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Isolation and Evaluation of Protective Effect against Fusarium Wilt of Sesame Plants of Antibiotic Substance from *Bacillus polymyxa* KB-8

Jae-Wook Hyun, Young-Hoon Kim[1], Yong-Se Lee[2] and Won-Mok Park[3,*]

Department of Agricultural Biology, Korea University, Seoul 136-701, Korea
[1]Department of Chemistry, Kon Kuk University, Seoul 143-701, Korea
[2]College of Natural Resources, Taegu University, Kyongsan 713-714, Korea
[3]Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

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An antibiotic compound was isolated from the culture of an antagonist against *Fusarium oxysporum* f. sp. *sesami*, *Bacillus polymyxa* strain KB-8, and tested for the control of Fusarium wilt of sesame in greenhouse conditions. Optimum conditions for culturing the antagonist to obtain the maximum antibiotic activity were determined using different culture media, initial medium acidity, and incubation periods, for which yeast -malt extract agar with the initial acidity of pH 5 and over 13 days culture were best. Antibiotic substances extracted by methanol had 2 main fractions, KB-8A and KB-8B, in thin layer chromatography (TLC) with Rf values of 0.35 and 0.67 in a solvent system of chloroform : methanol = 7 : 3. The fraction KB-8A was purified further by XAD-2, silica gel and Sephadex LH-20 column chromatography, and crystalization. Its minimum inhibitory concentrations (MICs) were 12.8 μg/ml for *F. oxysporum* and *Alternaria mali*, 6.4 μg/ml for *Colletotrichum gloeosporioides* and *Rhizoctonia solani*, and 3.2 μg/ml for *Phytophthora capsici*. Soil drenching of antibiotic KB-8A in the concentrations of 13.0 μg/ml and 26.0 μg/ml effectively inhibited the Fusarium wilt of sesame in a greenhouse test, which appeared to be comparable to the fungicide benlate of 6.5 μg a. i./ml.

Keywords : *Bacillus polymyxa* KB-8, *Fusarium oxysporum* f. sp. *sesami*, isolation of antibiotic, MIC, protective effect.

Fusarium oxysporum f. sp. *sesami* causes seedling blight and Fusarium wilt of sesame, which is one of the most important diseases reducing sesame yield in Korea (Cho and Choi, 1987). The control measures of the diseases comprise cultivation of resistant varieties, plastic-film mulching, rotation, use of healthy seeds, chemiacl control, and use of biological control methods.

In the chemical control of plant diseases, antibiotics of microbial origin have some advantages over organic synthetic pesticides; they are versatile in structure and activity, and biodegradable causing less hazards on the environments (Tanaka and Omura, 1993). So far many antibiotics from microbial metabolites have been developed, and used for the control of fungal diseases including blasticidine S (Larsen, 1989), polyoxin (Isono, 1988), kasugamycin (Suhara et al., 1972), validamycin (Kameda, 1988), and mildiomycin (Tashiro, 1984) from Actinomycetes, and strobilurin from Basidiomycetes (Leroux, 1996).

Bacillus species have been well known for their antagonistic activity against various plant diseases (Batinic et al., 1998; Berger et al., 1996; Ferreira et al., 1991; Korsten et al., 1997; Podile and Prakash, 1996; Utkhede and Sholberg, 1986). It is assumed that the antagonistic activities of *Bacillus* spp. are mainly attributed to the production of antibiotic compounds, including iturin group and peptide group (Buckingham, 1994).

In our preliminary experiment, *B. polymyxa* KB-8 showed a protective effect against Fusarium wilt of sesame, in which antibiosis might be involved the suppression of the disease. Therefore, this study was carried out to isolate antibiosis-related materials, antifungal compounds, from *B. polymyxa* KB-8, and to evaluate the control effect of the responsible material against Fusarium wilt of sesame in a greenhouse.

Materials and Methods

The antagonist. A strain of *B. polymyxa*, KB-8, isolated from non-cultivated soil in Ichun, Kyoungki-Do, which showed a antagonistic effect against Fusarium wilt of sesame in a preliminary experiment, was used in this study.

