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In vitro Antagonistic Activity of Chinese Isolates from Herbal Rhizosphere against Major Plant Fungal Pathogens

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Our results in the present study demonstrated that bacteria isolated from rhizosphere of plants had potential inhibitory activity against major fungal plant pathogens. Totally 116 bacterial isolates have been obtained from the rhizospheres of various herbal plants at various locations of Qinghai province in China. Some of the isolates have strongly inhibited the growth of the major plant pathogenic fungi, while, certain bacteria have not shown any antagonistic activity. The screening results indicated that tested bacterial species exhibited varying degree of antagonistic potential against most of the plant pathogenic fungi, and these strains can be used as promising biocontrol agents. Studies are under the way to find the possible action mechanisms and to seek the application of effective bacterial isolates in greenhouse conditions for biocontrol of various diseases in the vegetable crop plants.

Key words: Antagonism, Plant Fungal Pathogens, Rhizobacteria.

Biological control based on microorganisms to suppress plant disease, offers a powerful alternative to synthetic chemicals. A significant high number of fungal diseases have an influence on crop plants throughout the year when a farmer fails to take proper preventative measures. Plant disease control, therefore has become heavily dependent on fungicides to combat the wide variety of fungal diseases. The use of chemical pesticides or fungicides to cure or prevent plant diseases cause soil pollution and detrimental effects in humans. Over the past few decades, agricultural production has increased and the farmers rely on chemical pesticides as a relatively dependable method of protecting plants against soil-borne pathogens[1], and it has been found that the increasing use of chemical pesticides causes several negative effects on the environments as well as on human health[1]. Over the past decades, efforts have been directed towards developing new alternatives to chemical control to achieve better disease control[3].

The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been the subjects of many studies in the past two decades[4]. The search for new antimicrobial agents is a challenging in disease management. Soil is the abundant of beneficial microbes for the plant. The disease-suppressive soils have been described for several soil-borne plant pathogens[5].

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Although the abiotic factors of the soil are believed to play a role, there is also evidence that microbial activity contributes to disease suppression[6]. Thus, such soils are regarded as sources of natural, effective and valuable antagonistic for the purpose of biological control. Plant protection is an important area, which needs attention due to hazardous inputs of chemical control[7].

Biological control may be alternative to the chemicals in the control of some pathogenic fungi in order to reduce environmental pollution[8]. Many microorganisms have been studied for plant protection against plant pathogens for many years[9, 10]. Abundant antagonists have successfully controlled plant diseases in greenhouse trials, whereas field application seems to be less successful[11]. Many studies have reported on natural activity of some fungi and bacteria against fungal pathogens, and this is considered as an alternative to the use of chemical fungicides[2]. Additionally, an alternative advanced strategy for plant protection is using a systemic induced resistance on crops, which can be triggered by pre-inoculation of plant growth promoting rhizobacteria (PGPR) that are available around the root zone of the agricultural crops[12]. The aim of this study was to investigate the *in vitro* antagonistic activity of the bacterial isolates obtained from herbal rhizosphere at different locations of Qinghai province in China against important plant pathogenic fungi.

MATERIAL AND METHODS

Isolation of bacterial strains and culture conditions

Rhizobacteria were isolated from the rhizosphere of 25 different wild types of herbal plants at high altitude locations of Qinghai province in China. Nearly one gram of fresh root sample was surface disinfected with sodium hypochlorite (4%) for 5 min, followed by 70% ethanol for 2 min and finally rinsed with sterile distilled water (SDW) several times. The sample was ground by pulverization in sterilized phosphate-buffered saline (PBS, pH 7.3). Diluted soil samples (10^6 and 10^5) were spread on nutrient agar (0.3% beef extract, 0.5% peptone and 1.5% agar) plates. Plates were incubated for 48 h at 28°C to observe the colonies of bacteria. Bacterial colonies were streaked to other LB agar plates and the plates were incubated at 28°C for 48 h. Typical bacterial colonies were observed over the streak. Well isolated single colonies were picked up and re-streaked on LB agar plates. After 48 h of incubation, a total of 116 bacterial isolates were selected based on various characteristics of colonies such as shape, size, color, and pigmentation. Purified strains were stored on nutrient medium at 4°C for 10-15 days. These strains were maintained at -80°C in trypticase soy broth (TSB) with glycerol (20%) for long-term storage. For preparing bacterial suspensions, culture from -80°C was grown on trypticase soy agar (TSA) for 24 h at 28°C, and single colonies were transferred to TSB and incubated at 28°C for 24 h with shaking at 150 rpm. Bacteria were pelleted after centrifugation for 5 min at 8,000-xg and resuspended in SDW to a final concentration of 1×10^8 cfu/ml.

