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Isolation and Identification of Antagonistic Bacteria for Biological Control of Ginger Rhizome Rot Caused by *Pythium zingiberum*

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ABSTRACT

Sixteen isolates showing relatively strong antagonicity against the ginger rhizome rot pathogen, *Pythium zingiberum*, were selected among the 155 isolates from ginger rhizome surfaces and rhizospheres of ginger cultivation fields in Wanju, Chonbuk. The isolate, 'HB 26-5' showing the strongest antagonicity was finally selected by testing duration of inhibition effect and pathogenicity to ginger. The isolated antagonistic microorganism, 'HB 26-5', was rod shape, gram positive and formed endospore. The isolate produced acids utilizing glucose, arabinose, xylose and mannitol, and acetoin at VP test, and grew anaerobically. Temperature range for growth was from 10 to 40℃. Reaction to catalase and gelatin, hydrolysis were positive, and casein hydrolysis and indol production were negative. Based on the mycological characters and the fatty acid composition, it was identified as *Bacillus polymyxa*. The pathogenicity test of isolated *Bacillus polymyxa* 'HB 26-5' on 22 crop cultivars resulted that only the lettuce was influenced in germination, and the others were not affected.

Key Words : *Bacillus polymyxa*, Biological control, Ginger rhizome rot, *Pythium zingiberum*

INTRODUCTION

Rhizome rot of ginger caused by *Pythium zingiberum* has been a major limiting factor for ginger production (Takahashi, 1954) in major cultivation area of Chonbuk and Chungbuk in Korea, due to the difficulties in control.

To control this disease, seed disinfection, soil fumigation, and fungicide application were researched in Japan(Shinsu, 1980; 1984), but the effect of fungicide application at growing field is very low because of rice straw covering. And chemical control may cause soil contamination, and control effect is down, if the pathogen acquire resistance on that fungicide, and residual toxicity can be a great problem. So, biological control can be a good substitution for chemical control to cope with this disease.

The research on biological control using *Pythium* sp. was started by Sneh *et. al.*(1977), they reported parasitic fungus on oospores of *Pythium* sp. Hoch *et. al.*(1978) reported that damping-off of table beet was seriously suppressed when the *Corticium* sp. was applied in the soil contaminated by *Pythium ultimum*. Harman(1980)

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reported that the disease in carrot caused by *Pythium* spp. was suppressed when *Trichoderma hamatum* was applied on the seed. Hardar *et. al.*(1984) isolated *T. koningi*(T8) and *T. harzianum*(T12) as a biological control agent on the damping-off of pea caused by *Pythium* sp. But, the antagonistic fungi are thought to be difficult in use as a biocontrol agent of ginger rhizome rot.

We isolated antagonistic bacteria to *P. zingiberum*, and then investigated physiological characteristics and availbility as a biocontrol agent.

MATERIALS AND METHODS

Cultural practices

A ginger cultivar, Bongdong-jaerae(Chonbuk province) was used through out this study. The ginger rhizomes stored in the sand were used as needed. The experiment was conducted using 1/2,500a pot, and 3 ginger were planted in each pot, and then the surface of the pots was covered with rice-straw.

Isolation of antagonistic bacteria

Potential antagonistic bacteria were isolated from suppressive rhizosphere soil and ginger surface where ginger had been growing. Among the isolates, *in vitro* antagonistic activity was examined by dual culture; plates with potato dextrose agar(PDA) were inoculated with a potential antagonists, four in each petridish, by depositing a loopful of the antagonists on an area approximately 1 cm apart from the edge of the plate. After incubation for 2 days at 30℃, *P. zingiberum* was introduced by placing a 2mm diameter mycelial disk in the center of the plate. After 2 days of additional incubation, inhibition zone was measured. To investigate the pathogenicity of the antagonist on ginger, sliced ginger was placed on the water agar, and antagonist was inoculated on the ginger. After 5 days of incubation at 30℃, potential pathogenicity was investigated.

Antagogonistic activities of the potential bacteria on *Aspergillus niger, Magnaporthe grisea, A. oryzae, Alternaria alternata, Fusarium solani, Cladosporium cladospoioides* and *Penicillium* sp. were also investigated.

Identification of antagonists

Key characteristics of the antagonists were tested to identify them taxonomically. The tests were performed by Nishiyama and Ezuka(1975), and Goto and Yakikawa method(1984) for physiological characteristics, and Sasser(1990) method for the analysis of fatty acid. The taxonomic criteria for identification of the bacteria were followed the Bergy' s manual of systemic bacteriology, Vol. 2(1986).

