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Full Length Research Paper

Antagonistic mechanism and control effect of *Bacillus subtilis* Y2 against *Fusarium oxysporum* causing soybean root rot

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Soybean root rot caused by *Fusarium oxysporum* is a destructive soilborne pathogen and is of economic importance in all soybean producing areas. *Bacillus subtilis* strain Y2 is a potential bacterial biocontrol agent against *F. oxysporum*. An antagonistic mechanism of *B. subtilis* Y2 against *F. oxysporum* was conducted. The antagonistic mechanism of strain Y2 against *F. oxysporum* effectively inhibited mycelial growth, sporulation and germination. The activity of germfree filtrate of strain Y2 was sensitive to high-temperature (121°C). The results preliminary showed that *B. subtilis* strain Y2 had no effect on soybean seed germination and growth. Soybean root rot caused by *F. oxysporum* had effectively been prevented up to 98.77% by *B. subtilis* Y2 in potted plant experiments.

Key words: Soybean root rot*, Bacillus subtilis, Fusarium oxysporum,* antagonistic mechanism, control effect.

INTRODUCTION

Soybean is one of the important crops in the Northeast China. Monoculture soybean increased the numbers of soybean fungal pathogens, and caused an obvious allelopathic effect (Han et al., 2004; Xu et al., 2004) by reducing soybean yields up to 30% (Liu et al., 2001; He et al., 2003a; Li et al., 2010).

Soybean root rot, caused by soil-borne plant pathogen *Fusarium oxysporum* (Gordon and Martyn, 1997), is a widespread and destructive soybean disease in the Northeast China (Chen et al., 1997; Li and Ma, 2011). In recent years, with continuous cultivation of soybean, root rot became more serious, affecting the yield and the quality of soybean (Li et al., 2010).

Biocontrol of plant pathogens is one of the most promising options for the management of the soilborne diseases. However, the use of fungicides in the management of plant diseases not only pollutes the environment, but also is hazardous to human health (Bürger et al., 2012). Therefore, there is a need to explore alternatives to synthetic fungicides for controlling the soybean root rot (Li and Ma, 2012).

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Bacillus subtilis strains are a rich source of antifungal peptides. The lipopeptides surfactins (Jitendra and Ibrahim, 1997), the iturin family (Regine and Peypoux, 1994), and fengycins (Vanittanakom and Loeffler, 1986) are a prominent class of such compounds. The productions of these three antifungal peptides play an important role in the biocontrol activity against a variety of plant pathogens (Wang et al., 2007).

The objectives of the present work were to investigate the biocontrol activity of strain Y2 of *B. subtilis in vitro*, and study its antagonistic mechanism and control effect against *F. oxysporum.*

MATERIALS AND METHODS

Isolation and identification of strain Y2

The antagonistic bacteria Y2 was isolated from the surface of soybean root using the method of Wilson (Wilson and Chalutz, 1989). According to the Bergey's Manual of Determinative Bacteriology (Elisabeth et al., 1989), physiology and biochemistry analysis were made for identification of the strain Y2.

The *F. oxysporum* isolate was obtained from the Department of Plant Protection, Northeast Agricultural University, Harbin Heilongjiang, China. Soybean varieties (HeFeng 25) were obtained from Key Laboratory of Soybean Biology of Education Ministry.

Determination of antagonistic mechanism of strain Y2 against *F. oxysporum*

Effect of germfree filtrate of strain Y2 against F. oxysporum mycelium growth

Strain Y2 was inoculated into nutrient broth (NB) and incubated on a rotating shaker at 170 rpm for 24 h at 28°C. Then seed inoculum was added in NB (inoculation quantity 1% with 100 mL medium in 300 mL flask) and further incubated on a rotating shaker at 170 rpm for 48 h at 28°C for obtaining germfree filtrate. Sterilized filtrate was obtained by bacterial filter for testing antibacterial activity at 4°C until use.

