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Antagonistic Potentials of Rhizosphere-associated Bacterial Isolates Against Soil-borne Diseases of Tomato and Pepper Caused by *Sclerotinia sclerotiorum* and *Rhizoctonia solani*

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Abstract: By use of selective media, putative antagonist bacterial isolates were isolated from rhizosphere-associated soils from different parts of the Amik plain. Isolates to be tested were chosen in approximate proportion to their abundance on the plates. Selected 113 bacterial isolates were screened for *in vitro* antagonism towards two important soil-borne root infecting plant pathogens, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* by using dual culture test. From this test, significant numbers of bacterial isolates, but not the yeast, were found to produce inhibition zones by inhibiting the hyphal growth of fungal pathogens to a varying degree. On the basis of the result obtained from the preliminary screening test, isolates belonging to *Bacillus* spp. and *Pseudomonas* spp. found to be highly efficient against fungal pathogens used in the experiment. Among the inhibitory isolates, bacterial isolates AKB50 and AFP104 significantly suppressed hyphal growth of *S. sclerotiorum* and *R. solani* by 75.3 and 83.3%, respectively. This study indicates that selected bacterial strain have potential for controlling of soil-borne disease agents.

Key words: Antagonist, biological control, soil-borne pathogen, *Rhizoctonia*, *Sclerotinia*

INTRODUCTION

Sclerotinia sclerotiorum and *Rhizoctonia solani* are both soil-borne plant pathogenic fungi that are widely distributed in worldwide[1]. These pathogen causes damping off, root rot, stem canker, blight and wilt on a range of agronomic and vegetable crops growing in green house or open fields resulting in large substantial mortality of the plants and consequent reduction in yield every growing season[1]. Both pathogens survive in soils for several years by mass of specialized survival fungal structures such as sclerotia[2]. The increasing demand for a steady crop production to the growing world population will require controlling of plant disease that reduce crop yield substantially. Current practices for controlling plant diseases are based largely on development of resistant varieties and application of synthetic pesticides[3]. The use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens. Host resistance to the soil-borne pathogen is not usually available for most crops[4]. Crop rotations of 3 to 4 years may control some extend, but this is often not an option in many agronomically important crops growing areas in Hatay province, because of economic pressure to growers and high demands during winter period[5]. The high cost of pesticides, development of fungicides resistance pathogen isolates, governmental restriction on the use of chemicals, interest of environmental considerations and the difficulty in finding suitable rotation crops to reduce pathogen inoculum raises the need to find alternative control methods.

One such alternative is biological control, in which microorganisms are selected for their ability to antagonize plant pathogens. Biological control of soil-borne pathogens with antagonist microorganisms has been studied as an alternative or complementary approach to physical and chemical disease control measures for over 70 years[3,6].

The rhizosphere is the soil surrounding the root that is subject to the influence of root exudates[7]. Intense microbial activity and greater microbial populations occurs in this micro-environment because of the release of large amount of organic matter from roots. The rhizosphere is also the site for interactions between plants pathogenic microorganisms and antagonist rhizobacteria and fungi[8]. Plant growth-promoting bacterial species

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(collectively known as Plant-growth Promoting Rhizobacteria, PGPRs) exert their beneficial effects via several ways. Bacterial antagonism towards plant pathogenic fungi may be due to the production of antibiotics, nutrient competitions, or parasitism[9,10]. The relative importance of these mechanisms may differ considerably among rhizobacterial strains. Antagonistic PGPRs have often been the focus of research in sustainable agricultural system because of their ability to suppress diseases caused by soil-borne plant pathogens[3,11,12].

The objectives of this study were to isolate antagonist microorganisms from the rhizosphere of different agronomically important plants, such as pepper, tomato and cotton, growing in the different part of the region of Amik Plain; preliminary their characterization and to evaluate their antagonistic potential against soil-borne fungal pathogens *S. sclerotiorum* (an Ascomycete with a glucan containing cell wall) and *R. solani* (a Basidiomycete with a chitin containing cell wall) *in vitro* by using dual culture test.

