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Fusarium oxysporum Growth Inhibition by Endophytic Bacteria Isolated from *Sophora alopecuroides* Nodule

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Sixty endophytic bacteria strains isolated from healthy nodules of *Sophora alopecuroides* were studied by using surface spread plate experiment, confront antibiotic culture experiment, antagonistic activities of extracellular secretions determination and disease-control experiment in greenhouse. The results indicated that there were a lot of endophytic bacteria resources isolated from nodule of *S. alopecuroides* antagonized against *Fusarium oxysporum*. 48 strains with the relative inhibition rate more than 50% of total 60 endophytic bacteria were selected with surface spread plate method. 48 antagonistic strains were then screened in confront antibiotic culture trial. The results showed that 40 strains had inhibits against *F. oxysporum* with more than 20 mm inhibition zone, and 26 strains with more than 5 mm in extracellular secretions inhibitory activities testing experiment on 40 antagonistic strains. In disease-control experiments with *F. oxysporum* carried out in greenhouses, two isolates (KDRE12 and KDRE41) gave satisfactory results, with 67.11% and 72.65% average control effect, respectively, which have good applied potential.

Keywords: *Sophora alopecuroides*; Nodule Endophytic Bacteria; Cotton Fusarium Wilt; Biological Control; Antagonism

Cotton is one of the main crops in Xinjiang Uighur Autonomous Region of China that was planted in a total area of 1.10 million hectares and produced 3.18 million tons in 2012 year. In the last years, cotton fusarium wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* is becoming a severe threaten in the production of cotton in Xinjiang. Traditionally, selection of resistant cultivars and use of chemical fungicides are the methods to control cotton fusarium wilt. However, no genetic resources of resistance can protect the vascular system from infection (Colson-Hanks and Deverall, 2000). Therefore, other manages including biocontrol using the endophytic bacteria have been considered (Lin *et al*., 2009).

In the last decades, endophytic bacteria were been concerns as novel resource in biocontrol of plants diseases (Lin *et al*., 2009). The advantages to use endophytes as biocontrol agents are that they are well adapted to live inside the plants therefore they can provide reliable suppression of vascular disease (Misaghi and Dondelinger, 1990). They can benefit the host plants by fixing nitrogen, solubilizar phosphate, production of phytohormones, production of

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antibiotic compounds, or suppression of phytopathogens by competence of invasion sites etc. (Ryan *et al*., 2008).

In a recent study, 60 non-symbiotic endophytic bacterial strains, had antagonistic activities against *F. oxysporum* were (Lin *et al*., 2013), were isolated from the nodules of *Sophora alopecuroides,* a wild perennial legume plant with remarkable resistance against stress environment grown in Xinjiang. Considering that the nodule endophytic bacteria of *S. alopecuroides* might be valuable resource to search biocontrol agent for *F. oxysporum*, we decided to make an investigation about the potential of *S. alopecuroides* nodule endophytic bacteria in suppression of cotton *F. oxysporum*.

MATERIALS AND METHODS

Isolation of endophytic bacteria from nodules

A total of 60 strains which did not form nodule were designed as non-symbiotic endophytes and were used for this study. Among these 60 strains, 20 were from Wensu (strains KDRE1~KDRE20), 25 from Alar (strains KDRE21~KDRE45) and 15 from Wuqia (strains KDRE46~KDRE60).

Determination of antagonistic activity against *Fusarium oxysporum*

Screening of antifungal activity by plate spreading

An aliquot of 0.1 mL bacterial culture (approx.10^9~10^{10} cfu mL^{-1}) was spread onto the surface of PDA plate (9 cm in diameter) and 0.1 mL sterilized water instead of bacterial culture was also spread as control. And then, a disc of agar (in diameter of 0.70 mm) with the pathogenic fungus incubated at 28 °C for 5 days was placed in the center of plate and incubated at 28 °C in dark. After incubation for 5 days, the size of pathogen colony was measured. All the treatments and control were set in triplicates. In order to quantitatively evaluate the antagonistic activity of the endophytic bacteria, relative inhibition ratio (RIR) was adopted with the following formulae:

$$RIR\ (\%) = abs\ (D_T - D_{CK}) \times 100\% / D_{CK}$$

where D_T is the diameter of pathogen colony in treatment, D_{CK} is the diameter of pathogen colony in control. The isolates with RIR more than 50 % were considered to be significant of antagonistic activity.