Germfree filtrate heated to 121°C for 20 min was added in the quantitative potato dextrose agar (PDA) medium (concentration of 5%), adequately mixed and poured into sterile Petri dishes (9 mm) for 15 mL until medium cooling. To evaluate antagonistic activity of germfree filtrate of strain Y2 *in vitro*, 7-mm-diameter disks were cut from PDA plates of *F. oxysporum*, which were inoculated centrally onto pates. Sterile NB medium was used as a blank control in each treatment with three replicates. The colony diameter of *F. oxysporum* was measured at 26°C for 120 h and the inhibitory rate was calculated to detect the fractions containing inhibitory compounds.

Effect of germfree filtrate by strain Y2 on germination of F. oxysporum

To evaluate the effect of strain Y2 on spore germination of *F. oxysporum*, 7-mm-diameter disks of *F. oxysporum* were inoculated centrally in sterile Petri dishes at 26°C for 120 h. Spore suspensions of *F. oxysporum* were diluted to 10^8 spores/mL using hemacytometer counting method. Germfree filtrate of strain Y2 was added in spore suspension of *F. oxysporum* (concentration 5%) and sterile NB medium as a blank control in each treatment with three replicates. Spore germination of *F. oxysporum* was assessed microscopically by observing approximately 100 spores, in 5 field of vision per replicate. Percentage germination was calculated.

Effect of germfree filtrate and by strain Y2 on F. oxysporum sporulation

F. oxysporum was cultured in PDA medium at 26°C for 48 h. Then, 7-mm-diameter disks of *F. oxysporum* were inoculated centrally onto and incubated at 25°C. When the colony diameter of *F. oxysporum* was up to 4.00 cm, peripheral culture media without the colony was dug out using dissecting needle. Colony of *F. oxysporum* was submerged for 20 min with 20 mL germfree filtrate (concentration 5%) and sterile NB medium was added as a blank control in each treatment with three replicates. At last, germfree filtrate and sterile NB medium were removed. After 120 h, spore suspension was obtained by washing colony of *F. oxysporum* using 10 mL sterile water at 26°C. The concentration of spore suspension was measured using hemacytometer counting method. Then the inhibitory rate of *F. oxysporum* sporulation was calculated.

Effect of soybean germination and root length treated with Y2 bacterial suspension

Strain Y2 was inoculated into KB liquid medium and incubated on a rotating shaker at 170 rpm for 24 h at 28°C. Then inoculum was added in KB liquid medium (inoculation quantity 1% with 300-mL flask containing 100 ml medium) and incubated on a rotating shaker at 170 rpm for 48 h at 28°C.

Soybean seed (Hefeng 25) were treated with 75% alcohol for 2 s, and soaking with 0.1% corrosive sublimate for 3 min, and then the sample was washed twice with sterile water.

For seed coating, soybean seeds (Hefeng 25) were soaked in a 30 ml Y2 bacterial suspension for 20 min, and sterile medium was used as a blank control in each treatment with three replicates. Soybean seeds were removed and added to sterile Petri dishes (20 mm), soaked with sterile water (20 mL) for 20 min and 10 seeds had double gauze. In all tests, each treatment had three replicates with two replications per experiment. Soybean germination and root length treated with *B. subtilis* Y2 bacterial suspension were tested at 25 ± 3°C for 5 days.

Control effect of *B. subtilis* Y2 against soybean root rot caused by *Fusarium oxysporum* in pot trial

F. oxysporum was subcultured onto PDA medium and incubated at 26°C in the dark for 4 days. A fungal disk (7 mm) was added to 125 g of sorghum [*Sorghum bicolor* (L.) Moench.] seed, and autoclaved with 150 mL of water in 500-mL flasks for 40 min. The sorghum seed inoculum was incubated in stationary culture at 26°C for 5 days with periodic shaking to insure uniform colonization.