MATERIALS AND METHODS

Soils: Twenty nine soils used for pepper, tomato and cotton cultures throughout the Amik plain were selected and sampled during spring and summer period of 2003. Selection was based on a survey suggesting that pepper, tomato and cotton produced in those soils showed less symptoms of damping off stem and root rot caused by *S. sclerotiorum* and *R. solani*. Soil cores were randomly sampled to a depth of 10-20 cm from different regions.

Microbial populations and isolation of microorganisms: Microbial populations were evaluated using plate count serial dilutions on selective media in petri dishes. Ten gram of each soil was mixed with 20 mL of sterile distilled water containing peptone (10 g L^{-1}) and homogenized for 45 sec using an electric blender. Serial 1:10 dilutions were performed and 0.1 mL of each dilution was spread on selective media. Petri dishes were then incubated at 15 or 248°C for 3 and 2 days, respectively. Three replicates were performed for each dilution. Total microorganisms and gram-negative and positive bacteria were evaluated on Tryptic Soy Agar 10% (TSA).

Identification of putative antagonistic bacteria and fungal pathogen: Putative antagonistic bacteria and yeast appeared on the plates and were individually isolated as a single colony. All bacterial isolates were characterized according to Bergey's Manual of Systematic Bacteriology[13,14]. In brief, all isolates were examined for oxidase reaction, Gram's and KOH reactions, motility, spore formation and production of catalase. Plates of KB medium were examined under UV light (366 nm) for colonies producing diffusible fluorescent pigments to identify fluorescent *Pseudomonas* isolate.

The fungal isolates S. *sclerotiorum* and *R. solani* used in this study were isolated from the surface disinfected diseased pepper and tomato plants exhibiting typical disease symptoms[5]. Fungal isolates were plated on potato dextrose Agar (PDA, Merck) amended with penicillin and tetracycline (50 μg mL^{-1}). Replicate plates were incubated at 25°C. Fungal isolate was re-inoculated on to pepper and tomato seedling and found to be highly pathogenic. Fungal culture was maintained at 4°C and sub-cultured every second month.

Selection of bacteria for ability to inhibit *in vitro* fungal growth: Isolates to be tested as potential antagonists were selected randomly from the single colony present on agar plates such that representative organisms from all colony and morphological types present were collected in approximate proportion to their abundance on the plates.

Presumptive antagonistic bacterial isolates were screened on PDA media for their ability to inhibit fungal growth in dual petri dish culture test as described by Landa *et al.*[15]. Briefly, two bacterial isolates were streaked at equidistant points along the perimeters of the each petri dishes. Plates subsequently incubated at 27°C for 2 days. Following bacterial growth, mycelial plug (5 mm) from the advancing edge of the 7 days old fungal culture on PDA were deposited in the centre of the plates, approximately 4 cm away from the each edges of the bacterial mass. Plates without bacteria were used as control. Plates were then incubated at 25°C and checked after 4-7 days when the fungal mycelium had reached the edge of the plate in control petri dishes. The radius of fungal growth towards the bacteria and control petri dishes were measured 4-7 days after inoculation and % inhibition of fungal growth was calculated in relation to the control treatment. SPSS statistic program was performed for all calculations and the significance was determined by means of Duncan's Multiple Range Test ($p<0.01$).

RESULTS

Using the combination of techniques for the isolation procedures described above, total of 113 bacterial and yeast isolates were obtained from the rhizosphere-associated soils from different regions of Amik Plain. All collected isolates were screened individually for their ability to suppress fungal growth in dual culture test.