Gram reaction was observed by staining crystal violet and safranin after fire-fixing smear of 24 hours after incubation on nutrient agar.

Endopore was observed as follows; individual bacteria was smeared on the slideglass, and allowed to air dry and fire-fixing. Flooded 7.5% of malachite green for 10 min, and washed under running tap water, and counterstained with 0.25% safranin for 15sec, and then examined under oil immersion(X1,000).

Oxidation and fermentation test was performed by the method of Hugh and Leifson(1953), and oxigen demand by the method of Iizuka and Komakata(1962). Catalase test was investigated by bubbles of free oxygen gas after adding 1% H_2O_2. Nutritional requirement on sugar was investigated by using Ayers' minimal synthetic agar(1919) after adding 40*ml* of 0.2% BTB gradually. Gas production was examined by cracking of agar, and the other tests were performed by the method of Sakazaki(1974), Goto and Takikawa(1984).

Fatty acid was analyzed by using gas chromatography(Varian star, 3600cx) on the manufacturer' s manual. When the similarity index was above 0.5, we accepted that identification.

RESULTS AND DISCUSSION

Isolation of antagonistic bacteria

Total 155 bacterial strains were isolated at the rhizosphere of ginger in ginger cultivated fields and surfaces of growing ginger rhizomes in Wanju , Chonbuk, Korea. In the first step of screening process to select useful antagonistic strains the 155 strains were cultivated dually with the recognized pathogens, *P. zingiberum, Fusarium oxysporum*, which is major pathogen on ginger rhizome rot. The isolates involving HB 26-5, which shown antagonism against both the pathogens or relatively strong antagonism against *P. zingiberum*(Table 1) were selected.

The isolate which lost the least of the antagonistic activity against *P. zingiberum* for 15 days was HB 26-5 compared with the other two isolates, HB 26-3 and HB 102-3(Fig 1). The antagonistic mechanisms of the six isolates including HB 26-3 were tested and appeared that the mycelial growth inhibition was resulted from lysis of mycelium HB 128-5 and HB 149-3 showed in the course of the lysis the distal end of mycelium swelled and eventually exploded to ooze out the cytosol, but the HB 85-4, inhibited mycelial growth by parasitism(Table 2).

Table 1. Antagonistic ability of bacteria isolated from ginger rhizome or rhizosphere soil

Isolate	Inhibition zone(mm)	
	Pythium zingiberum[1]	*Fusarium oxysporum*[2]
HB 9-3	3.5	6.9
HB 26-3	8.5	7.0
HB102-3	6.9	8.0
HB144-3	6.5	6.3
HB149-3	4.0	5.6
HB158-3	3.0	9.5
HB 61-4	11.8	7.5
HB 85-4	8.0	0
HB110-4	3.5	8.2
HB142-4	7.5	0
HB154-4	6.0	6.8
HB 26-5	13.5	9.9
HB 75-5	6.7	0
HB 87-5	5.0	9.0
HB128-5	10.7	10.5
HB140-5	5.5	9.0

[1]Pathogen was inoculated 2 days after incubation of isolated bacteria, incubated for 2 days at 30℃, and observed.

[2]Pathogen and bacterial isolates were inoculated simultaneously, and observed 4 days after inoculation.

In the pathogenicity test against ginger itself, all the isolates tested were appeared not to be pathogenic on the following test, the section inoculation, the injection inoculation to growing ginger rhizome and the soaking

