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Potential of endophytic bacteria isolated from *Sophora alopecuroides* nodule in biological control against Verticillium wilt disease

Tianxing Lin[1,†], Longfei Zhao[2,†] Yazhen Yang[3], Qinlan Guan[1], Mingfu Gong[1*]

[1]Key Laboratory of Special Biological Resources in Mountain Emei, School of Life Sciences, Leshan Normal Universlty, Leshan, Sichuan 614004, China
[2]College of Life Sciences, Shangqiu Normal University, Shangqiu, Henan, 476000, China
[3]College of Life Sciences,Yangtze University, Jingzhou, Hubei, 434025, China
[†] Equally contributed to this work.

***Corresponding author: gongmingfu98@163.com, sxylwsw2006@yahoo.com.cn**

Abstract

Antagonistic activities against *Verticillium dahliae* were screened in sixty endophytic bacteria isolated from root nodules of *Sophora alopecuroides* by plate spreading, plate confrontational culture and examining the antifungal activity of extracellular products methods. The results showed that inhibitory rate of more than half of the 60 studied strains is 50%, fungistasis zone of 41 strains is beyond 15 mm and those of 36 strains is over 5 mm, respectively, through the above-mentioned method. In addition, 17 strains showed remarkable seed germination-promoting activity. Mean control effect of strains *Bacillus subtilis* KDRE01 and *Bacillus megaterium* KDRE25 in the prevention against cotton *Verticillium* wilt under pot-growing conditions was 84.91% and 78.82%, respectively, and showed significant difference ($p<0.05$) from the CK1 and CK2. Moreover, these two strains showed plant growth promoting characteristics (such as IAA and ACC deaminase activity, etc.) and both exhibited obvious promoting effects ($p<0.05$) on cotton growth. These results suggested that the nodule endophytic bacteria of *S. alopecuroides* were valuable biological resources, which might be potential biocontrol agents.

Keywords: *Sophora alopecuroides*; Endophytic bacteria; Cotton verticillium wilt; Plant growth; Biological control.
Abbreviations: YMA-yeast-extract-mannitol agar; MGIS-mycelia growth inhibited size; RIR-relative inhibition ratio; ID-Incidence of disease; RCE-Relative control effect; CAS-Chrome azurol-S; PDA- Potato Dextrose Agar.

Introduction

Sea island cotton (*Gossypium barbadense* L.) 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 1.8 million tons in 2005. The cotton in Xinjiang is mainly planted in the oases of the Tarim Basin, which is centered by the Taklamakan Desert and has alkaline-saline soils and drought climate. In the last years, *Verticillium* wilt caused by *Verticillium dahliae* is becoming a severe threaten in the production of cotton in Xinjiang. As a fungal genus of the division Ascomycota, *Verticillium* includes diverse groups comprising saprobes and parasites of higher plants, which cause *Verticillium wilts* on over 300 different cultivated trees and herbaceous plants, with the symptoms of midsummer wilting, 'Black Heart and death', etc. (Barbara and Clewes, 2003; Klosterman et al., 2009). Traditionally, selection of resistant cultivars and use of chemical fungicides are the methods to control cotton *Verticillium wilt*. However, no genetic resources of resistance can protect the vascular system from infection (Colson-Hanks and Deverall, 2000), while the repeated uses of chemical fungicides caused the development of resistance in the fungi, as well as environmental contamination. Therefore, other manages including biocontrol using the endophytic bacteria have been considered (Lin et al., 2009; Marois et al., 1982). In the last decades, endophytic bacteria have attracted more and more concerns as novel resource in biocontrol of plants diseases and in promotion of plant growth (Lin et al., 2009; Marois et al., 1982). 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) and that they do not cause environmental contamination. Generally, the endophytic bacteria live inside the plant tissues and do not cause visible damage or morphological change on their hosts. They can benefit the host plants by production of phytohormones, fixing nitrogen, solubilizar phosphate, production of antibiotic compounds, or suppression of phytopathogens by competence of invasion sites etc. (Khan et al., 2008; Ryan et al., 2008). In a recent study, we collected and characterized rhizobia associated with *S. alopecuroides*, a wild perennial legume plant with remarkable resistance against stress environment grown in Xinjiang. Nine genomic species corresponding to *Mesorhizobium alhagi*, *Mesorhizobium gobiense*, and so on were identified among 75 symbiotic strains (Zhao et al., 2010). As by-products of that work, 60 non-symbiotic endophytic bacterial strains were also isolated from the nodules. As a symbiotic structure, the root nodules of legume plants are induced by rhizobia, kinds of symbiotic nitrogen fixing bacteria. However, many non-symbiotic bacteria, like *Agrobacterium*, *Bacillus*, *Burkholderia*, *Enterobacteria*, etc., have been isolated from root nodules in various leguminous plants since 1902 (Beijerinck and Van Delden, 1902; De Lajudie et al., 1999; Kan et al., 2007; Manninger and Antal, 1970; Oehrle et al., 2000; Zakhia et al., 2006), and they are considered as endophytes without causing any obvious pathogenic symptom (Bacon and White, 2000). These root nodule endophytes may produce IAA

