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Production, Purification and Antifungal Activity of Antibiotic Substances Produced by *Pseudomonas aeruginosa* Strain B5

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***Pseudomonas aeruginosa* strain B5 with antagonistic activity against *Phytophthora capsici* and *Magnaporthe grisea*, was isolated from pepper-growing soil. From the culture of *P. aeruginosa* strain B5 grown on King's medium B, antibiotic substances were purified using XAD-2 column chromatography. XAD-2 eluates inhibited not only the mycelial growth of *P. capsici* and *M. grisea*, but also the development of Phytophthora blight on pepper plants. The crude antibiotic substances were further purified by using silica gel column chromatography, Sephadex LH-20 column chromatography, thin layer chromatography on silica gel plates, and high performance liquid chromatography. Silica gel column chromatogrphy gave good separation of the four antibiotic substances. The pure antibiotics P1, P2, and P3 finally purified by preparative HPLC inhibited the mycelial growth of *P. capsici*, at concentrations from 7 to 10 μg/ml. Only P1 and P2 had antifungal activity against *M. grisea* at 8 μg/ml. P1 and P3 were highly inhibitory to the mycelial growth of *Botryosphaeria dothidea* and *Botrytis cinerea* at relatively low concentrations. However, the three antibiotics had no antifungal activity against *Rhizoctonia solani*. The chemical structures of these antibiotics are being identified.**

Metabolites such as antibiotics, cyanide, and fluorescent siderophores produced by *Pseudomonas* sp. were studied as the biocontrol agents against many plant pathogens (6, 20, 22, 23). Most of the antibiotics isolated from *Pseudomonas* culture filtrates, such as phenazines (10, 12), pyrrolnitrin-type antibiotics (13, 14), pyo compounds (9), and indole derivatives (4), fall into the class of N-containing heterocycles. Another substantial class of *Pseudomonas* secondary metaboltes is comprised of unusual amino acids and peptides. In addition, some glycolipids, lipids, aliphatic compounds, and pseudonomic acid have been isolated from *Pseudomonas* cultures (7, 11, 15). Compared to variety of antibiotics produced by *Actinomycetes* and fungi, the antibiotics produced by pseudomonads cover a more restricted range of chemical structure. Macrolides, aminoglycosides, polyenes, quinone-type antibiotics, oxygen-containing heterocycles, and alicyclic antibiotics have not yet been found from pseudomonads. Among many antibiotic substances obtained from pseudomonads, only pyocyanine (2), pyrrolnitrin, and pseudonomic acid (7) have been produced commercially. A number of their derivatives also were developed to increase their antibiotic activity (19).

Antibiotics produced by *Pseudomonas aeruginosa* have been studied extensively, since Hays *et al.* (9) first detected an antibacterial activity against gram-positive bacteria during an ethanol extraction of *P. aeruginosa* culture. From the bioactive preparation, they separated a number of antibiotics named pyo compounds. The four pyo-compounds were structurally related. Since the research of Hays *et al.* (9), pyo compounds have been rediscovered by many other researchers (3, 24). Several glycolipids such as pyolipic acid and rhamnolipid which also have antibiotic activity were known to be produced by *P. aeruginosa*. A glycolipid was isolated by Jarvis and Johnson (16) from the culture fluid of *P. aeruginosa* grown on glycerol. It was identified as L-rhamnosyl-(1,2)-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxy-decanoic acid. Itoh *et al.* (15) reported that a rhamnolipid was produced by *P. aeruginosa* grown on n-paraffin. The compounds showed antibacterial activity against gram-positive bacteria. Further, Scannell *et al.* (21) demonstrated that an amino acid antimetabolite named as AMB

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(L-2-amino-4-methoxy-*trans*-3-butenoic acid) produced by *P. aeruginosa* inhibits the growth of gram-positive and gram-negative bacteria on minimal media. Secondary antibiotic metabolites such as pyocyanine, and other phenazine compounds were isolated not only from *P. aeruginosa* but also from other *Pseudomonas* sp. (19). Among the phenazine compounds, hemipyocyanine and chlororaphin produced by *P. aeruginosa* showed antifungal activity against *Fusarium oxysporum* f.sp. *adzukicola* on Adzukibean (5). Other phenazine compound, phenazine-1-carboxylic acid, has been found to have antifungal activity against *Helminthosporium oryzae* and *Phytophthora cactorum* (18). Pyoluteorin produced by several *Pseudomonas* speices showed antifungal activity against *Pythium ultimum* and *Phytophthora cactorum* (14, 18).