Table 1: *In vitro* Fungal Growth Inhibition (FGI) of *S. sclerotiorum* and *R. solani* by selected antagonistic bacterial isolates

Bacterial isolates	Fungal pathogens: *S. sclerotiorum* FG (mm)	*S. sclerotiorum* FGI (%)	*R. solani* FG (mm)	*R. solani* FGI (%)	Bacterial isolates	Fungal pathogens: *S. sclerotiorum* FG (mm)	*S. sclerotiorum* FGI (%)	*R. solani* FG (mm)	*R. solani* FGI (%)
Control	59.6r	-	58.7y	-	Control	59.6r	-	58.7y	-
ASP3	18.7d	68.7	24.8mn	57.7	AKB51	17.8d	70.2	18.9de	67.8
AFP4	59.3r	0.0	28.7p	51.1	AKB53	21.8e	63.5	15.7b	73.3
AKB5	15.7b	73.7	18.3cd	68.8	AFP55	34.8n	41.7	37.7t	35.8
ABP7	25.3fgh	57.5	22.8kl	61.1	AKB57	24.6fg	58.8	20.3efg	65.3
ABP8	30.3n	49.2	24.7mn	57.9	AKB58	30.7n	48.5	19.3cde	67.1
ABP9	40.3o	32.4	59.3y	0.0	AKB60	27.9jk	53.2	26.7o	54.5
AKB12	26.6ij	55.4	20.3fgh	65.3	AKB61	25.1fgh	57.9	21.8ijk	62.8
AKB13	24.7fg	58.7	21.3hij	63.6	AKB62	26.0ij	56.4	22.4jk	61.8
AKB14	28.8l	51.7	21.7ijk	63.1	ASP64	25.6ghi	57.1	28.9r	50.7
AKB21	16.7c	72.1	14.8b	74.8	AFP65	59.3r	0.0	25.1no	57.2
AKB24	22.3e	62.6	20.1fg	65.7	AKB70	24.9fg	58.3	22.8kl	61.1
AKB26	24.7fg	58.7	25.8no	56.0	ASP75	28.6l	52.1	36.7t	37.4
ASP28	59.8r	0.0	28.9p	50.7	AFP85	28.7l	51.9	22.8kl	61.1
ASP31	24.7fg	58.7	30.9r	47.3	AKB89	26.5ij	55.6	17.2c	70.7
ABP32	59.9r	0.0	43.7u	25.6	ASP97	40.1o	32.8	59.6y	0.0
AKB36	25.4fgh	57.4	18.9de	67.8	AKB101	27.2jk	54.4	19.7ef	66.5
AKB37	30.3m	79.7	20.1fg	65.7	AFP104	30.9m	48.2	9.8a	83.3
AFP40	28.1k	52.9	24.8mn	57.7	AKB108	18.8d	68.5	14.8b	74.8
AKB44	24.8fg	58.4	22.2jk	62.2	AKB110	27.1jk	54.6	24.7mn	58.0
AKB45	22.9e	61.6	19.8ef	66.3	AKB113	28.7l	51.9	20.2fgi	65.6
AKB46	30.1m	49.6	23.9lm	59.3	AKB126	30.1m	49.6	24.7mn	58.0
ASP49	50.8p	14.9	46.8v	20.2	AKB129	26.8ij	55.1	25.1no	57.2
AKB50	14.7a	75.3	21.0gh	64.2					

Bacterial isolates were applied to PDA plates 2 days before inoculation with *S. sclerotiorum* or *R. solani*. The length of the fungal growth toward to the antagonistic bacterial colony was assessed after 4 days of incubation at 25°C. The isolates written in bold character indicate the most efficient isolates against to tested fungal pathogen (i.e., AKB50 against *S. sclerotiorum*, AFP104 against *R. solani*). Values are the mean of 5 replicate plates. The means, for each fungal pathogen, in a column followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test ($p<0.01$)

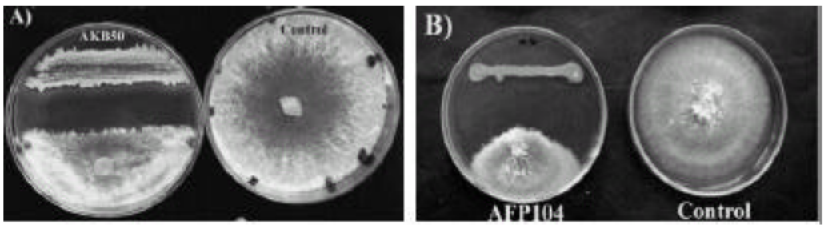

Fig. 1: Hyphal growth inhibition of *Sclerotinia sclerotiorum* (A) and *Rhizoctonia solani* (B) by the most efficient antagonist bacterial isolate *Bacillus* spp. AKB50 and *Pseudomonas* spp. AFP104