Culturing of plant pathogenic fungi

The plant fungal pathogens namely, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora capsici*, *Colletotrichum acutatum*, *Botrytis cinerisa*, *Alternaria alternate* and *Pythium ultimum* were obtained from plant pathology laboratory, NAAS, Korea. They were maintained in potato dextrose agar (PDA).

Assessment of the *in vitro* antagonistic activity

The antifungal activities of the Chinese isolates were assessed against the major plant pathogenic fungi stated above. One day old grown cultures were dropped onto a sterile disc (6 mm diameter), and placed at the edges of a Petri dish with equal spacing around the plate containing PDA and TSA (Difco, Detroit, MI, USA) in 1:1 ratio; then a fully grown mycelium disk (6 mm diameter) prepared with sterile cork borer was placed at the center of each PDA+TSA plate. All fungi were grown on the medium at room temperature for 7 days. Suppression of the fungal growth was observed by the appearance of a clear zone between the bacterial disc and the fungal mycelium.

RESULTS AND DISCUSSION

In the present study, screening of the antagonistic activity of 116 bacterial isolates was carried out against seven major plant fungal pathogens. Our screening results showed that

Table 1. Preliminary screening of antagonistic activity using Chinese isolates against plant fungal pathogens by disc diffusion method (mm)

Bacterial strains from China		Plant fungal pathogens						
		RS	PC	FO	CA	BC	AA	PU
Control	-	-	-	-	-	-	-	
1	CP1-1	-	-	-	+++	-	++	-
2	CP1-2	-	-	-	-	-	++	-
3	CP1-3	-	-	-	-	-	-	-
4	CP2-1	-	-	-	++	-	-	-
5	CP2-2	-	-	-	-	-	++	+
6	CP2-3	-	-	-	++	-	-	-
7	CP2-4	-	+	-	++	+	++	-
8	CP3-1	-	-	-	+++	+	-	-
9	CP3-2	+	-	-	+++	++	++	++
10	CP3-3	++	-	-	+++	++	++	-
11	CP3-4	-	-	-	++	-	-	++
12	CP4-1	-	-	-	++	-	-	-
13	CP4-2	-	-	-	+++	-	-	-
14	CP4-3	-	-	-	+++	-	-	++
15	CP4-4	-	-	-	++	-	-	-
16	CP5-1	-	-	-	++	-	-	-
17	CP5-2	-	-	-	+	-	-	-
18	CP5-3	-	-	-	++	-	-	-
19	CP5-4	-	++	+	++	++	++	-
20	CP6-1	+	++	-	+++	++	-	-
21	CP6-2	-	+	-	+++	++	-	+
22	CP6-3	-	-	-	++	-	-	-
23	CP6-4	-	-	-	+++	++	-	-
24	CP7-1	++	+	-	+++	++	-	-
25	CP7-2	-	-	-	-	-	-	-
26	CP7-3	-	-	-	++	-	-	-
27	CP7-4	-	-	-	-	-	-	-
28	CP8-1	++	+++	-	+++	+++	+++	+++
29	CP8-2	-	-	-	++	++	++	+
30	CP8-3	++	-	-	+++	+	-	-
31	CP8-4	++	-	-	++	+++	++	-
32	CP9-1	-	-	-	+++	+++	+++	+
33	CP9-2	+	-	-	+++	+	-	-
34	CP9-3	++	-	-	+++	+++	+++	-
35	CP9-4	+	-	-	++	+	++	+
36	CP9-5	++	-	-	++	++	-	-
37	CP10-1	+	-	-	++	+++	+++	++
38	CP10-2	+	-	-	++	++	++	+
39	CP11-1	++	-	-	++	++	+++	-
40	CP11-2	-	-	-	++	++	++	+
41	CP11-3	++	-	-	++	++	+++	++
42	CP13-1	+	-	-	+++	++	++	++
43	CP13-2	+	++	-	++	++	+	-
44	CP13-3	-	-	-	+++	++	-	-
45	CP13-4	-	-	-	+	-	-	-
46	CP13-5	-	++	-	++	-	-	-
47	CP14-1	+	+	-	+++	++	+++	-
48	CP14-2	++	++	-	+++	++	-	++