Screening of antifungal activity by plate confrontational culture

Agar disc with pathogen was inoculated on PDA plate as described above. Tested isolates were streaked in triplicates nearby the pathogenic disc with a distance of 1.5 cm. Sterile water was streaked as control. When the mycelia of the pathogen fully covered the Petri dish in control, the size of the fungistatic zone was examined in the treatments in order to measure the antifungal activity. Therefore, the size of inhibition zone was computed by subtracting the diameter of pathogen colony in treatment from that in the control.

Screening of antifungal activity by extracellular products

The test strain was inoculated into 100 mL of NB broth (including beef extract 5.0 g/L, Peptone 10.0 g/L, Sodium chloride 5.0 g/L) and incubated with shaking at 160 rpm at 28 °C for 2 days. The culture broth was centrifuged at 12,000 rpm for 10 min and the supernatant was filtered (0.22 μm membrane filter). The obtained culture filtrate was the primary extracellular product and an aliquot of 0.1 mL was spread on the surface of NA agar (add agar 18.0 g/L to NB broth) to test sterility.

Four small holes with diameter of 7 mm were pressed on the surface of PDA agar when the mycelia of *F. oxysporum* covered approximate one third surface of agar, and an aliquot of 50 μL culture filtrate was poured into each of the three holes. The last one was filled with sterilized distilled water as the control. Every treatment was set in triplicate and incubated at 28 °C for 2 to 7 days. The inhibitory zone of mycelia growth in the vicinity of small holes was check and measured to estimate the antagonistic capability.

The antagonistic capability of the extracellular products of tested bacteria was indicated by mycelia growth inhibited size (MGIS), which was defined as: MGIS= the radius of the control mycelia colony – the radius of the treated mycelia colony.

The pot tests for cotton disease prevention

The tests of the cotton disease prevention capability of test strain were conducted under pot-growing condition in a greenhouse. In this experiment, the cotton plants were divided into three categories: the blank infected cotton group (inoculated with *F. oxysporum,* CK1), the healthy

cotton group (not infected, CK2) and treatment group (inoculated with both *F. oxysporum* and test strain). Each treatment was set in 3 pots with 10 cotton seedlings in each pot.

The soil sample was sieved with 2 mm mesh and sterilized by autoclave at 121 °C for 1 h and repeated once again after 24 h. Full development and healthy cotton seeds of cultivar Zhongmian 21# were selected. Ten surface-sterilized seeds were planted in a depth of 2 cm into a pot filled with sterilized soil adjusted with sterilized water to 60% of capacity of water retention. The pots were maintained at 30±2 °C in greenhouse under natural sunlight in May of 2009 and were weighted two times each week. The lost weight was supplemented by pouring aseptic water to maintain the humidity. Four weeks later, when 2 to 3 real leaves appeared and the height of seedlings were about 7 to 8 cm, 2 mL of bacterial suspension (10^9 CFU mL^{-1}) for each seedling in treatment group and 2 mL of aseptic water for CK1 and CK2 were inoculated. Two days later, 50 ml of spore suspension (10^9 $CFUmL^{-1}$) of pathogenic fungus were introduced to the pots of CK1 and treatment groups. Equal amount of aseptic water instead of pathogenic spore were used in CK2.

When the diseased cottons were diagnosed, the number of diseased plant, the syndrome and seriousness of cotton fusarium wilt were recorded and identified. In general, from the first diseased cotton was diagnosed after the inoculation of pathogenic spores, the observation and record were conducted with 10 days interval and lasted for 100 days.

The seriousness of disease was classified into five categories according to the following standard: Class zero for plants grown normally without obvious symptoms; Class one for plants with partially infected one or two leaves; Class two for plants with partially infected cotyledon and one leaf; Class three for plants with two infected leaves and only the central leaf maintained healthy; Class four for the plants with wilted growth point or withered entire plant.