(indoleacetic acid, phytohormone), dissolve phosphate, help the symbiotic rhizobia to form nodules with unspecific hosts (Liu et al., 2010), or inhibit nodulation of rhizobia on the host (Mrabet et al., 2006). Meanwhile, they also could occupy the normal root tissues (Liu et al., 2010). These data demonstrated that the nodule endophytes of the legumes have the potential of biocontol and biofertilizers. Considering that the nodules endophytic bacteria of *S. alopecuroides* have not yet studied, that the association of endophyte with their host plants is not stringently specific, and that *S. alopecuroides* and cotton plants have the same distribution in Xingjiang, the nodule endophytic bacteria of *S. alopecuroides* might be valuable resource to search biocontrol agent for *Verticillium dahlia*. Based upon these considerations, we decided to make an investigation about the potential of *S. alopecuroides* nodule endophytic bacteria in suppression of cotton *Verticillium wilt* and in promotion of the plant growth. The objectives of the present study were (i) to investigate the antagonistic activities of endophytic bacteria against the pathogen of cotton *verticillium wilt*, (ii) to examine the potential of the screened endophytic bacteria in plant disease control and (iii) to evaluate the plant growth promoting capability of the screened endophytic bacteria.

Results

Screening of endophytic bacteria with antagonistic activity by plate spreading and plate confrontational culture

The 53 of total 60 endophytic bacteria showed the RIR over 50% in plate spreading cultures. The antagonistic activity of the 53 endophytic bacteria against *V. dahlia* 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 53 bacteria produced certain antagonistic activity to the pathogenic fungus, but this activity (size of fungistasis zone) varied significant amongst 9-32 mm. The 41 strains with size of fungistasis zone over 15 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 41 strains illustrated obvious difference. The maximal fungistasis zone was produced by extracellular products of strain KDRE25 (29 mm), followed by that of KDRE01 (26.3 mm). In contrast, the minimum fungistasis zone was 3.33 mm produced by strains KDRE10 and KDRE15. Therefore, the two strains KDRE01 and KDRE25 were used for cotton disease prevention assay. In addition, all the 17 bacteria with fungistasis zone over 20 mm were used for cotton seed germination assay.

Cotton disease prevention test and cotton seed germination tests

The strains KDRE01 and KDRE25 exhibited distinct prevention effects on cotton *verticillium wilt* disease (Table 3), with RCE 84.91% and 78.82% 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 KDRE25 showed a decline trend and the maximal RCE 92.36% were found on the 80th day, whereas the RCE of KDRE01 showed a fluctuating variation, the maximum 95.04% emerged on the 80th day, the minimum 75.9% emerged on the 90th day but its RCE restored to 83.79% on the 100th day.