In our previous screening studies, we isolated antagonistic bacteria from pepper growing soils at various locations in Korea (17). Among the antagonistic bacteria with antifungal activity against *Phytophthora capsici* and *Magnaporthe grisea*, strain B5 identified as *Pseudomonas aeruginosa* exhibited the best antagonistic activity *in vitro* and *in vivo*. We demonstrated that the suppressive activity that *P. aeruginosa* strain B5 has on Phytophthora blight in pepper plants may be due mainly to the production of antibiotic substances. The purpose of the present study is to further purify antibiotic substances active against *P. capsici* and *M. grisea* from *P. aeruginosa* strain B5, and then to evaluate their potential in the Phytophthora blight control in pepper plants.

MATERIALS AND METHODS

Identification of Antibiotic-Producing Bacteria Strain B5

The antibiotic-producing bacteria strain B5 was selected for further study and identified by using Bergey's manual of determinative bacteriology (1) and the manual of methods for general bacteriology (8). The strain B5 was characterized with respect to fluorescence on King's medium B, gram reaction, growth at pH 3.5, oxidase reaction, Xanthomonadian production, arginine dehydrolase, gelatin liquefaction, growth at 41℃, levan production, poly-β-hydroxybutyrate accumulation, and growth on minimal medium containing one of nutrient sources such as glucose, arginine, D-arabinose, L-rhamnose, D-mannose, D(−)tartrate, and D(+)tartrate.

Production of Antibiotics

A few loopfuls of bacterial suspension from the stock culture of *P. aeruginosa* strain B5 were transferred into a 500-m*l* flask containing 100 m*l* of King's medium B [proteose peptone #3 (Difco) 20.0 g, glycerol 15.0 m*l*, $MgSO_4 \cdot 7H_2O$ 6.0 g, $K_2HPO_4 \cdot H_2O$ 2.5 g per 1 L H_2O]. The seed culture incubated at 28℃ for 24 hr was inoculated into a 10-liter jar fermentor containing 6 liters of King's medium B. The fermentation was carried out at 27℃ for 48 hr under agitation of 150 rpm and aeration of 2 liters per minute.

Purification of Antibiotics

The cultures (18 L) of the strain B5 were centrifuged at 7,000 g for 15 min to remove bacterial cells. The remaining supernatants were extracted three times with n-butanol. The butanol phase was taken to dryness under reduced pressure at 40℃ on a rotary evaporator. The resulting residue was dissolved in a minimum volume of methanol and purified by chromatography on Amberlite XAD-2 using a H_2O-methanol stepwise gradient (100% H_2O; H_2O-MeOH (50:50); H_2O-MeOH (20:80); 100% MeOH). The active fractions (80% and 100% methanol eluates) from this chromatography were combined and lyophilized.

The 9 g portion of the crude antibiotics from the XAD-2 column chromatography was dissolved in a 25 m*l* butanol-methanol (95:5) solution, and chromatographed on silica gel (silicagel $60F_{254}$, Merck) column (9×50 cm) using a butanol-methanol gradient step [BuOH-MeOH (95:5); BuOH-MeOH (60:40); BuOH-MeOH (40:60); 100% MeOH] at a flow rate of 1 m*l*/min. The 10 m*l* fractions were collected from a fraction collector (Pharmacia Redi-Frac), and bioassayed by using paper disk method. The active fractions were pooled for further purification. The silica gel column fractionation yielded four active antibiotic regions P1, P2, P3, and P4. Each active fraction was evaporated and lyophilized.

Each of the lyophilized antibiotic substances P1, P2, P3, and P4 was dissolved with methanol and chromatographed on Sephadex LH-20 column (1.6×95 cm) with methanol at flow rate of 0.05 m*l*/min. The 3 m*l* fractions were collected from the fraction collector (Parmacia Redi-Frac) and bioassayed for antifungal activity. The active fractions were pooled and concentrated *in vacuo*. The residue was dissolved with a minimum volume of methanol and further purified by thin layer chromatogrphy (TLC) on preparative silica gel plates (Silica gel $60F_{254}$, Merck, 20×20 cm, 2.0 mm-thickness) using a solvent system of either chloroform-methanol (9:1, v/v) or butanol-acetic acid-water (4:1:2, v/v/v). The antibiotic active bands were scraped from the TLC plates, followed by the elution with butanol and methanol. For further purification of the silica gel eluates, high performance liquid chromatography (HPLC) was carried out using a Waters HPLC system with semi-preparative C-18 reverse phase column (LiChrosorb, 10 mm×25 cm, 7 μm, Merck) at a flow rate of 3 m*l*/min. The separation was

