2 bacterium	1.40 cd	35
5	B3	soil with fungal inoculum + seeds treated with B3 bacterium	2.03 b	51
6	B4	soil with fungal inoculum + seeds treated with B4 bacterium	2.81 a	70
7	B5	soil with fungal inoculum + seeds treated with B5 bacterium	0.74 e	19
8	B6	soil with fungal inoculum + seeds treated with B6 bacterium	1.11 de	28
9	B7	soil with fungal inoculum + seeds treated with B7 bacterium	1.78 bc	44
10	B8	soil with fungal inoculum + seeds treated with B8 bacterium	2.90 a	73

*each figure is the average of four replicates. Figures marked with the same letters are not statistically different ($p < 0.05$)

lates in controlling and reducing Verticillium wilt and other fungal pathogens causing different plant diseases have been documented in previous studies (Thomashow and Weller 1990; Pierson and Weller 1994; Amer and Utkhede 2000; Manjula *et al*. 2000; Collins and Jacobson 2003; Jataraf *et al*. 2004; Jorjani *et al*. 2011).

In this study, *P. fluorescens* isolates performed relatively more effectively than Bacillus isolates in reducing wilt disease of cotton. This could be due to the various antagonistic mechanisms of *P. fluorescens* bacteria such as antibiosis, siderophore production, hormone production, and inducing systemic resistance in host plants that has been shown in some previous studies (Kloepper *et al*. 1980; Thomashow and Weller 1990; Pierson and Weller 1994; Amer and Utkhede 2000; Chen *et al*. 2000; Manjula *et al*. 2000; Collins and Jacobson 2003; Jataraf *et al*. 2004; Jorjani *et al*. 2011). Although Bacillus bacteria have shown some of the above-mentioned new mechanisms such as antibiotic production and spore formation, they do not show others, including siderophore production (Chen *et al*. 2000; Bharathi *et al*. 2004).

The results obtained in this study are promising, and suggest that bacterial antagonists are potential candidates for biological control of Verticillium wilt disease in cotton and its causal agent (*V. dahliae*). As was mentioned previously, cotton is an economically important crop around the world, including Iran, and Verticillium wilt is an important disease of cotton with no effective control methods available. The results of this study could, therefore, be helpful in the formulation of an effective and ecologically-friendly strategy for the management of this destructive disease. Such a formulation could play an important role in the integrated management of Verticillium wilt disease and possibly other cotton diseases.

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