The 17 strains used in cotton seed germination test showed different degree promotion effects (Table 4). Compared with the control, the treatments of cotton seeds immersed in endophytes culture significantly improved germination rate. The germination rate of treatment with KDRE25 was the highest (76%), following by that of treatment with KDRE01 (75.75%), 28% and 27.75% higher than that of the control, respectively. Even the lowest germination rate 53.75% in treatments also showed 5.75% higher than that of the control. Hence, endophytic bacteria KDRE25 and KDRE01 exhibited pronounced promotion effects to cotton seed germination.

Potential of plant growth promotion

Both strains KDRE01 and KDRE25 had statistically similar ACC-deaminase activity and produced IAA in the presence and absence of L-tryptophan (Table 5). With regard to siderophore production of strains KDRE01and KDRE25, positive reactions by the CAS method presented yellow halo surrounding the bacterial colonies under iron-limiting conditions, the colonies were sorted in the range of big diameter. Besides, both strains KDRE01 and KDRE25 were negative in the Arnow assays, indicating that these strains could not produce catechol-type siderophores. But they were able to produce hydroxamate-type siderophores to various degrees in the Csáky assay, the intensity of production of siderophores were high.

Identification of strains KDRE01 and KDRE25

Based on the alignment of 16S rRNA gene sequences among KDRE01, KDRE25 and others, the phylogenetic tree was constructed. Two major clades were formed in the tree (Fig. 1); the two strains KDRE01 and KDRE25 were grouped to the *Bacillus* genus. The first clade included strain KDRE01 (EU835568) and reference strains *B. mojavensis* BCRC17124^T, *B. subtilis* NRRL NRS-744^T, *B. atrophaeus* NRRL NRS-213^T and related strain *B. licheniformis* ATCC14580^T. Strain KDRE01 to them showed higher similarity, 99.6%, 99.7%, 99.4% and 91.8%, respectively. However, Strain KDRE25 (EU835562) and reference strains *B. megaterium* SB3112, *B. megaterium* IAM13418^T, *B. amyloloquefaciens* NRRLB-14393^T and other strains formed another clade. The similarity of strain KDRE25 to reference strains showed 99.9%, 99.6% and 90.4%, respectively. Therefore, their sequences exhibited a high similarity with those of bacteria classified in *Bacillus* sp. in GenBank. Similarity analysis combined with physiological and biochemical characteristics (data not shown) indicated that strain KDRE01 was affiliated with *B. subtilis* and KDRE25 was affiliated with *B. megaterium*.

Discussion

The association of endophytic microorganisms and plants do not cause visible damage, but can benefit the plants with different mechanisms (Khan et al., 2008; Ryan et al., 2008; Strobel et al., 2004). 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.

In this study, 60 endophytic bacteria were obtained from root nodules of *S. alopecuroides.* By the preliminarily screening by

Table1. Antifungal activity of antagonistic nodule endophytic bacteria against *Verticillium dahliae* in confrontational culture tests.

Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)
KDRE01	30.5±0.2121f	KDRE20	26.5±0.0707e	KDRE42	14.5±0.3536b
KDRE02	26±0e	KDRE21	23±0.1414d	KDRE43	14±0.2828b
KDRE03	17.5±0.2121c	KDRE22	17.5±0.6364c	KDRE44	22±0d
KDRE04	24±0.2828d	KDRE23	21±0.1414d	KDRE45	17±0.7071c
KDRE05	29.5±0.0707e	KDRE24	23±0.1414d	KDRE46	23±0.1414d
KDRE06	24.5±0.3536d	KDRE25	32±0.7071f	KDRE47	28±0.1414e
KDRE07	24±0d	KDRE26	27±0e	KDRE48	27±0e
KDRE08	18±0c	KDRE28	18±0.1414c	KDRE49	23.5±0.495d
KDRE09	19±0.1414c	KDRE29	26±0.2828e	KDRE50	19.5±0.0707c
KDRE10	17±0c	KDRE31	20±0c	KDRE51	22±0d
KDRE11	14.5±0.9192b	KDRE32	11.5±0.0707b	KDRE53	9±0.2828a
KDRE12	20±0.2828d	KDRE33	21.5±0.6364d	KDRE54	19.5±0.3536c
KDRE13	29±0.9899e	KDRE34	25±0.1414e	KDRE55	18.5±0.2121c
KDRE14	27±0.7071e	KDRE37	21±0.1414d	KDRE56	20±0c
KDRE15	28.5±0.2121e	KDRE39	21±0.1414d	KDRE57	10.5±0.3536b
KDRE18	10.5±0.2121b	KDRE40	21±0.1414d	KDRE60	24±0d
KDRE19	14.5±0.3536b	KDRE41	9.5±0.3536a		

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

the spreading method, plate confrontational culture method and examining the antifungal activity of extracellular products method, antagonistic activities were verified in 53 of the 60 strains. 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 (de Lajudie et al., 1999), their affects on the symbiosis (nodulation and fixation of nitrogen)(Zhao et al., 2011), and potential to stimulate plant growth (Liu et al., 2010; Mrabet et al., 2006). 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 *V. dahliae* in our strains indicated that the nodule endophytes are an important source for scanning the biocontrol agents and biofertilizer microorganisms. The great proportion (53 in 60, 88.3%) 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 present study, the most effective strains were two *Bacillus* strains, that was consistent with the fact that a wide range of antimicrobial compounds have been found in this genus (Moyne et al., 2001; Patel et al., 2009). To our knowledge, this is the first report to verify that nodule endophytic *B. subtilis* and *B. megaterium* of *S. alopecuroides* have antagonistic effect *in vitro* against phytopathogenic fungus *V. dahliae*. In particular, Endo-spore-forming *Bacillus* have properties that make them suitable to be developed as biocontrol agents, such as good stress resistance and producing diverse secondary metabolic products (Patel et al., 2009), eliciting ISR (induced systemic resistance) to reduce disease severity by a broad range of pathogens, promoting plant growth (Kloepper et al., 2004) and being easy cultivated and stored, as well as being applied as spores on plant seeds or in inoculants. Based on our result, the screened strain KDRE01 was identified as shown by *B. subtilis*. Previously, *Bacillus subtilis* has been isolated as endophyte, which has strong antifungal activity by producing fungicidal or fungistatic peptides synthesized nonribosomally via a multi-enzyme-catalyzed synthesis (Li et al., 2008). Moreover, Zhao et al. (2010) studies has showed that the antifungal effect

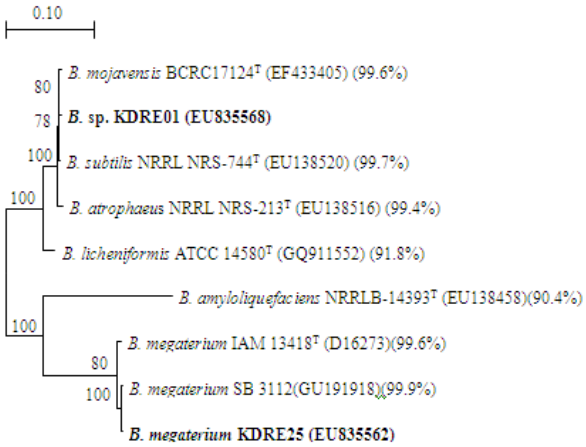

Fig 1. Phylogenetic NJ tree based on alignment of nucleotide sequences of the16S rRNA gene from representative strains (shown in bold) and reference strains. Bootstrap values greater than 50% were indicated. Scale bar represents the number of substitutions per site.