monitored at 246 nm for P1, 219 nm for P2 and 299 nm for P3. The mobile phase solution systems were methanol-water (80:20, v/v) for P1 and acetonitrile-methanol-water (60:25:15, v/v/v) for P2 and P3. Column output from individual peaks was manually collected, and then bioassayed by paper disk method. The antibiotic P4 was purified only by preparative TLC. The UV absorption spectrum of the purified antibiotic substances in methanol was measured with a spectrophotometer from 200 to 500 nm.

All the antibiotic substances P1, P2, P3, and P4 were monitored for purity by using a thin layer chromatography (silica gel $60F_{254}$ pre-coated TLC aluminium sheets, 0.2 mm-thickness, Merck) at various solvent systems during each of the purification steps and Waters HPLC system with an analytical C-18 reverse phase column (LiChrosorb, 4 mm×25 cm, 5 μm, Merck).

Bioassay of Antibiotics for Antifungal Activity

Antifungal activity of the crude antibiotic substances against *P. capsici* and *M. grisea* was tested on V_8 juice agar containing a series of concentrations in the range of 0~1000 μg/m*l* media. A mycelial disk of *P. capsici* and *M. grisea* was placed on the each test plate. The percentage of inhibition of mycelial growth was calculated, and compared with the diameter of mycelial growth on the control plates without crude antibiotic substance. Disease suppressive activity of the crude antibiotic substances against *P. capsici* was tested on pepper plants under greenhouse conditions. The pepper plants at six leaf-stage were soil-drenched with 30 m*l* of zoospore suspension (10^6 zoospores/m*l*). Six hours later, the crude antibiotic substance dissolved in methanol was mixed with water at a series of concentrations (0~1000 μg/m*l*). The crude antibiotic solutions were soil-drenched. Disease severity in pepper plants was rated daily after inoculation, based on a scale of 0~5, where 0= no visible disease symptoms; 1= leaves slightly wilted with brownish lesions beginning to appear on stems; 2= 30~50% of plant diseased; 3= 50~70% of plant diseased; 4= 70~90% of plant diseased; 5= plant dead.

To detect the antifungal active fractions from the column chromatography, each fraction was applied to sterile filter paper disks (7 mm in diameter). The zoospore suspensions (10^6 zoospores/m*l*) of *P. capsici* mixed with molten V_8 juice agar were poured into 9-cm petri-dishes. The fragmented mycelial suspensions of *M. grisea* mixed with molten potato dextrose agar (PDA) were also used for paper disk bioassay. The bioassay disk soaked with each fraction was placed in the center of the agar plate seeded with the test fungi. After incubation for 3 days at 28℃, clear zones originating from the inhibition of fungal growth, were measured.

The active antibiotic band was identified by bioassay on TLC plates (silica gel $60F_{254}$, pre-coated TLC aluminium sheets, 0.2 mm-thickness, Merck) as follows. The antibiotic substance dissolved in methanol was chromatographed on TLC plates with the same solvent system as used in preparative TLC. The TLC plate was placed on a 2% water agar plate after elimination of solvent. A zoospore suspension of *P. capsici* (10^6 zoospores/m*l*) was mixed with molten V_8 juice media (1:1, v/v) or the mycelial suspension of *M. grisea* was mixed with molten potato dextrose agar (1:1, v/v). The 5 m*l* of molten seeded agar was poured on the TLC plates. After incubation for 2~3 days, the TLC plate spread with P. capsici zoospore suspension was stained with the naphtol blue-black solution (0.1 g naphtol blue-black in 100 m*l* of 5% acetic acid). Clear inhibition zones appearing on the blue-black coloured backgrounds indicated the presence of antibiotic activity at the band of the Rf value.

The antibiotic activities of P1, P2, and P3 were tested on V_8 juice agar containing a series of concentrations (0~10 μg/m*l*) of each antibiotic substance. The mycelial disks of various plant pathogenic fungi were placed on each test plate. The lowest concentration that prevents visible mycelial growth of plant pathogenic fungi was determined after incubation for 2~8 days.