49	CP15-1	-	-	-	++	-	++	-
50	CP15-2	+	++	-	+++	-	-	-
51	CP15-3	++	++	-	+++	+++	-	++
52	CP15-4	-	++	-	-	++	++	-
53	CP15-5	-	+	-	+++	-	-	-
54	CP16-1	++	+++	-	+++	-	+++	-
55	CP16-2	-	+	-	+++	-	+++	-
56	CP16-3	-	-	-	+++	-	-	-
57	CP16-4	-	-	-	++	++	-	-
58	CP16-5	+	+	-	-	++	+++	-
59	CP17-1	-	-	-	-	+	-	-
60	CP17-2	+	-	-	-	++	-	++
61	CP17-3	-	-	-	-	++	-	-
62	CP18-1	-	+	-	-	++	-	-
63	CP18-2	-	-	-	-	-	-	-
64	CP18-3	-	-	-	+++	++	-	-
65	CP18-4	-	-	-	+++	-	-	-
66	CP19-1	+	+++	-	+++	+++	-	-
67	CP19-2	-	-	-	++	++	-	-
68	CP19-3	-	-	-	+	-	-	-
69	CP19-4	-	-	-	+++	++	-	+
70	CP20-1	-	-	-	+++	+++	-	-
71	CP20-2	+	+	-	+++	+++	-	-
72	CP20-3	-	+	-	++	++	-	-
73	CP20-4	-	-	-	-	+	-	-
74	CP20-5	+	-	-	-	+	-	-
75	CP21-1	+	-	-	-	+++	++	-
76	CP21-2	+++	+++	-	++	+++	+++	+
77	CP21-3	-	+	-	++	++	-	+
78	CP21-4	-	-	-	++	+++	-	+
79	CP21-5	+	++	-	+++	+++	+++	-
80	CP22-1	-	-	-	++	++	-	-
81	CP22-2	-	-	-	++	++	-	-
82	CP22-3	+	++	-	++	++	-	+
83	CP22-4	-	-	-	++	++	++	-
84	CP23-1	++	++	++	+++	++	++	++
85	CP24-1	-	-	-	++	+++	++	-
86	CP24-2	-	+++	-	++	++	++	++
87	CP24-3	-	-	-	++	+++	++	-
88	CP24-4	-	-	-	-	+++	-	-
89	CP24-5	-	-	-	++	-	-	-
90	CP25-1	-	-	-	++	+	++	-
91	CPS10-2-1	-	-	-	++	-	-	-
92	CPS10-3-1	-	-	-	++	+	-	-
93	CPS10-4-1	+	+++	-	++	++	-	+++
94	CPS10-5-1	-	-	-	++	-	-	-
95	CPS10-9-1	-	-	-	++	-	-	-
96	CPS10-11-1	++	+++	-	++	++	++	+
97	CPS10-13-1	-	-	-	-	-	-	-
98	CPS10-14-1	-	++	-	-	-	-	-
99	CPS10-15-1	++	+++	-	++	++	-	++
100	CPS10-16-1	+	++	-	++	++	-	++
101	CPS10-16-2	-	-	-	-	-	++	-
102	CPS10-17-1	-	-	-	-	++	-	-
103	CPS10-17-2	-	-	-	-	-	-	-

104	CPS10-17-3	-	-	-	-	-	-	-
105	CPS10-18-1	++	++	-	++	-	-	+++
106	CPS10-18-2	+	++	-	++	-	-	+++
107	CPS10-19-1	-	-	-	-	-	-	+++
108	CPS10-20-1	-	-	-	-	-	-	-
109	CPS10-20-2	-	+	-	-	++	++	-
110	CPS10-21-1	-	++	-	-	++	++	-
111	CPS10-22-1	-	-	-	-	-	-	-
112	CPS10-23-1	+	++	-	-	+++	++	++
113	CPS10-24-1	-	-	-	-	-	-	-
114	CPS10-25-1	++	++	-	++	-	-	-
115	CP14-3	-	++	+	+++	-	++	++
116	CP14-4	-	++	+	+++	-	++	++