Optimum culture conditions for the antibiotic production. Five media consisting of various nutritional elements were tested for the antibiotic production by *B. polymyxa* KB-8. Five media used in this study were as follow: yeast extract-malt extract agar medium (YMA; 4 g yeast extract, 10 g malt extract, 4 g glucose, and 15 g agar in 1 L distilled water), tryptic soy agar medium (TSA; 20 g tryptic soy broth, and 15 g agar in 1 L distilled water), potato dextrose agar medium (PDA), King's B agar medium

*Corresponding author.
Phone) +82-2-3290-3412, Fax) +82-2-923-9923
E-mail) wmpark@kuccnx.korea.ac.kr

(KBA; 20 g proteous peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$ $7H_2O$, 15 ml glycerol, and 15 g agar in 1 L distilled water), nutrient broth-yeast extract agar medium (NYA; 8.0 g nutrient broth, 2.0 g yeast extract, 2.0 g K_2HPO_4, 0.5 g KH_2PO_4, 2.5 g glucose, 0.062 g $MgSO_4$ $7H_2O$, and 15 g agar in 1 L distilled water). The optimal culture period and initial medium pH were also determined using YMA medium. Culture times tested were 3-13 days, and initial pHs 4-10 at intervals of pH 1.

The optimum culture conditions of *B. polymyxa* KB-8 for the antibiotic production were determined on the basis of antifungal activity of the bacterial culture extracts against *F. oxysporum* f. sp. *sesami*. For the production of antibiotic materials at different media, the bacterium was cultured on 20 Petri plates contained 25 ml agar media each other for 13 days using the inoculum grown in YM broth for 48 hr at 27°C on a rotary shaker (150 rpm). The agar cultures (500 ml) of *B. polymyxa* KB-8 were homogenized in a blender, and extracted in methanol for 2 days. The methanol extract was centrifuged at 10,000 g for 20 min to remove cells and medium debris, and dried *in vacuo*. Each culture extract was dissolved in methanol (with the concentration equvalent to 1 culture extract of 500 ml medium in 5 ml), and tested for antifungal activity against *F. oxysporum* f. sp. *sesami* using the agar dilution bioassay method.

For the agar dilution bioassay method, 0.2 ml of the culture extract of 500 ml medium concentrated in methanol 5 ml was incorporated in 20 ml PDA (in a Petri plate), and a 5-day-old fungal culture disk (8 mm in diameter) of *F. oxysporum* f. sp. *sesami* was placed on the medium. After incubated at 27°C for 7 days, diameters of the fungal colonies were measured to evaluate the inhibitory effect of the crude antibiotics on the fungal pathogen.

Purification of antibiotics produced by *B. polymyxa* KB-8. Antibiotic compounds responsible for the antibiosis against *F. oxysporum* f. sp. *sesami* were initially extracted with methanol from YMA cultures of the antagonistic bacterium grown for 13 days at 27°C. The methanol extract was purified by Amberlite XAD-2, silica gel, and Sephadex LH-20 column chromatography, and the final crude substances were eluted through Sep-Pak C-18 cartridge.

The culture extract of *B. polymyxa* KB-8 was dissolved in distilled water and purified by chromatography through Amberlite XAD-2 (nonionic polymeric adsorbent, Serva Chemical Co.) which had been activated by extracting with dichloromethane and methanol. After stepwise-elution with H_2O, 30% MeOH, 80% MeOH, 100% MeOH and acetone, each fraction was concentrated *in vacuo* and bioassayed by the agar diffusion bioassay method. Active fractions against *F. oxysporum* f. sp. *sesami* were combined. The crude antibiotics from the XAD-2 column chromatography were dissolved in a small volume of chloroform-methanol (8 : 2) and chromatographed on silica gel (silica gel 60F254, Merck; 7.0×70 cm) using a chloroform-methanol step gradient [$CHCl_3$-MeOH (8 : 2); $CHCl_3$-MeOH (7 : 3); 100% MeOH] at a flow rate of 1 ml/min. The elutes were concentrated *in vacuo* and bioassayed against *F. oxysporum* f. sp. *sesami* by the agar diffusion method. Active fractions were combined and stored at 4°C. The crude antibiotics from silica gel column chromatography were dissolved in a minimum volume of methanol, and chromatographed on a Sephadex LH-20 column (2.6×100 cm) with methanol at a flow rate of 0.1 ml/min. Fractions of 5 ml each were collected using a fraction collector (Korea Manhathan Co.). Each fraction concentrated in 1 ml of methanol was bioassayed by the agar diffusion method. Active fractions were combined and stored at 4°C. The crude antibiotics from Sephadex LH-20 column chromatography were dissolved in H_2O and applied to Sep-Pak C-18 cartridge using 12 ml syringe. After stepwise-elution with H_2O, 30% MeOH, 50% MeOH, 80% MeOH and 100% MeOH, each eluate was concentrated and bioassayed by the agar diffusion method. Active fractions were pooled and crystalized in H_2O at 4°C.