RESULTS

Identification of Bacterial Strain B5

Several biochemical and carbohydrate utilization tests were performed to identify the antagonistic bacterial strain B5 (Table 1). The strain B5 was aerobic Gram-negative bacteria which produced diffusable fluorescent yellow-green pigment. Poly-β-hydroxy butyrate did not accumulate in the bacterial cell. The bacteria did not grow at pH 3.6. Xanthomonadians were not produced in the yeast extracts-dextrose $CaCO_3$ agar (YDC). These characteristics differentiated genus *Pseudomonas* from other genera of *Pseudomonadaceae*. The strain B5 could grow at 41℃. Arginine was dehydrolyzed, turning the arginine medium to alkaline condition. Oxidase test came out positive. Levan was not formed in the sucrose-supplemented media. Gelatin was liquified. The strain B5 utilized sucrose and arginine as nutritional sources, but did not utilize D-arabinose, rhamnose, and mannose. Based on these diagnostic data, the strain B5 could be identified as *Pseudomonas aeruginosa*.

Antifungal Activity of Crude Antibiotics

The culture filtrates (18 L) of *P. aeruginosa* strain B5 were extracted with butanol. The antifungal activity against *P. capsici* and *M. grisea* were retained in the butanol phase. No antifungal activity was detected in

Table 1. Diagnostic tests for identification of the antagonistic bacteria strain B5

Characteristics	Strain B5	*Pseudomonas aeruginosa* in Bergey's manual
Gram stain	–[a]	–
Fluorescent pigment	+	+
Xanthomonadian production	–	–
Growth at pH 3.6	–	–
Growth at 41℃	+	+
PHB accumulation	–	–
Arginine dehydrolase	+	+
Oxidase reaction	+	+
Levan formation	–	–
Gelatin liquefaction	+	+
Utilization of:		
Glucose	+	+
Arginine	+	+
D-Arabinose	–	–
L-Rhamnose	–	–
D-Mannose	–	–
D(–)Tartrate	–	–
L(+)Tartrate	–	–

[a]Symbols: +, positive reaction; –, negative reaction.

the pigmented aqueous phase. The 80% and 100% methanol fractions obtained from the chromatography of the butanol-extrcted substances on Amberlite XAD-2 polystyrene resin exhibited a high level of antifungal activity against the two fungi. The crude antibiotics completely inhibited the mycelial growth of *P. capsici* and *M. grisea* at 1 mg/m*l* (Fig. 1). Soil drench of the crude antibiotics remarkably suppressed the development of Phytophthora blight in pepper plants inoculated with *P. capsici* (Fig. 2). No disease was observed in pepper plants treated with 1 mg/m*l* crude antibiotics. In the bioassay test on TLC plates with *P. capsici*, three zones of inhibition were produced by the crude antibiotics from XAD-2 chromatography (no data presented), indicating the biosynthesis of at least 3 antibiotic substances in the bacteria.

Purification of Antibiotics

Silica gel column chromatography of XAD-2 eluates could well separate the antibiotic substances into four active fractions, designated P1, P2, P3 and P4, by using different elution systems with butanol and methanol (Fig. 3). The active fractions P1 and P2 eluted with butanol and methanol of 95:5 and 60:40, respectively, had a high inhibitory effect on the mycelial growth of *P. capsici* and *M. grisea*. However, fractions P3 and P4 obtained with butanol and methanol (60:40) and 100% methanol, were highly inhibitory only to *P. capsici*.

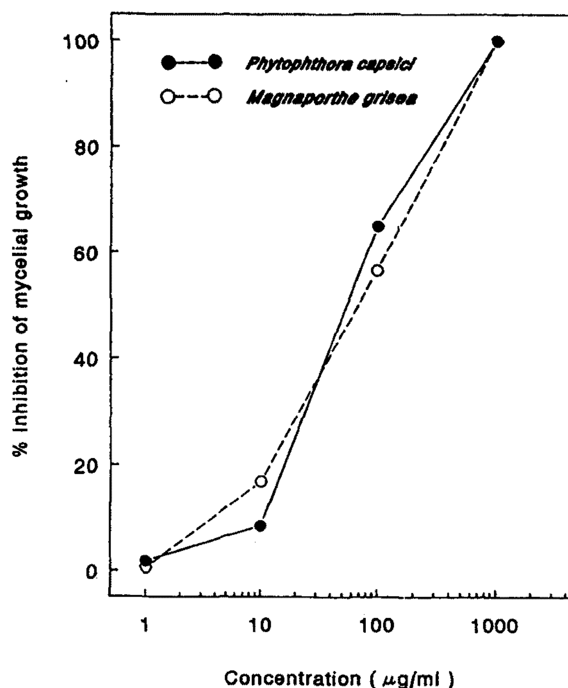

Fig. 1. Inhibition of the mycelial growth of *Phytophthora capsici* and *Magnaporthe grisea* by different concentrations of XAD-2 eluates from cultural extracts of *Pseudomnas aeruginosa* strain B5.