From this test, 45 bacterial isolates (39.8%) were found to produce inhibition zones by inhibiting the hyphal growth of both fungal pathogens to a varying degree. Among the antagonistic bacterial isolates, 17 isolate (37.8%) originated from 13 different fields that were developed under wheat-cotton polyculture, 12 isolates (26.7%) from 5 different fields that were developed under wheat-maize polyculture, 10 isolates (22.2%) from 6 different fields that were developed under carrot-tomato polyculture and 6 isolates (13.3%) from 5 different fields that were developed under wheat-pepper polyculture. The 28 isolates were all gram-positive and, the greater number, able to produce endospore (*Bacillus* spp.). The 17 isolates were gram negative that six of them produced fluorescent siderophore on KB plates (fluorescent *Pseudomonas* spp.).

Antagonistic activities of selected bacterial isolates were screened against *S. sclerotiorum and R. solani*. The extent of hyphal growth inhibition was significantly ($p<0.01$) influenced by bacterial isolates tested (Table 1). Among the inhibitory bacterial isolates, 41 bacterial isolates were able to inhibit the growth of *S. sclerotiorum*, 43 isolates were able to inhibit the growth of *R. solani in vitro* conditions. The 39 isolates were able to inhibit both fungal growths. In the case of *S. sclerotiorum*, isolate of *Bacillus* spp., AKB50, showed the strongest antagonistic activity among the bacterial isolates tested (Fig. 1A) and suppressed the mycelial growth by 75.3%. In the case of *R. solani,* isolate of fluorescent *Pseudomonas* spp., AFP104, showed the strongest antagonistic activity among the all-antagonistic bacterial isolates tested (Fig. 1B). This isolate suppressed the mycelial growth by 83.3%.

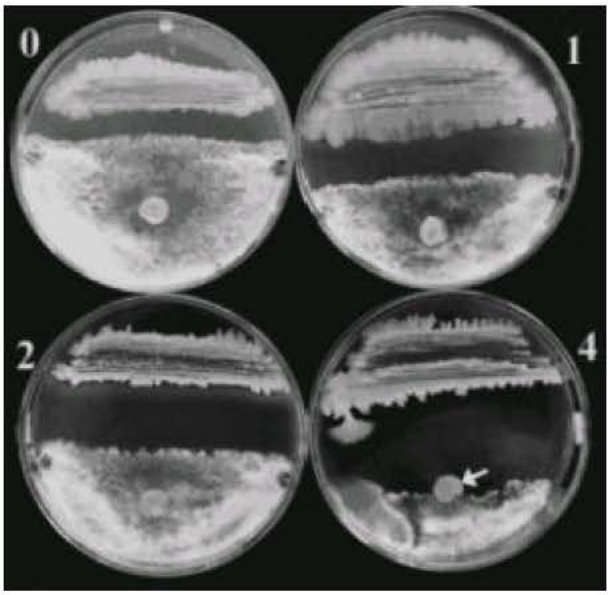

Fig. 2: Effect of pre-incubation time on the antagonistic activity of *Bacillus* spp. AKB50 *in vitro* dual culture test against *S. sclerotiorum*. Bacterial isolate was applied to PDA plates 0, 1, 2 and 4 days before inoculation with *S. sclerotiorum*. The length of the inhibition zone, away from the antagonistic bacterial colony, was assessed after 4 days of incubation at 25°C. Note complete hyphal growth inhibition (arrow) towards bacteria on which bacterial isolate was streaked 4 days before fungal inoculation

Inhibition zone observed with *Bacillus* isolate increased proportionally with the preincubation time of bacteria prior inoculation with the fungal pathogen. Complete fungal growth inhibition was observed on PDA plate in which antagonistic bacteria was streaked 4 days before fungal inoculation (Fig. 2).