After Sep-Pak C-18 cartridge, the antifungal-active compound in crude antibiotic substances was identified using the bioautographic technique on TLC plate. The fractions with antifungal activity were spotted on a TLC plate (layer thickness of 0.1 mm, 60 F254, Merck), and developed in a solvent system of chloroform-methanol (7 : 3, v/v). After air-drying to remove solvent, the TLC plate was placed on the water agar plate (2% agar in a 9 cm-diameter Petri plate). Molten PDA seeded with 10^5 spores/ml of *F. oxysporum* f. sp. *sesami* was uniformly spread onto the TLC plate. After incubating for 36 hr at 27°C, the agar plate was stained with Coomassie brilliant blue solution for 30 min (staining solution: 800 ml distilled water, 200 ml methanol, 70 ml acetic acid, 60 g trichloro acetic acid, and 25 ml 2% coomassie brilliant blue R-250 solution), and destained (destaining solution: 28 ml distilled water, 12 ml methanol, and 2 ml acetic acid). The Rf value of antifungal-active compound was confirmed by comparing the inhibition zone with the bands on TLC plates visualized by UV irradiation or iodine vapor.

To detect antibiotic activity of purifying substances in the purification procedures, respective fractions were tested by the agar diffusion bioassay method. For the agar diffusion bioassay method, each prepared fraction was concentrated and diluted in proper concentrations, and then placed into the central well (7 mm in diameter) of PDA seeded with fungal spores (10^5/ml) of *F. oxysporum* f. sp. *sesami*. The sizes of inhibition zones around the wells were mearsured after 36 hr incubation at 27°C.

Antifungal activity of the purified antibiotic. The activity of the finally purified antibiotic was tested on PDA supplemented with a series of the antibiotic concentrations (0-50 μg/ml) dissolved in same volume of methanol using 5 cm-diameter Petri plates. The mycelial disks of *F. oxysporum* f. sp. *sesami*, *Alternaria mali*, *Colletotrichum gloeosporioides*, *Phytophthora capsici* and *Rhizoctonia solani* were placed on the each test plates and incubate at 27°C. The lowest concentration that prevents visible mycelial growth of plant pathogenic fungi was determined after incubation for 4-5 days.

Control effect of the antibiotic on Fusarium wilt of sesame plants in the greenhouse. Disease suppressive effect of the purified antibiotic produced by *B. polymyxa* KB-8 against Fusarium wilt of sesame was tested in greenhouse conditions. Ten ml spore suspension (10^7 spores/ml) containing the antibiotic with a series of concentrations (6.5, 13.0 and 26.0 μg/ml) was poured into the rhizosphere of sesame plants at the six leaf stage for both inoculation and antibiotic treatment. A commercial fungicide,

benlate (6.8 μg a.i./ml), was used as a positive control to compare the control effect with the antibiotic.

Disease occurrence was examined daily after inoculation, and disease severity was scaled based on the following indices: 0= no symptom, 1= slightly wilted with yellowish lesions on leaves, 2= stem wilted, and 3= whole plant wilt and death.

Results

Optimum culture conditions for antibiotics production. Five culture media were tested to determine the culture conditions for maximum production of antibiotic substances against *F. oxysporum* f. sp. *sesami*. The methanol extracts of the bacterial cultures were assayed for their inhibitory effects on *F. oxysporum* f. sp. *sesami* by the agar dilution bioassay method. Among the culture media tested, YMA was evaluated the best antibiotic-production medium against *F. oxysporum* f. sp. *sesami* having the inhibitory effect of 69.4%, while the other media relatively were less effective in producing antibiotics, although no difference in the bacterial growth was noticed (Table 1). Especially no antibiotic activity against *F. oxysporum* f. sp. *sesami* was observed in the NYA culture of the bacterium.

To determine the optimal culture period for the production of antibiotics from *B. polymyxa* KB-8, the methanol extracts of the bacterial culture cultured on YMA for 3, 5, 9, 11 and 13 days were assayed for their inhibitory effect on the *F. oxysporum* f. sp. *sesami* by the agar dilution bioassay method. Increase in culture period of *B. polymyxa* KB-8 resulted in the production of more antibiotic substances in the culture. The antifungal activity of the bacterial culture increased with days of incubation periods, having inhibitory effect of 10.7%, 11.2%, 13.0%, 21.9% and 69.4% for 3, 5, 9, 11 and 13 days, respectively (Table 2).