of the crude preparation of strain *B. subtilis* attribute to Bacillomycin D. Furthermore, Han et al. (2005) pointed that the supernatant of *Bacillus* sp. sunhua showed no inhibitory activity towards beneficial bacteria, such as *Rhizobium meliloti*, a symbiotic nitrogen-fixing microorganism, or *Pseudomonas fluorescens*, which can produce a toxic insecticidal compound.
In the present study, the endophytic bacterial strain KDRE25 was identified as *B. megaterium*. Liu et al. (2006) has reported that *B. megaterium* is a Gram-positive nitrogen fixer, which was originally isolated from the maize rhizosphere, the bacterium has nitrogenase activity and *nifH* gene amplified by polymerase chain reaction. *B. megaterium* could colonize root surface or resides in the interiors of roots, stems, and leaves by GFP-labeled and provides ammonia to plants as a diazotrophic endophyte. In addition, *B. megaterium* could produce schizokinen, which being a citrate derivative was easily cross-utilized by *Rhizobium* sp. IC3123 since it is known to utilize ferric-citrate (Rajendran et al., 2007). In the assay of plants grown in pots, mean relative control effect of KDRE01 and KDRE25 reached to 84.91% and 78.82%, respectively.

They exhibited distinct prevention effects on cotton verticillium 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. Similar study reported that some endophytic bacteria of *B. vallismortis* isolated originally from red pepper have been proved to possess strong antagonistic activity against cotton wilt caused by *V. dahlia*. Moreover, strain was an efficient plant growth promoting and disease controlling agent in many different crops against multiple pathogens (Zhang et al., 2008).

Bacteria having the characteristics of producing IAA, siderophores, ACC deaminase may have the potential for the promotion of plant growth (Sun et al., 2010). Such as *Bacillus* species, positive for IAA production and ACC deaminase activity, increased the ability of pepper plants to cope with abiotic stress (Sziderics et al., 2007). Our experiment results showed that strains KDRE01 and KDRE25 possessed plant growth-promoting characteristics such as producing siderophore, IAA production and ACC deaminase activity (Table 5). Moreover, the observed results from antifungal activity of endophytic bacteria or its culture extracellular products to *V. dahliae*, control effects of endophytic bacteria to *V. dahliae* and cotton seed germination assay, evidenced that strains KDRE01 and KDRE25 have exhibited obvious promoting effect. Therefore, strains KDRE01 and KDRE25 have great potential as a biological control agent for the control of *Verticillium* Wilt disease, and as a biofertizer.

Materials and methods

Plant materials

Sophora alopecuroides is a legume, wild perennial herb of the xerophyte species and is widely distributed in northwestern China. Especially, most of Xinjiang Uighur Autonomous Region of China belongs to arid and semi-arid areas. *S. alopecuroides* shows excellent performance in drought and alkaline tolerance as well as sandstorm resistance due to its well-developed root system. In addition, *S. alopecuroides* plays a vital role in environmental protection in northwest of China (Zhao et al., 2010). It also has promising prospects through its utilization in pharmaceutics and pesticides, as a source of livestock feed, and its role as a natural windbreaker and nectar source. As a traditional Chinese medicine, this plant also is used to treat fever and diarrhea; some studies even suggest it has the potential to inhibit cancer cells (Zhao et al., 2011).

Isolation of endophytic bacteria from the root nodules

Healthy *S. alopecuroides* plants were collected from various habitats. Over 100 nodules were obtained from plants grown in Wensu county, Alar city and Wuqia county. Three nodules were randomly selected from each plant and surface sterilized with 0.1% $HgCl_2$, following by rinsing in sterilized distilled water for 6 times. The surface sterilized nodules were crushed and streaked on yeast-extract-mannitol agar (YMA) plates for isolation of endophytic and symbiotic bacteria with the standard methods described previously (Vincent, 1970). The plates were incubated at 28°C for 5 days and single colonies were further purified by repeatedly streaking on the same medium. Thoroughness of surface-sterilization and the nodulation ability were checked as described previously (Zhao et al., 2011). 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

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:

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

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

After being streaked on NA (Nutrient agar culture media, including beef extract 5.0 g/L, Peptone 10.0 g/L, Sodium chloride 5.0 g/L, Agar 15.0 g/L) plate and incubated for 24 h, a full loop of the test strain was inoculated into 100 mL of NB broth (same as NA medium, but without agar) 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 to test sterility. Four small holes with diameter of 7 mm were pressed on the surface of PDA agar when the mycelia of *Verticillium dahliae* 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.