Sterile vermiculite was placed in plastic planting trays (8× 8 cm) with one-third vermiculite, and ten sorghum seeds with *F. oxysporum*. Then, the inoculum was covered with sterile vermiculite (0.5 cm thick), and 15 seeds (Hefeng 25) with sterile vermiculite (2 cm) in turn without inoculated plant acted as the control group. Y2 bacterial liquid (10 mL) was poured into plastic planting trays.

In all tests, each treatment was had three replicates; there were two replications per experiment. After emergence, plants were thinned to 10 plants per row and grown with water spreading and normal management in the greenhouse at 25 ± 3°C for 10 days.

Disease was rated using a standard disease index covering five categories by reference to Wang and Wen's Standard (2011) with slight difference. The plants were removed from the vermiculite. After washing roots, a visual rating following the 0 to 7 rating scale was used (in this scale, 0 = healthy plant; 1=slightly darkening fibrous root, the plant of aboveground grew well; 3= slightly darkening taproot, the plant of aboveground grew well; 5= seriously darkening taproot or hypocotyls erosion, the plant of aboveground grew poor; 7= root necrotized and the death of infected plants). The visual assessment was carried out between January and March 2012 to facilitate direct comparison.

RESULTS

Determination of antagonistic mechanism of strain Y2 against *F. oxysporum*

Biological activity of germfree filtrate of strains Y2 with treatment of high temperature (121°C) and normal temperature, were measured by using mycelial growth rate (Table 1). Inhibitory rate of 5% germfree filtrate against *F.oxysporum* mycelium growth had significant difference between normal temperature and 121°C ($P<0.05$). The antibacterial substance was sensitive to heat.

Effect of strain Y2 on germination of *F. oxysporum* was observed. The germination number of 100 spores was investigated with each dealing after more than 60% spore germination in the blank control. The results (Table 2) show that the percentages of the spore germination of treated with 5% germfree filtrate were 66.33% ($P<0.05$).

The spores of *F. oxysporum* were susceptible to germfree

Table 1. Effect of germfree filtrate of strain Y2 against *F. oxysporum* mycelium growth with different temperature treatment.

Temperature (°C)	Germfree filtrate	
	Colony diameters mean ± standard error (cm)	Inhibitory rate (%)
CK	6.08±0.12[a]	
121	5.74±0.27[a]	5.59
25	4.45±0.27[b]	26.81

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at P<0.05 according to Duncan's multiple range tests.

Table 2. Effect of Y2 germfree filtrate on germination of *F. oxysporum.*

Treatment	Germinative rate mean ± standard error	Inhibitory rate (%)
CK	67.33±2.73	
5%	22.67±2.03	66.33

Data are treatment means of pooled data ± standard errors.

Table 3. Effect of Y2 germfree filtrate on *F. oxysporum* spores bearing.

Treatment	Mean ± standard error (%)	Inhibitory rate (%)
CK	1166667±180422 [a]	
germfree filtrate	270833.3±43368.04[b]	76.79

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at P<0.05 according to Duncan's multiple range tests.

Table 4. Effect of soybean seeds germination treated with Y2 bacterial suspension.

Treatment	Germination rate (%)			Mean ± standard error (%)
	1	2	3	
Y2 bacterial suspension	80	70	70	73.33±3.33[a]
CK	80	70	70	73.33±3.33[a]

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at P<0.05 according to Duncan's multiple range tests.

filtrate.

The count of spore suspensions was enumerated by blood count method (Table 3). The results show that there was a significant difference between control and treatments treated with 5% germfree filtrate with respect to spores bearing of *F. oxysporum*. *F. oxysporum* spores were more susceptible to germfree filtrate with inhibitory rate for 76.79%.

Effect of soybean germination and root length treated with *B. subtilis* Y2 bacterial suspension

Under similar conditions and similar duration of treatment, Y2 bacterial suspension was used to treat the soybean varieties (Hefeng 25). The results show that there was not a significant difference between control and treatments with Y2 bacterial suspension with respect to soybean seeds germination and root length (Tables 4 and 5). The results preliminary showed that *B. subtilis* Y2 had no effect on soybean seeds germination and growth.