Table 1. Inhibitory effects of culture extracts of *Bacillus polymyxa* KB-8 produced at different culture media against *Fusarium oxysporum* f. sp. *sesami*[a]

Medium	% inhibition[b]
TSA[c]	43.5[d]
YMA	69.4
PDA	59.6
KBA	30.6
NYA	0.0

[a] The inhibitory effects was examined by agar dilution bioassay method, as described in Materials and Methods.
[b] [1-(diameter of mycelium on 20 ml PDA + the 0.2 ml of culture extract of 500 ml medium concentrated in 5 ml methanol/diameter of mycelium on PDA)] × 100
[c] TSA: tryptic soy agar, YMA: yeast extract-malt extract agar, PDA: potato dextrose agar, KBA: King's B agar, NYA : nutrient broth-yeast extract agar
[d] Each value is the mean of 3 replicates.

Table 2. Optimum incubation periods for the production of antibiotic substances from *Bacillus Polymyxa* KB-8 examined by the inhibitory effect of the cultural extract against *Fusarium oxysporum* f. sp. *sesami*[a]

Incubation period (days)	% inhibition[b]
3	10.7[c]
5	11.2
9	13.0
11	21.9
13	69.4

[a] The antifungal activity was bioassayed by agar dilution bioassay method, as described in Materials and Methods.
[b] [1-(diameter of mycelium on 20 ml PDA + the 0.2 ml of culture extract of 500 ml medium concentrated in 5 ml methanol / diameter of mycelium on PDA)] × 100
[c] Each value is the mean of 3 replicates.

In initial pH of YMA for the antibiotic production, antifungal activity against *F. oxysporum* f. sp. *sesami* decreased with increase of pH from inhibitory effects of 67.4% at pH 5 to 48.4% at pH 10 (Table 3). No bacterial growth was observed on YMA at pH 4.

Purification of antibiotics. The antifungal activity was found in the 80 and 100% methanol eluates in Amberlite XAD-2 column chromatography. In silica gel column chromatography, the fractions eluted with two void volumes of chloroform -methanol (7 : 3) had the inhibitory effect on mycelial growth of *F. oxysporum* f. sp. *sesami* by agar diffusion bioassay method.

The crude antibiotics partially purified by silica gel column chromatography were chromatographed on Sephadex LH-20 column chromatography, and the antifungal activity of each fraction was assayed by agar diffusion bioassay

Table 3. Effect of initial pH of yeast extract-malt extract agar medium on production of antibiotic substances from *Bacillus polymyxa* KB-8[a]

pH	% inhibition[b]
4	[c]
5	67.4[d]
6	66.0
7	61.4
8	56.3
9	51.2
10	48.4

[a] The production of antibiotic substances was examined by the inhibitory effect of the cultured extract against *Fusarium oxysporum* f. sp. *sesami* by agar dilution bioassay method, as described in Materials and Methods.
[b] [1-(diameter of mycelium on 20 ml PDA + the 0.2 ml of culture extract of 500 ml medium concentrated in 5 ml methanol / diameter of mycelium on PDA)] × 100.
[c] No bacterial growth
[d] Each value is the mean of 3 replicates.

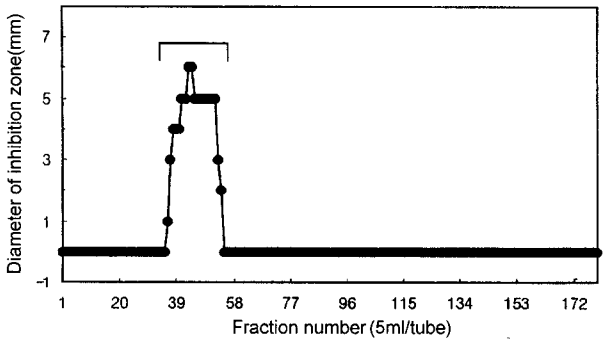

Fig. 1. Sephadex LH-20 column chromatogram of the crude antibiotic obtained from silica gel chromatography. The column (2.5×100 cm) was developed with methanol at a flow rate of 0.1 ml/min. Each fraction was bioassayed for antifungal activity to *Fusarium oxysporum* f. sp. *sesami* by agar diffusion method, as described in Materials and Methods. The bar represents the pooled active fractions.

method. Fractions from 36th through 54th showed inhibitory effect on the *F. oxysporum* f. sp. *sesami* (Fig. 1).