Table 2. The antifungal activity of extracellular products of endophytic bacteria against *Verticillium dahliae*.

Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)	Strain	Size of fungistasis zone (mm)
KDRE01	26.333±1.1547f	KDRE20	1.667±0.2887a	KDRE40	13.333±0.7638c
KDRE02	20.667±0.5132e	KDRE21	5±0.5b	KDRE44	25±0e
KDRE03	21.667±0.2887e	KDRE22	14.333±0.1155c	KDRE45	9.333±0.4041b
KDRE04	21.667±0.2887e	KDRE23	20±0.5e	KDRE46	15±0c
KDRE05	17.333±0.4041d	KDRE24	22.333±0.1528e	KDRE47	15±0c
KDRE06	18±0.5196d	KDRE25	29±1.7321f	KDRE48	20.667±0.1155e
KDRE07	20.333±0.5033e	KDRE26	24.333±0.1155e	KDRE49	18.333±0.2887d
KDRE08	22.333±0.2517e	KDRE28	11.667±0.5774c	KDRE50	16.333±0.5774d
KDRE09	20±0.5e	KDRE29	11.667±0.5774c	KDRE51	21±0.1732e
KDRE10	3.333±0.2887a	KDRE31	20±0d	KDRE54	20.667±0.4933e
KDRE12	4±0.3606a	KDRE33	21.667±0.2887e	KDRE55	1.667±0.2887a
KDRE13	13.333±0.5774c	KDRE34	19.333±0.2082d	KDRE56	6.667±1.1547b
KDRE14	16.667±0.2887d	KDRE37	15±0.866d	KDRE60	6.667±0.7638b
KDRE15	3.333±0.5774a	KDRE39	20.333±0.0577e		

Note: 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 *Verticillium dahliae* in pot tests under greenhouse condition.

Treatment	Disease index			Relative control effect (%)			Mean control effect (%)
	80 d	90 d	100 d	80 d	90 d	100 d	
KDRE01	4.44	22.5	15.7	95.04	75.9	83.79	84.91a
KDRE25	6.85	21.53	31.81	92.36	76.94	67.16	78.82b
CK_1	93.29	94.21	98.61	--	--	--	
CK_2	0	0	0	--	--	--	

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

The cotton disease prevention tests with selected endophytes

The tests of the cotton disease prevention capability of endophytic bacteria 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 *Verticillium dahlia*, CK1), the healthy cotton group (not infected, CK2) and treatment group (inoculated with both *Verticillium dahlia* and nodule endophytic bacteria). Each treatment was set in 3 pots with 10 cotton seedlings in each pot. To perform the experiments, soil was collected from Wensu county, Alar city and Wuqia county. At each region, soil cores were sampled at five locations with 15-20 cm depth and 5 cm away from tap roots, and were bulked and thoroughly mixed to get a composite sample that is sandy loam with pH 8.11, organic material of 14.52 g kg^{-1}, total N of 0.787 g kg^{-1}, available P of 7.62 mg kg^{-1} and K of 293.75mg kg^{-1}. 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 Junhai I 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 *verticillium wilt* were recorded and identified.

Plant with obvious diagnosable symptoms was identified as diseased cotton. 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 (healthy) for plants grown normally without obvious symptoms; Class one (slightly sick) for plants with partially infected one or two leaves; Class two (intermediate sick) for plants with partially infected cotyledon and one leaf; Class three (serious sick) for plants with two infected leaves and only the central leaf maintained healthy; Class four (wilt) for the plants with wilted growth point or withered entire plant.