Control effect of *B. subtilis* Y2 against soybean root rot caused by *F. oxysporum* potted experiments

The control effect of Y2 germfree filtrate and bacterial suspension on soybean root rot caused by *F. oxysporum* was conducted in a pot trial (Table 6). Germfree filtrate and suspension of *B. subtilis* Y2 exhibited control effects

Table 5. Effect of soybean root length treated with Y2 bacterial suspension.

Treatment	Root length (cm)			Mean ± standard error (%)
	1	2	3	
Y2 bacterial suspension	9.59	7.18	10.70	9.16±1.04[a]
CK	9.38	9.64	6.15	8.39±1.12[a]

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at P<0.05 according to Duncan's multiple range tests.

Table 6. Control effect of *B. subtilis* Y2 on *F. oxysporum* causing soybean root rot.

Treatment	Replicate	Incidence (%)	Disease index	Disease index mean ± standard error (%)	Control effect (%)
CK	1	100.00	94.29	77.03±21.91[b]	
	2	100.00	84.42		
	3	100.00	52.38		
Y2 germfree filtrate	1	21.06	12.86	7.89±5.95[a]	92.35
	2	9.09	1.30		
	3	16.67	9.52		
Y2 bacterial suspension	1	0.00	0.00	0.95±1.65[a]	98.77
	2	0.00	0.00		
	3	6.67	2.86		

Data are treatment means of pooled data ± standard errors. Values of each column followed by different letters are significantly different at P<0.05 according to Duncan's multiple range tests.

significantly reducing disease by 92.35 and 98.77%, respectively.

DISCUSSION

Soybean root rot, caused by a complex infection of many soil inhabitants, has become an increasing problem for soybeans (Roy et al., 2000; Wang et al., 2004). In Heilongjiang province, the dominant strain was *F. oxysporum* (Xing et al., 2009). Environmental pollution caused by excessive use and misuse of chemical pesticides has led to considerable awareness and changes in people's attitudes toward the use of pesticides in agriculture (Wang et al., 2007). Biological control of *F. oxysporum* using antagonistic microorganisms may become an important way to reduce the damages brought about soybean root rot in practice.

The *B. subtilis* strains produce a wide variety of peptide antibiotics, such as subtilin, subtilosin A, TasA and sublancin of ribosomal origin (Klein and Entian, 1994; Hyronimus et al., 1998; Ye et al., 2012). Fengycins also show antifungal properties and play a role in the biocontrol effect of *B. subtilis* by direct antagonism against pathogen as well as by induction of plant defence mechanisms (Ongena et al., 2005). The antagonistic mechanism of *B. subtilis* Y2 against *F. oxysporum* could effectively inhibit mycelial growth, sporulation and germination in the study. But, the main reason, which had lower inhibitory action on mycelial growth of *F. oxysporum,* was sterilized filtrate obtained from only 48 h bacterial filter for testing antibacterial activity. The antibacterial activity of bacterial filter reach highest at 7 to 8 days.

The temperature was an important factor affecting antibacterial activity. Antibacterial substance containing macromolecular protein may be sensitive to heat. Because of *B. subtilis* Y2 isolated from the surface of soybean root, the *B. subtilis* Y2 would be adapted to micro-ecological environment of soybean root, which would provide a certain foundation on suppressing soybean root rot in future (Li and Ma, 2012).

Biological seed treatment provides a means of reducing infections of soil-borne pathogens. Root rot in mungbean was significantly suppressed with a seed treatment with *B. subtilis* (Siddiqui et al., 2001). The study support the results obtained in this study using germfree filtrate and bacterial suspension of *B. subtilis* which reduced disease by 92.35 and 98.77% , respectively. So, *B. subtilis* Y2 has good application prospects.

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