The active fractions from Sephadex LH-20 column chromatyogrphy were pooled in H_2O and further purified by stepwise-elution with H_2O, 30% MeOH, 50% MeOH, 80% MeOH and 100% MeOH in the Sep-Pak C-18 cartridge. Only 50 and 80% MeOH fractions had inhibitory effects on *F. oxyporum* f. sp. *sesami* by agar diffusion bioassay method (Table 4).

When the active fractions were chromatographed on TLC plate by using a chloroform-methanol (7 : 3) and bioassayed with *F. oxysporum* f. sp. *sesami*, 50% MeOH fraction produced one antifungal region at Rf 0.67 and 80% MeOH fraction produced one antifungal region at Rf 0.35 on the chromatogram, and we designated antibiotic KB-8A and KB-8B, respectively (Fig. 2).

The 50% fraction was pooled and crystallized in H_2O at 4°C, and total 50 mg of white powders were finally produced from 56 L culture.

Fig. 2. Bioautography test of antibiotic substances from *Bacillus polymyxa* KB-8 against *Fusarium oxysporum* f. sp. *sesami* on TLC plate developed with chloroform-methanol (7 : 3).
Lanes 1 and 2: 80% MeOH fraction from C-18 cartridge
Lane 3: 50% MeOH fraction from C-18 cartridge

Minimum inhibitory concentrations (MICs) against plant pathogenic fungi. The antifungal activity of antibiotic KB-8A was examined on PDA containing a series of concentrations (0-50 μg/ml). The MICs of the antibiotic were at 12.8 μg/ml for *F. oxysporum* f.sp. *sesami* and *Alternaria mali*, at 6.4 μg/ml for *Colletotrichum gloeosporioides* and *Rhizoctonia solani*, at 3.2 μg/ml for *Phytophthora capsici* (Table 5).

Evaluation of antifungal activity in greenhouse condition. Protective effect of antibiotic KB-8A against Fusarium wilt of sesame plants was daily evaluated until the plants treated with the pathogen alone died. The Fusarium wilt was effectively controlled by treatment with 13 μg/ml and 26 μg/ml of antibiotic KB-8A, but not with 6.5 μg/ml. At 24 days after inoculation, disease severity of plants

Table 4. Antibiotic activities of fractions from C-18 cartridge of the crude antibiotics obtained from Sephadex LH-20 column chromatography to the *Fusarium oxysporum* f. sp. *sesami* by agar diffusion method[a]

Fraction (Methanol : Water)	Diameter of inhibition zone (mm)
0 : 10	0
3 : 7	0
5 : 5	5
8 : 2	13
10 : 0	0

[a] Each fraction was placed in a well (7 mm diameter) of PDA seeded with fungal spores (10^3/ml) of *Fusarium oxysporum* f. sp. *sesami*. After incubated at 27°C for 36 hr, the plates were evaluated for mycelium-free zone around the wells.

Table 5. Minimum inhibitory concentrations (MICs) against plant pathogenic fungi of antibiotic KB-8A from *Bacillus polymyxa* KB-8[a]

Plant pathogenic fungus	Minimum inhibitory concentration (μg/ml)[b]
Alternaria mali	12.8
Colletotrichum gloeosporioides	6.4
Fusarium oxysporum	12.8
Phytophthora capsici	3.2
Rhizoctonia solani	6.4

[a] A mycelial disk of tested plant pathogenic fungi was placed on PDA containing a series of concentrations (0-50 μg/ml PDA).
[b] The lowest concentration that completely inhibits the growth of test fungus was determined after incubation for 3-5 days.

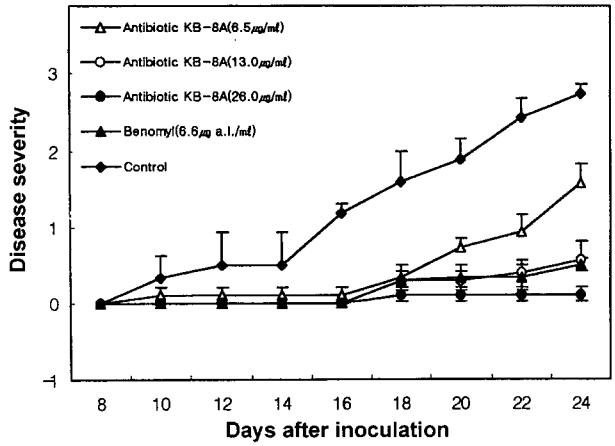

Fig. 3. *In vivo* protective effects of antibiotic KB-8A from *Bacillus polymyxa* KB-8 against Fusarium wilt of sesame plants. Disease severity based on a 0-3 scale, as described in Materials and Methods. Each value represents a mean and vertical bars represent standard deviation of nine plants.