Incidence of disease (ID) was defined as

ID (%) = ∑ (number of diseased plants/number of investigated plants)×100%

Disease index (DI) was calculated by the following equation

DI=∑[(∑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

RCE (%) =DI in the control×100%/DI in the treatment

Influence of selected endophytes on the germination of cotton seeds

Considering the future application, we also evaluated the potential influence of the selected endophyties broth culture on cotton seed germination. The cotton seeds of Junhai I was used for this experiment. First, each of the endophytic bacteria was incubated in 5 mL of NB broth for 2 days with shaking and the seeds were immersed into the endophyties broth culture for 24 h. The treated seeds then were placed separately on an aseptic filter paper moistured with sterilized water in diameter of 9 mm Petri dish for germination. Seven days later, the germinated seeds were counted and the germination ratio was calculated. In

Table 4. The antagonistic effects of nodule endophytic bacteria on cotton seed germination.

Strain	Seeds germination rate (%)				Mean value (%)
	1	2	3	4	
KDRE01	75	74	76	78	75.75aA
KDRE02	62	64	72	60	64.5bcB
KDRE03	56	72	62	64	63.5bcB
KDRE04	61	62	62	61	61.5bcBC
KDRE07	50	56	56	57	54.75efD
KDRE08	71	62	62	70	66.25bB
KDRE09	55	56	72	66	62.25bcBC
KDRE23	57	59	60	62	59.5cdeBCD
KDRE24	58	59	62	61	60cdBCD
KDRE25	73	77	78	76	76aA
KDRE26	59	62	63	63	61.75bcBC
KDRE33	60	62	61	58	60.25cdBCD
KDRE39	50	55	56	54	53.75fDE
KDRE44	52	54	57	56	54.75efD
KDRE48	56	56	58	55	56.25defCD
KDRE51	54	53	58	52	54.25fDE
KDRE54	51	54	56	55	54fDE
CK	45	48	50	49	48gE

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

Table 5. Identification and plant growth promoting characteristics of selected strains KDRE01 and KDRE25

Strain	ACC deaminase Activity (nmol g^{-1} h^{-1})*	IAA production (mg L^{-1})#		CAS-Agar test§	Csáky test†	Arnow test‡
		L-try (-)	L-try (+)			
KDRE01	186.35±8aA¢	4.2±0.5aA	8.9±0.6aA	+++¶	++	-
KDRE25	175.00±5bA	7.2±0.8bB	10.3±0.7bA	+++	++	-
Control	/	-	-	-	-	-

*. ACC=1-aminocyclopropane-1-carboxylic acid, activity=nmol α-ketobutyrate g^{-1} biomass h^{-1}. #. L-try (-)=without L-tryptophan; L-try (+)=with L-tryptophan. §. CAS-Agar test= universal test. †. Csáky test= hydroxamate-type. ‡. Arnow test= catechol-type. ¶. The symbols represent the relationship between the average diameter of halo and the average diameter of the colony growth (+: small; ++: medium; +++: big) for CAS-Agar univesal test and the intensity of production of siderophores (–: none; +: low; ++: high; +++: very high) for Csáky and Arnow tests.
¢The same letter means no significant difference between treatments, the capital letter indicates significant level at 0.01 while lowercase letter indicates significant level at 0.05. The data in columns is average values of three repetitions. - negative action; / blank; Control for IAA assay was LB (10g NaCl/L) without inoculated bacterial suspension under same incubation condition.

this experiment, parallel tests were conducted in tetrad with 100 seeds for each treatment. Seeds immersed in sterilized broth served as the control.