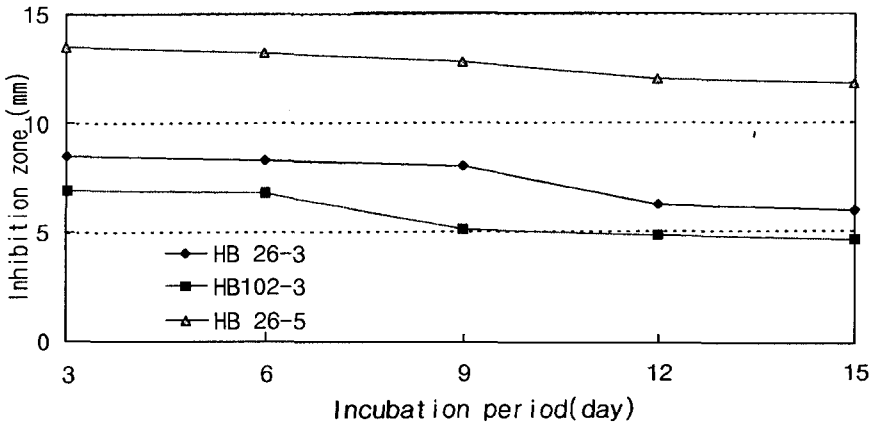

Fig. 1. Inhibitory effect of antagonistic isolates on *Pythium zingiberum*

Table 2. Antagonistic aspects of the isolate against *Pythium zingiberum*

Isolate	Inhibition of growth	Lysis of hyphae	Hyperparasitism
HB 26-3	+[1]	-	-
HB 85-4	-	-	+
HB102-3	+	-	-
HB 26-5	+	-	-
HB128-5	+	+	-
HB149-3	+	+	-

1 + : Positive response - : negative response

Table 3. Pathogenicity of the antagonistic isolates to ginger rhizome

Isolate	Pathogenicity according to inoculation method		
	Drenching[1]	Dipping[2]	ginger section[3]
HB 26-3	-[4]	-	-
HB102-3	-	-	-
HB 26-5	-	-	-
Control (*Pythium zingiberum*)	+	+	+

[1]Antagonistic bacteria suspension(10^8cfu/ml) and *P. zingiberum* suspension(10^3propagule/ml) were drenched to ginger growing pot.

[2]Ginger was dipped in suspension of 10^8cfu/ml for one hour and seeded.

[3]Antagonistic isolates and pathogen were inoculated on sliced ginger.

[4]+: positive response, -: negative response.

inoculation by dipping the ginger into the culture solution(Table 3). Among the isolates shown relatively strong antagonism and without pathogenicity against ginger, HB 26-5 was finally selected and tested for whether it was antagonistic to other pathogenic fungi and saprophytes or not. *Magnaporthe grisea, A. oryzae, A. alternata, Fusarium solani, Cladosporium cladospoi-oides* and *Penicillium* sp. were inhibited in growth excepting Aspergillus niger.

Identification and physiological characteristics of antogonist

HB 26-5 showed remarkable control effect in the small scale indoor test where ginger rhizome rot pathogen and the antagonist were simultaneously inoculated to the ginger planted in a stainless steel tray(Fig 2).

HB 26-5, the most promising isolate which shown strongest control effect on the ginger rhizome rot, was characterized in physiological and morphological properties(Table 4). It is gram positive bacillus and forming endospore(Fig 3). Acids were produced when glucose, arabinose, xylose and mannitol was used as

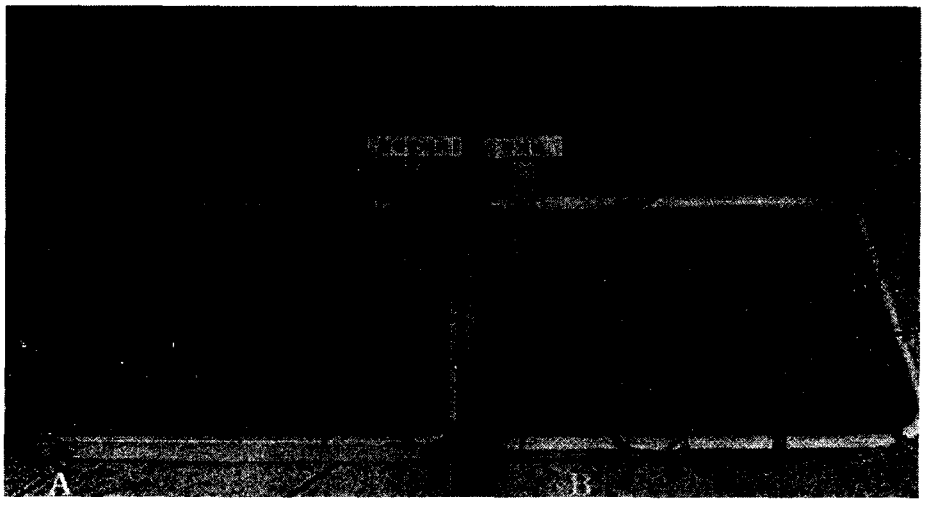

Fig. 2. Control effect on ginger rhizome rot with antagonistic bacteria.
Antagonistic bacteria 'HB 26-51' was treated to soil(A) and not treated(B)

Table 4. Comparison of the characteristics of the antagonistis isolate 'HB 26-5' with the *Bacillus* spp. in Bergey's manual