+: less activity (d”12 mm); ++: moderate activity (13-16 mm); +++: high activity (e”17 mm); -: No activity; RS: *Rhizoctonia solani*; PC: *Phytophthora capsici*; FO: *Fusarium oxysporum*; CA: *Colletotrichum acutatum*; BC: *Botrytis cineria*; AA: *Alternaria alternata* and PU: *Pythium ultimum*.

bacterial isolates exhibited varying degree of biological potential against pathogenic fungi compared to control (Table. 1). Out of 116 isolates tested, only one isolate (CP21-1) exhibited high antagonistic activity, 17 isolates showed moderate activity, and 20 isolates showed less activity against *R. solani*. Several workers have also found the successful control of *R. solani* under *in vitro* conditions using biocontrol agents[13, 14, 15, 16]. In case of pathogen, *P. capsici*, 8 strains have exhibited high antagonistic activity, 18 isolates have possessed moderate, and 11 isolates have possessed less activities. So far, various antifungal compounds against oomycete plant pathogen Phytophthora have been isolated and characterized from actinomycetes[17]. From the previous study, microorganisms producing antifungal antibiotics useful for the control of plant diseases, *Streptomyces humidus* strain S5-55 was isolated from soils in Korea, which showed substantial antagonistic activity against plant pathogens[18].

The pathogen, *F. oxysporum* was found to be mostly resistant to all the isolates, and no antagonistic activity was observed except the isolate, CP23-1 which possessed moderate antagonistic activity and 3 isolates have inhibited the growth of fungi at minimum level. Previous research by van Peer et al[19] showed that the strain *Pseudomonas* sp CS417 suppressed *Fusarium* wilt moderately. It has been observed that antagonistic fungi are specific in their antagonistic activity against specific fungi[20]. Previously, antifungal potential of *Bacillus* sp, *Pseudomonas* sp. and *Escherichia* sp. have also been reported to inhibit the mycelial growth of many species of *Aspergillus, Penicillium* and *Fusarium*[20, 21]. In the case of antagonism of *C. acutatum*, among 116 isolates tested, 34 isolates have showed high antagonistic activity, 49 isolates showed moderate activity and 3 isolates have showed less activity. Several mechanisms are responsible for the suppression of fungal pathogens by bacteria, including competition, antibiotic and metabolite production[1]. The inhibition of growth of fungus of *C. acutatum* and *C. gloeosporoides* was considered as antibiosis, whereby the antibiotic metabolites may penetrate the pathogen cell and inhibit its activity by chemical toxicity. *Bacillus subtilis* produced several kinds of antimicrobial peptide substances such as subtilin, bacilysin, mycobacillisyn, and iturin[23].

In the case of *A. alternata*, 12 isolates have exhibited high inhibition activity, 29 isolates possessed moderate activity and only one isolate exhibited less activity. Only five isolates have exhibited high antagonistic activity against *P. ultimum*, while, 16 isolates exhibited moderate and 11 isolates exhibited less activities. A large number of bacterial strains were found to protect rice plants from sheath blight disease[24]. The exploitation of these biocontrol agents for the management of sheath blight at field level in the long run is an exciting possibility. Generally, it has been found that the bacteria strains isolated from the rhizosphere of legumes have been found to be more efficient in solubilizing phosphates than those from the non-rhizosphere or from the root zone of non-legumes[25, 26]. Furthermore, *Bacillus* can act as

friendly bacteria by strengthening natural host defenses that acts as biocontrol against invading pathogens either directly by antagonistic activity against both gram-negative and gram-positive bacteria[27] or indirectly through induced systemic resistance. *Bacillus* spp. are considered as safe biological agents[28] based on different antagonists studies carried out. Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens[29, 30]. The ability to produce multiple classes of antibiotics, that differentially inhibit different pathogens, is likely to enhance biological control. Results of this study indicated that the potential of these antagonistic organisms to produce antimicrobial compounds could be useful for biological control of field crops and will be better explored in future.

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