Characterization of potential for plant growth promotion

After the screening procedure mentioned above, KDRE01 and KDRE25 were selected for this analysis as the best strains of antifungal activity. The two strains were separately inoculated in 150 mL of the Dworkin and Foster (DF) minimal salt medium (Dworkin and Foster, 1958) containing ACC (1-aminocyclopropane-1- carboxylate) as the sole nitrogen source and incubated at 28 °C for 24 h. The production ACC-deaminase was determined by monitoring the amount of α-ketobutyrate formed by the bacteria as described by Honma and Shimomura (1978). The production of IAA in vitro by the tested endophytic bacteria was determined according to the protocol described by Khalid et al. (2004).

The production of Siderophore by tested endophytes was measured according to the Chrome azurol-S (CAS) analytical method described by Schwyn and Neilands (1987) and modified by Silva-Stenico et al. (2005). Briefly, 60.5 mg of CAS was dissolved in 50 mL deionised water, and mixed with 10 mL of $FeCl_3$ solution. With stirring, this solution was slowly mixed with 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) previously dissolved in 40 mL of water. Then, the resulting dark-blue solution was autoclaved at 105 °C for 20 min, cooled to 50 °C and mixed with 900 mL sterile MM9 medium (Silva-Stenico et al., 2005) containing 15g L^{-1} agar (also kept at 50 °C). This medium was poured in Petri dishes, subsequently inoculated with bacterial strains and incubated in the dark at 28 °C for 5 days. In addition, Catechol-type siderophores were measured with culture supernatants through Arnow assay (Arnow, 1937), while hydroxamate-type siderophores were measured according to Csáky (1948). Positive results were indicated by the formation of a clear halo around the colonies, showing a visual change in color from darkblue to yellow. Each assay was performed with triplicate.

Identification of selected endophytes

Strains KDRE01 and KDRE25 were identified on the basis of 16S rRNA gene sequence similarity and its physiological characteristics for the corresponding bacteria described in Bergey's Manual of Systematic Bacteriology (second edition) (George et al., 2004). The total genomic DNA of each strain was extracted and purified as described (Sambrook et al., 1989). The forward primer (5'-CgggATCCAgAgTTTgATCCTgg-CTCAgAACgAACgCT-3') and reverse primer (5'-CgggATC-CTACggCTACCTTgTTACgACTTCACCCC-3') (Van Berkum et al., 1996) respectively corresponding to the positions of 8~37 and 1479~1506 in *E. coli* 16S rRNA gene were used for amplification of the 16S rRNA gene. Then, the PCR products were purified and sequenced on a SQ5500E DNA sequencer

(Hitachi Tokyo, Japan). The acquired nucleotide sequences have been deposited in NCBI database under the accession numbers EU835562 and EU835568. All reference sequences obtained from the NCBI database were aligned using the multiple sequence alignment software, Clustal-X1.81. Neighbor-joining (NJ) phylogenetic tree was constructed with the Jukes–Cantor model by using the TREECON package. Sequences similarities were computed using DNAMAN application (version 6.0.3.40, Lynnon Corporation).

Statistical analysis

Antifungal activity of nodule endophytic bacteria and extracellular products of endophytic bacteria against *Verticillium dahliae*, the antagonistic effects of nodule endophytic bacteria against *Verticillium dahliae* in pot tests and on cotton seed germination, plant growth promoting characteristics of selected strains KDRE01 and KDRE25, these parameters were examined with ANOVA analysis using the SPSS 17.0 package (by the Data Theory Scaling System Group, Faculty of Social and Behavioral Sciences, Leiden University, The Netherlands).

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 phytopathogenic fungus *V. dahlia*. 2) Some strains stimulated the cotton seed germination and had plant growth-promoting characteristics by different methods. 3) Strains *B. subtilis* KDRE01 and *B. megaterium* KDRE25 exhibited prominent prevention effects on cotton *verticillium wilt* and exhibited *in vitro* obvious plant growth-promoting effects. These findings suggested that the nodule endophytic bacteria from *S. alopecuroides* are potential biological resources for selection of biocontrol agents and biofertilizers.

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