Incidence of disease (ID) was defined as

$$\text{ID (\%)} = \frac{\text{number of diseased plants}}{\text{number of investigated plants}} \times 100$$

Disease index (DI) was calculated by the following equation

DI=Σ[(Σ-number of disease plants within every disease category×class number)/(number of total investigated plants×maximal class number ocured in investigation)]

Relative control effect (RCE) was expressed as

$$\text{RCE (\%)} = \frac{\text{DI in the control}}{\text{DI in the treatment}} \times 100$$

RESULTS

Screening of antagonistic activity by plate spreading and plate confrontational culture

The 48 of total 60 endophytic bacteria showed the RIR over 50% in plate spreading cultures. The antagonistic activity of the 48 endophytic bacteria against *F. oxysporum* was further confirmed in the plate confrontational culture. The results showed that different sized fungistasis zones were distributed around the tested endophytic bacteria (Table 1). All the 48 bacteria produced certain antagonistic activity to the pathogenic fungus, but this activity (size of fungistasis zone) varied significant amongst 10-30 mm. The 40 strains with size of fungistasis zone over 20 mm were used for the fungal inhibition test by extracellular products.

The antifungal activity assay of extracellular products

The results in Table 2 showed that the antifungal activities of extracellular products of the 40 strains illustrated obvious difference. The maximal fungistasis zone was produced by extracellular products of strain KDRE41 (19.6 mm), followed by that of KDRE12 (17.6mm). In contrast, the minimum fungistasis zone was 0 mm produced by strains KDRE21, KDRE24 and KDRE25. Therefore, the two strains KDRE41 and KDRE12 were used for cotton disease prevention assay.

Cotton disease prevention test and cotton seed germination tests

The strains KDRE41 and KDRE12 exhibited distinct prevention effects on cotton fusarium wilt disease (Table 3), with RCE 76.65% and 67.11% respectively, which were significant difference ($p<0.05$) from the CK1 and CK2. Furthermore, within the three investigated groups, the relative control effect of strain KDRE12 showed a decline trend and the maximal RCE 72.01% were found on the 80th day, whereas the RCE of KDRE41

showed a fluctuating variation, 76.52% emerged on the 80th day, the minimum 60.95% emerged on the 90th day but its RCE restored to 80.49% on the 100th day.

DISCUSSION

The association of endophytic microorganisms and plants do not cause visible damage, but can benefit the plants with different

Table 1. Antifungal activity of antagonistic nodule endophytic bacteria against *Fusarium oxysporum* in confrontational culture tests

Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)
KDRE01	22.0±0.0c	KDRE21	27.5±0.2d	KDRE44	25.0±0.0c
KDRE02	30.0±0.0d	KDRE22	29.5±0.1d	KDRE45	17.5±0.1b
KDRE03	26.0±0.0d	KDRE23	25.5±0.5d	KDRE46	13.5±0.5a
KDRE04	24.0±0.6c	KDRE24	29.5±0.2d	KDRE47	21.0±0.1c
KDRE05	22.0±0.3c	KDRE25	26.5±0.1d	KDRE48	21.5±0.2c
KDRE06	29.5±0.2d	KDRE26	25.5±0.4d	KDRE49	12.5±0.1a
KDRE07	30.0±0.0d	KDRE28	27.0±0.4d	KDRE50	24.0±0.0c
KDRE08	23.5±0.1c	KDRE29	19.0±0.1b	KDRE51	27.0±0.4d
KDRE09	29.5±0.5d	KDRE31	28.5±0.1d	KDRE53	19.0±0.1b
KDRE11	21.5±0.1c	KDRE32	25.5±0.5d	KDRE54	22.5±0.1d
KDRE12	28.5±0.1d	KDRE33	22.0±0.3c	KDRE55	18.0±0.0b
KDRE13	23.5±0.1c	KDRE34	29.0±0.1d	KDRE56	24.0±0.1c
KDRE14	22.0±0.3c	KDRE37	22.5±0.4c	KDRE57	18.0±0.3b
KDRE15	28.5±0.2d	KDRE39	25.0±0.4d	KDRE59	18.0±0.8b
KDRE18	17.0±0.1b	KDRE41	22.5±0.1c	KDRE60	22.5±0.1d
KDRE19	22.5±0.1d	KDRE42	24.0±0.0c	CK	“
KDRE20	23.5±0.5c	KDRE43	25.0±0.1d		

Same letters presented behind the data means no significant difference among tested isolates while different letters means the difference was significant ($p<0.05$).