Characteristics	*B.circulans*	*B.licheniformis*	*B.polymyxa*	*B.pumilus*	HB26-5
Cell diameter(>1.0㎛)	-[1]	-	-	-	d
Endospore	+	-	+	-	+
Catalase test	+	+	+	+	+
Anaerobic growth	d	+	+	-	d
Voges-proskauer test	-	+	+	+	+
Acid from; D-glucose	+	+	+	+	+
" ; L-arabinose	+	+	+	+	+
" ; D-xylose	+	+	+	+	+
" ; D-mannitol	+	+	+	+	+
Gas from; glucose	-	-	+	-	+
Casein hydrolysis	-	-	-	-	-
Gelatin hydrolysis	d	+	+	+	+
Starch hydrolysis	+	+	+	-	+
Tyrosine denature	-	-	-	-	-
Nitrate to nitrite	d	+	-	-	-
Indol formation	-	-	-	-	-
NaCl, KCl requirement	-	-	-	-	-
Growth at pH6.8	+	+	+	+	+
" pH5.7	d	+	+	+	+
Growth in NaCl; 2%	ND	+	ND	+	+
" ; 5%	d	+	-	+	+
" ; 7%	d	+	-	+	-
" ; 10%	-	ND	-	ND	-
Growth at ; 5℃	-	-	d	-	-
" ; 10℃	d	-	+	+	+
" ; 30℃	+	+	+	+	+
" ; 40℃	+	+	+	+	+
" ; 50℃	-	+	-	d	-
" ; 55℃	-	-	-	-	-
" ; 65℃	-	-	-	-	-

[1]+; ≥90% positive, -; ≥90% negative, d; 11～89% positive

Fig. 3. Light microscopy of *Bacillus polymyxa* 'HB 26-5'. Typical rod-shaped cells, 2.2 to 4.5㎛ in length and 0.7 to 0.8㎛ in width. Bar indicates 5㎛

carbon sources and aceotoin anaerobically at VP test. Air bubbles were generated by treating H_2O_2. It showed positive response to the catalase, gelatin hydrosis and starch hydrosis but not casein. And it did not produce indol and demand bases. And it grew well at the pH range between weak alkaline(pH6.8) and weak acid(pH 5.7), and could grow at temperature range between 10 ℃ and 40℃. The bacteria was rod form as shown in Fig.3, and was 4.5㎛ long and 0.7㎛～0.6㎛ wide. Considering the above results and referring to Bergey's manual of systematic bacteriology, Vol. 2(1986), HB

Table 5. Pathogenicity of *Bacillus polymyxa* 'HB 26-5' to 22 crops

Common name	% germination			% healthy seedlings
	Seed treatment[1]	Seeding after bacteria removed[2]	Control	
Rice	100	100	100	100
Wheat	100	100	100	100
Barley	100	100	100	100
Corn	90	85	80	90
Barnyard millet	90	80	85	90
Green gram	100	100	100	90
Chinese cabbage	100	95	100	90
Radish	100	100	100	100
Rape	100	90	100	90
Lettuce	65	55	100	70
Red pepper	100	95	100	90
Tomato	95	100	95	95
Eggplant	30	40	40	45
Tobacco	85	90	90	85
Chinese chive	90	80	85	90
Spinach	80	75	75	80
Carrot	100	100	100	85
Cucumber	95	100	100	100
Orientalmelon	100	100	95	95
Water melon	100	95	95	100
Onion	100	100	100	95
Melon	100	95	100	90

[1]The seeds treated with bacterial suspension were placed on water agar. Initial concentration of bacterial suspension was 10^8cfu/*ml*.

[2]The seed viabilities were checked on NA(Difco) media covered with a sheet of the sterilized dialysis membrane and the filter paper soacked in bacterial suspension previously, and the dialysis membranes were removed after two days of incubation at 25℃.

26-5 was identified as *Bacillus polymyxa*.

Pathogenicity of HB 26-5 to the crops other than ginger

The pathogenicity test of isolated *Bacillus polymyxa* HB 26-5 on 22 crop cultivars by inoculation of the isolate or treatment of filtered culture solution resulted that the lettuce was only influenced in germination, but the other crops were not(Table 5). According to Sneath(1988) some microbes in genus *Bacillus* were pathogenic to certain mammals or insects, but until recently it has been unknown that they might cause disease in plants. Robert(1998) used *B. polymyxa* to produce alcohols from fiber, sugar, and xylan. As the microbes secret various catalases involved in decomposition of the structural substances of plants, it may facilitate the microbe itself to enter through the wound made by the action of the catalases and affect the plants.

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