DISCUSSION

Soil-borne fungal pathogens cause considerable damage to crop plants and they have been often targeted in biocontrol. The region of soil surrounding and including the plant root (the rhizosphere) is of crucial importance for plant health and nutrition[8]. The rhizosphere harbors a large and diverse community of prokaryotic and eukaryotic microbes that interact and compete with each other and with the plant root. Activity of any one member of this community affects the growth and the physiology of the others and also affects the physical and chemical properties of the soil. Together these interactions set up gradients along individual roots for mineral nutrients, pH, redox potential, reducing processes, root exudates and microbial process. Rhizosphere-inhabiting bacteria, belonging to genus of *Bacillus*, *Pseudomonas* and *Streptomyces*, have been shown to play an important role in the biological control of soil-borne fungal pathogens[6].

In the present study, using standard methods, 113 bacterial isolates were isolated from the different rhizosphere-associated soils, screened and selected putative isolate for biological control of two important soil-borne fungal pathogens, *S. sclerotiorum* and *R. solani*. From this screening, forty-five gram-negative and gram-positive bacterial isolates have shown antagonistic activity against the both fungi. Several species of the gram-negative bacteria, especially fluorescent *Pseudomonas* spp. such as *P. fluorescence* and *P. aeruginosa,* isolated from the rhizosphere several plant species have demonstrated antagonistic activity against sclerotial pathogens such as *Rhizoctonia solani*, *Macrophomina phaseolina* and *Sclerotinia rolfsii*[15-19]. Different mechanisms, such as competition for nutrients and space, antibiosis, production of siderophores and lytic enzymes, are involved in the suppression of these soil borne plant pathogens[9,20-22]. Involvement of siderophore production in inhibition of fungal growth by fluorescent *Pseudomonas* used in this study is ambiguous, since we observed inhibition zone in dual culture test on PDA medium, which is iron rich media. This result indicates that not only siderophore production but also other mechanism(s) that affect the growth of the fungus may be involved in the inhibition of hyphal growth of fungal pathogens tested. Antibacterial, antifungal and anti-nematode activity has been identified

in the antibiotic producing strains of a wide range of bacterial genera, but most notably from *Pseudomonas* spp. (2,4-diacetyl phloroglucinol, phenazine derivatives, pyoleutorin and pyrrolnitrin), *Erwinia* sp. (herbicolin A) and *Bacillus* sp. (iturin A, surfactin)[10,23,24]. In addition, induction of resistance by fluorescent Pseudomonads is an additional mechanism by which these bacteria protect several crop plants against pests and diseases[25,26]. Several gram-positive bacterial isolates of the genus *Bacillus* also displayed strong antagonism against fungal isolates tested. *Bacillus* species are characterized by their ability to form highly resistant endospore and to produce a wide range of antibiotics, which have been shown to suppress the growth of several plant pathogen fungi[27]. Unlike to *Pseudomonas*, the gram-positive bacteria, like *Bacillus* spp., however, have been studied less intensively than the gram-negative bacteria. *Bacillus* spp. is considered to be a safe biological agent[16] and their dormant structures, such as endospores, are resistant to desiccations, heat and several stress conditions, which allow this microbial agent for further formulation and commercialization procedures[28]. *Bacillus* species are therefore ideal candidates for use as biocontrol agents in seed treatment programs against soil-borne pathogen[29].

The results of this study indicate that fluorescent *Pseudomonas* and *Bacillus* spp. are the dominant antagonists in soils of Amik Plain, although other organisms may contribute to disease suppression. Selected isolates showing strong fungal antagonism are promising for future applications of these bacteria in biological control of soil-borne pathogens. Further studies is being undertaken to identify efficient bacterial isolate into species level; to study which mechanism(s) are involved in *in vitro* suppression of fungal growth and their possible use in greenhouse and/or field conditions. Subsequent analytical studies would lead to identification of compounds involved in disease suppression *in vitro* studies and to develop these antagonists as biocontrol agents.

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