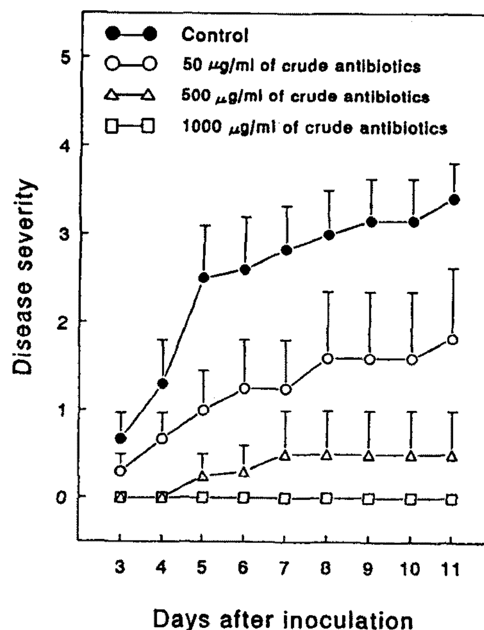

Fig. 2. Effect of XAD-2 eluates of cultural extracts of *Pseudomnas aeruginosa* strain B5 at different concentrations on the disease development in pepper plants inculated with *Phytophthora capsici*.

Disease severity rating based on a 0~5 scale, as described in Materials and Methods. Each value represents a mean± standard deviation of 6 plants infected.

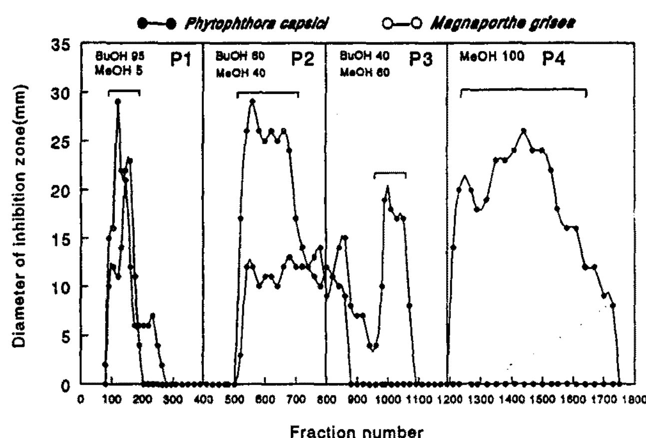

Fig. 3. Silica gel column chromatogaphy of the pooled fractions of Amberlite XAD-2 column chromatography. Each of all fractions (10 m/fraction) was bioassayed for antifungal activity to *Phytophthora capsici* and *Magnaporthe grisea* by a paper disk method. The bars represent the pooled active fractions for further purification.

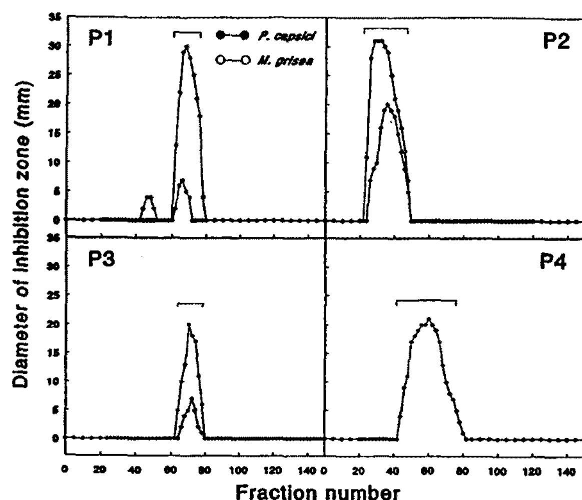

Fig. 4. Sephadex LH-20 column chromatography of antibiotic substances P1, P2, P3 and P4 obtained from silica gel column chromatography.
Each of the lyophilized antibiotic substances was dissolved with a minimum volume of methanol and chromatographed on Sephadex LH-20 column (1.6×95 cm) with methanol at a flow rate of 0.05 ml/min. The 3 ml fractions were collected from the fraction collector (Pharmacia Redi-Frac). Each of all fractions was bioassayed for antifungal activity to *Phytophthora capsici* and *Magnaporthe grisea* by paper disk method. The bars represent the pooled active fractions for further purification.