treated with 13.0 μg/ml and 26.0 μg/ml of antibiotic KB-8A and 6.5 μg a.i./ml of benlate was 0.56±0.51, 0.11±0.19 and 0.50±0.17, respectively. However, severity of plants treated with the antibiotic of 6.5 μg/ml was relatively high, 1.57± 0.51 and the plants with pathogen alone was 2.73±0.25 (Fig. 3).

Discussion

Bacillus spp. are one of the biological control agents that have been shown inhibitory effects against a number of plant pathogens, and the antibiotics produced from them were generally assumed to be responsible for the control activities (Helbig et al., 1998; Krebs et al., 1998; Leifert et al., 1995; Phae et al., 1990).

In a preliminary test, *B. polymyxa* KB-8 showed a protective effect against Fusarium wilt of sesame, suggesting that antibiosis may be involved in the disease control. In this study, it is evident that *B. polymyxa* KB-8 produced at least two antibiotics, KB-8A and KB-8B, having Rf 0.67 and Rf 0.35 on TLC developed in a chloroform-methanol (7 : 3) system. However, these antibiotics produced *in vitro* cannot provide a sufficient proof for the involvement of the antibiotics in the biocontrol activity *in vivo* because *Bacillus* spp. produce other metabolites including biosurfactants, chitinase and other fungal cell wall-degrading enzymes, volatiles and compounds which elicit plant resistance mechanisms, and are involved in a number of mechanisms of biological control not only a antibiosis but also competition, PGPR and etc. (Helbig et al., 1998; Leifert et al., 1995).

The inhibitory efficacy of culture extract from the strain KB-8 against *F. oxysporum* f. sp. *sesami* increased as the culturing time was prolonged. In general, most bacterial antibiotics are produced at the begining of idiophase when cell multiplication ceases, but the strain KB-8 was included in a different case, though the reason is not known.

It is known that kinds or quantities of produced antibiotics may vary according to the components of media or culture conditions (Gupta and Utkhede, 1987; Leisinger and Margraff, 1979). In our study, *in vitro* test showed YMA adjusted to initial pH 5 was the best culture medium for the antibiotics production. Antibiotic substances extracted from culture medium inoculated by streaking showed higher inhibitory efficacy than by spreading (data not presented). These suggest that various conditions including nutrition, pH, inoculation method, and so on are very important in the culture of the strain KB-8 for antibiotics production.

Many antibiotic substances from *Bacillus* spp. were known, including antibiotics of iturin group (Ohno et al., 1993; Phae et al., 1990; Winkelmann et al., 1983), cyclic lipopeptide group (Kajimura et al., 1995), peptide group (Bechard et al., 1998; Chatterjee et al., 1992; Kajimura and Kaneda, 1997), ansamycin group (Hosokawa et al., 1996), polyene class (Nishikiori et al., 1978) and antiviral compounds (Lampis et al., 1995).

In this study, we confirmed that *B. polymyxa* KB-8 produced at least two antibiotics which could not be purified by HPLC using C-18 column. We assume that the two antibiotics, which could not be applied to HPLC C-18 column using methanol or acetonitrile, were different from iturin group antibiotics because antibiotics of iturin group could be readily seperated by HPLC using C-18 column with methanol or acetonitrile.

The pure antibiotic KB-8A was finally obtained by crystalization. The antibiotic KB-8A produced one band on TLC plate visualized by UV irradiation and iodine vapor, and we confirmed that the antibiotic KB-8A was pure by analysis of ^{1}H NMR and FAB mass (data not presented).

In greenhouse test, the antibiotic KB-8A shown the effective inhibitory activity against Fusarium wilt of sesame plants though the control activity was some lower than benomyl, suggesting the possibility that the antibiotic has the effective control activity against Fusarium wilt of sesame plants in field conditions.

Therefore, the structure of the antibiotic KB-8A needs to be elucidated in future and the practical disease control potential of KB-8A should also be evaluated in field conditions.

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