Table 2. The antifungal activity of extracellular products of endophytic bacteria against *F. oxysporum*

Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)
KDRE1	11.7±0.6c	KDRE19	8.3±0.3b	KDRE39	4.7±0.3a
KDRE2	8.0±0.0b	KDRE20	3.3±0.3a	KDRE41	19.3±0.3d
KDRE3	6.3±0.3b	KDRE21	-	KDRE42	0.3±0.3a
KDRE4	6.3±0.3b	KDRE22	2.3±0.3a	KDRE43	7.3±0.1b
KDRE5	8.0±0.0b	KDRE23	3.3±0.3a	KDRE44	5.3±0.4b
KDRE6	8.0±0.0b	KDRE24	-	KDRE47	5.0±0.6b
KDRE7	7.7±0.1b	KDRE25	-	KDRE48	9.3±0.3b
KDRE8	7.3±0.1b	KDRE26	11.5±0.3c	KDRE50	7.7±0.1b
KDRE9	11.7±0.6c	KDRE28	12.7±0.4c	KDRE51	7.7±0.1b
KDRE11	14.0±1.7c	KDRE31	4.0±0.2a	KDRE54	5.0±0.9b
KDRE12	17.3±0.3d	KDRE32	4.7±0.3a	KDRE56	6.0±0.8b
KDRE13	13.0±0.7c	KDRE33	5.0±0.0a	KDRE60	8.3±0.8b
KDRE14	11.7±1.3c	KDRE34	3.7±0.1a		
KDRE15	4.7±0.1a	KDRE37	4.0±0.2a		

Same letters presented behind the data means no significant difference among tested isolates while different letters means the difference was significant ($p<0.05$).

Table 3. The antagonistic effects of nodule endophytic bacteria against *Fusarium oxysporum* in pot tests under greenhouse condition

Treatment	Disease index			Relative control effect (%)			Mean control
	80 d	90 d	100 d	80 d	90 d	100 d	effect (%)
KDRE12	23.89	26.76	38.80	72.01	70.02	59.30	67.11
KDRE41	20.04	34.85	18.60	76.52	60.95	80.49	72.65
CK_1	82.65	87.59	94.62	-	-	-	-
CK_2	0	0	0	-	-	-	-

Same letters presented behind the data means no significant difference among tested isolates while different letters means the difference was significant ($p<0.05$).

mechanisms (Ryan *et al*., 2008). In many cases, this association offered the plants antimicrobial ability (Ryan *et al*., 2008; Verma *et al*., 2009). In traditional agriculture of China, extracts of *S. alopecuroides* have been used to protect crops from attack by phytopathogens, it is also used as medicine against bacteria and cancer cells (Sato *et al*.,1995; Song *et al*., 1999). The antimicrobial activity of *S. alopecuroides* implies the possibility that this plant might contain endophytic microorganisms with antimicrobial activities. The results in the present study confirmed this possibility.

Previously, some endophytes are thought to protect their host from attack by fungi and insect by producing secondary metabolites (Zhang, 2007), and are potential biological control agents in sustainable crop production (Sturz and Nowak, 2000). However, previous studies on the nodule endophytic bacteria have been focused on their diversity, their affects on the symbiosis (Mrabet *et al*., 2006), and potential to stimulate plant growth (Liu *et al*., 2010). Therefore, our present study explored another research field for these bacteria: to reveal their biocontol values. The verification of the ability to inhibit the pathogenic fungus *F. oxysporum* in our strains indicated that the nodule endophytes are an important source for scanning the biocontrol agents.

The great proportion (48 in 60, 80%) of antifungal strains in the studied bacteria also evidenced that the nodule endophytic bacteria are a good resource for study of biocontrol agents. At this moment, we are not sure if this is related to the antimicrobial activity of this plant, but it is worthy to make further study.

In the assay of plants grown in pots, mean relative control effect of KDRE41 and KDRE12 reached to 72.65% and 67.11%, respectively. They exhibited distinct prevention effects on cotton fusarium wilt, the control effect was far better than that of any practical chemical fungicides used in this region. It was therefore assumed that strong antifungal activity is caused by producing various biologically active metabolites, offering the further advantage of forming endospores that are resistant to salinization, heat, dry, organic solvents and UV-radiation (Liu *et al*., 2006). Another possible mechanism (Berg *et al*., 2005) responsible for antagonistic activity is competition for colonization sites, nutrients and minerals.

CONCLUSIONS

Based upon the results of the present study, the following conclusions could be draw: 1) Most of the endophytic bacteria isolated from root nodules of *S. alopecuroides* had antagonistic effect against *F. oxysporum*. 2) Strains KDRE41 and KDRE12 exhibited prominent prevention effects on cotton *F. oxysporum*. These findings suggested that the nodule endophytic bacteria from *S. alopecuroides* are potential biological resources for selection of biocontrol agents.

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