Sephadex LH-20 column chromatography of antibiotic fraction P1 produced inhibition zones at two regions, in which fraction numbers 44-46 were found active against *P. capsici* and 66-69 active against *M. grisea*. The antifungal activity on *P. capsici* was weak, but 66-69 fractions produced relatively large inhibition zones against *M. grisea* (Fig. 4). After the fractions were pooled and crystallized at 4℃, 20 mg of yellow needle type crystals were produced. Although P1 generated a single spot on TLC plates which had been developed with different solvent systems of chloroform-methanol (9:1, v/v), ethyl acetate-methanol (87:13, v/v) and butanol-acetic acid-H_2O (4:1:2, v/v/v), it was further purified by using a HPLC system with a MeOH-H_2O (8:2, v/v) solvent system. Five discrete peaks appeared at the retention time (Rt)- 3'37, 4'72, 5'93, 8'26, 9'10, and 11'06. The peak at Rt-9'10 corresponded to the 90% portion of total area at UV absorbance of 246 nm, exhibiting inhibition zone against *M. grisea* in bioassay test. The collected eluates at Rt-9'10 produced a single peak at the same retention time in a further HPLC analysis. 4.5 mg of yellow-needle type crystals were yielded. The UV absorption spectra of P1 showed maximum absorbance at 246 nm (no data presented).

The antibiotic fraction P2 chromatographed on Sephadex LH-20 column showed antifungal activity against *P. capsici* and *M. grisea* at fraction numbers 26-46 (Fig. 4). The pooled active fractions produced two bands on TLC plates. To find active band, a bioassay test on TLC plates was performed with chloroform-methanol (9:1). An inhibition zone was produced on the band at Rf-0.05. The methanol eluent obtained from the band was concentrated and crystallized to give 812 mg of white crystals. In the chromatography on a HPLC system, several peaks were produced. The only peak at Rt-11'58 showed inhibition zones on the plates seeded with *P. capsici* and *M. grisea*. The analysis of the collected peak with a HPLC system using a solvent system of acetonitrile-methanol-water (60:25:15, v/v/v) showed a single peak at the same retention time. Twenty five mg of white crystals were generated. The UV absorption spectra of P2 showed maximum absorbance at 219 nm (no data presented).

Sephadex LH-20 column chromatography of the antibiotic fraction P3 yielded one active region (fraction no. 63-78) which had antifungal activity against *P. capsici* and *M. grisea* (Fig. 4). The pooled fractions were rechromatographed on silica gel plates, and the bioactive Rf-0.97 band (chloroform-methanol, 9:1, v/v) was eluated. The eluates obtained from silica gel plates were crystallized to yield 8 mg of brownish powder. The purified antibiotics P3 produced a single peak at Rt-3'42 in an analytical HPLC system using a solvent system of acetonitrile-methanol-water (60:25:15, v/v/v) (no data presented). The antibiotic P3 showed the characteristic UV absorption spectra with maximum absorbance peaks at 215

Table 2. Minimum inhibitory concentration (MIC) against various plant pathogenic fungi of antibiotic substances P1, P2, and P3 from *Pseudomonas aeruginosa* strain B5[a]

Plant pathogenic fungus	Minimum inhibitory concentration (μg/ml)[b] of		
	P1	P2	P3
Altfernaria brassicae	3	0.1	>10
Alternaria solani	8	10	8
Botryosphaeria dothidea	0.05	5	0.1
Botrytis cinerea	5	5	1
Cylindrocarpon destructans	>10	9	>10
Mycosphaerella melonis	5	7	>10
Magnaporthe grisea	8	8	>10
Phytophthora capsici	10	7	8
Rhizoctonia solani	>10	>10	>10
Sclerotinia sclerotiorum	3	>10	7

[a] A mycelial disk of various plant pathogenic fungi was placed on V_8 juice agar containing a series of concentrations (0~10 μg/ml media) of each antibiotic substance.
[b] After incubation for 2~8 days, the lowest concentration (MIC) that prevents mycelial growth of plant pathogenic fungi was determined.
[c] >10 represents that the mycelial growth of treated fungi was not inhibited at 10 μg/ml.

nm, 281 nm and 324 nm (no data presented).

The antibiotic P4 chromatographed on Sephadex LH-20 column showed antifungal activity against *P. capsici* at 45-78 fractions (Fig. 4). The pooled fractions were chromatographed on TLC plates by using butanol-acetic acid-H_2O (4:1:2). The antifungal active band at Rf-0.46 was scraped from the TLC plates and eluted with methanol and then with butanol. The eluates from the antifungal active band were concentrated and crystallized to produce 108 mg of white powder. Further purification of this antibiotic by HPLC was not successful.

Antifungal Activity of Antibiotics

The antifungal activity of antibiotics P1, P2, and P3 were examined on V_8 juice agar containing different concentrations of antibiotic substances (Table 2). These antibiotics inhibited the mycelial growth of *P. capsici* at concentrations from 7 to 10 μg/ml. P1 and P2 had antifungal activity against *M. grisea* at 8 μg/ml, whereas P3 could not inhibit the fungus even at 10 μg/ml. P1 and P3 were highly inhibitory to the mycelial growth of *Botryosphaeria dothidea* and *Botrytis cinerea* at relatively low concentrations. However, the three antibiotics had no antifungal activity against *Rhizoctonia solani*.

DISCUSSION

Pseudomonas aeruginosa strain B5 selected from pepper growing soils by our previous screening studies not only inhibited the mycelial growth of *P. capsici* and *M. grisea*, but also suppressed the development of Phytophthora blight on pepper plants (17). *P. aeruginosa* strain B5 seemed to produce antifungal substances, suggesting a potential mode of biocontrol and their compatibility to environments they are introduced to. Therefore, to isolate pure antifungal substances from culture filtrates of *P. aeruginosa* strain B5, we tested their antifungal activity *in vitro* and *in vivo* by using the antibiotics partially purified by different steps.

The bioassay on TLC plates revealed the presence of at least three antibiotic substances. Silica gel column chromatography gave good separation of the compounds to produce 4 discrete antibiotic active regions. The four bioactive regions named P1, P2, P3, and P4, showed different band patterns on TLC plates. P1 showed strong antifungal activity against *M. grisea*, but it was weak against *P. capsici*. The Rf values of P1 on TLC plates were 0.92 (chloroform-methanol, 9:1, v/v) and 0.77 (ethyl acetate-methanol, 87:13, v/v). The Rf value of P2 on TLC plates was 0.05 (chloroform-methanol, 9:1, v/v). P3 which had antifungal activity against *P. capsici* produced single bands on TLC plates at Rf 0.89 (ethyl acetate-methanol, 87:13, v/v) and 0.97 (chloroform-methanol, 9:1, v/v). More recently, Kim *et al.* (18) reported that antifungal phenazine compounds such as phenazine-1-carboxylic acid and oxychlororaphine produced by *P. aeruginosa*, produced single bands on the TLC plates developed with chloroform-methanol (9:1, v/v) at Rf 0.58 and 0.57, respectively. Pyoluteorin had Rf value at 0.35 (18). Therefore, the antibiotics P1, P2, and P3 isolated from *P. aeruginsa*, strain B5 in the present study should have chemical structures quite different from such phenazine compounds and pyoluteorin, indicating the possible synthesis of structurally new antibiotics in *P. aeruginosa*. It is suggested that *P. aeruginosa* may produce different antibiotic secondary metabolites dependent on the strains, the nutrient compositions and the culture conditions (19).

P1, P2, and P3 were highly soluble in methanol, but P4 showed very low solubility in methanol and insolubility in water. Consequently, the antibiotic P4 gave some

difficulties in the purification process using HPLC system, suggesting the application of other possible purification methods in future.

In conclusion, we isolated antibiotic substances P1, P2, and P3 from *P. aeruginosa* strain B5. P1 and P2 showed antifungal activity against *P. capsici* and *M. grisea*. P3 was active against *P. capsici*. P1 and P3 were also highly inhibitory to *B. dothidea* and *B. cinerea in vitro*. These antibiotic substances have the potential to suppress Phytophthora blight in pepper plants. Chemical structures of the purified antibiotics P1, P2, and P3 are being identified. Their potential in the practical control of plant diseases should also be further evaluated in greenhouses and fields to determine the possibility of their use as leading compounds in the development of fungicides.

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