J. Microbiol. Biotechnol. (2000), **10**(6), 811–816

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Isolation of Bacterial Strain Antagonistic to *Pyricularia oryzae* and Its Mode of Antifungal Action

BAE, DONG-WON[1], JOON-TAEK LEE[1,2], DAE-YOUNG SON[2,3], EUN SOOK LEE[4], AND HEE-KYU KIM[1,2]*

[1]*Department of Agricultural Biology,* [2]*Genetic Engineering Institute*
[3]*Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Chinju 660-701, Korea*
[4]*Department of Preparatory Oriental Medicine, Kyungsan University, Kyungsan 712-240, Korea*

Received: July 24, 2000
Accepted: November 1, 2000

Abstract An antagonistic bacterium PM-1 which strongly inhibits the growth of *Pyricularia oryzae* was isolated and identified as *Paenibacillus macerans*. The antifungal substances of the strain PM-1 showed the broad antifungal spectra against *P. oryzae* races. Relating to the localization test, it was found that the antifungal substances existed not only in the cytoplasm but also in the culture supernatant, and importantly the antifungal activity of the latter was stronger than that of the former. The extracellular antifungal substances were extremely heat-stable up to 121°C for 15 min. The substances were optimally produced at 20°C and pH 10.0 in a potato dextrose broth. The culture filtrate of the strain PM-1 caused a partial swelling of the mycelia of *P. oryzae*, and it prevents the normal growth of the fungus as well. This result suggested that the antifungal substances secreted by the strain PM-1 potentially inhibited the germination of *P. oryzae*.

Key words: *Pyricularia oryzae*, *Paenibacillus macerans*, antifungal substances, mycelial growth

The blast disease of rice, occurring throughout the cultivation period by *P. oryzae*, has been one of the most destructive rice diseases [16, 18]. Many attempts to overcome this disease problem have been done. These include chemical control by using fungicide [1] and breeding of the disease resistant races [2]. Unfortunately, these fungicides are adversely effecting the quality of the rice production and environment [7, 21], and breeding rice in the consecutive use also brings about a problem due to the differentiation of *P. oryzae* races which cause the emergence of a new mutants.

Recently, an environment-friendly method with biocontrol agents is recognized as an alternative approach to control this disease [10, 13, 19, 20]. The use of natural fungicides [25] and the biological control by antagonistic microorganisms [22] are a plausible ways to take this approach. Blasticidin S [23], kasugamycin [24], polyoxin [5], and validamycin [6] have been isolated and used as the antifungal antibiotics. These antibiotics are the metabolites of actinomycete or fungi [8, 10], however, they have several defects such as toxicity and mass use.

In this report, we describe physiological properties of the metabolites produced by the antagonistic bacterium PM-1, the growth inhibition of *P. oryzae* races, and the antifungal mechanism.

Materials and Methods

Isolation of Antagonistic Microorganisms

Soil and plant samples were collected from locations near to Chinju city. An appropriate amount of soil samples was suspended in sterile water and then diluted. The plant samples were cut to the size of 1 mm^2 and then treated with the same procedures as the soil samples. The diluted samples were inoculated in the nutrient agar (NA, Difco) as described in the routine spreading method. The plates were incubated at 28°C for 2 days and pure isolates were obtained. Twenty-two isolates of *P. oryzae*, representing the races KI 101, KI 201, KI 301, KJ 101, KJ 201, KJ 301, and KJ 401, were inoculated in the left side from the center of potato dextrose agar (PDA, Difco) plates and incubated at 28°C for 2 days. The bacterial isolates were streaked in the right side, 30 mm away from colonies of *P. oryzae* races. Inhibition of mycelial growth of each race was measured after further incubation for 5 days.

Identification of Antifungal Strain PM-1

Microbiological properties were investigated by the methods described in the Bergey's Manual of Systematic Bacteriology

*Corresponding author
Phone: 82-55-751-5443; Fax: 82-55-751-5199;
E-mail: heekkim@nongae.gsnu.ac.kr

[12]. Analysis of cellular fatty acid was performed with automated microbial identification system (MIDI, Inc., Newark, U.S.A.).

Culture Conditions and Growth Curve

To determine the optimal culture conditions for the production of antifungal substances, the isolate PM-1 was cultured in 500 ml-Erlenmeyer flasks containing 100 ml of PD broth (pH 3.0–11.0) at 20–40°C with shaking at 160 rpm. Sampling for investigating growth profiles and antifungal activities was made every 3 h for 39 h. Growth profiles were plotted with absorbance values at 600 nm.

Antifungal Activity

P. oryzae races were inoculated in the center of PDA plates and incubated at 28°C for 3 days. Culture supernatant was filtered through a cellulose acetate membrane (pore size, 0.22 μm), that was later applied to sterile filter paper disks (7 mm in diameter). The disks were placed in both sides, 30 mm distance from the growing cultures of *P. oryzae* races. After incubation for 5 days, clean zones resulting from inhibition of fungal growth were measured.

Localization and Thermostability of Antifungal Substances

Culture filtrates were used as extracellular fraction and incubated at 80°C for 20 min and at 121°C for 15 min. Culture pellet was sonicated for 30 min in ice-bath (Sonic 300V/T, IPI, Japan), and the suspension was centrifuged at 8,000 rpm for 20 min. The supernatant was filtered with the membrane filter (pore size, 0.22 μm) and used as an intracellular fraction. Each sample was mixed with PDA media at a volume of 10% (v/v). Then the mixtures were poured into petri-dishes (9 cm in diameter). *P. oryzae* races were inoculated in the center of the plates and incubated at 28°C for 7 days. PDA plates, with untreated culture filtrates added were used as the control.

Mode of Action of Antifungal Substance

Culture filtrate was treated in the same way as for the PDA plates mentioned in the previous page where the culture filtrate was not added and used as the control. Each region of the hyphal tip was cut in the size of 1 cm^2. Morphological changes of each hypha were observed under a light microscope (Carl Zeiss Axiovert 135M, × 400).

Inhibition of Conidial Germination

Each conidial suspension (2.5×10^6 conidia/ml) was mixed with culture filtrates at the ratio of one to three (v/v). The mixtures were placed on the hole of slide glasses. These slide glasses were placed into the glass plates equipped with two glass rods on the moistened filter paper. After an incubation of the plates at 28°C for 24 h, conidial germination was observed of the platesunder a light microscope (Carl Zeiss SF, ×100).

$$\text{Ratio of conidial germination (\%)} = \frac{\text{germinated conidia}}{\text{total conidia}} \times 100$$

RESULTS

Isolation of Antifungal Microorganisms

To obtain antifungal microorganisms, we first isolated approximately one hundred strains from soil and plant samples. The isolates, incubated on PDA plates, were screened against twenty-two races of *P. oryzae*. Six strains were selected as the producer of antifungal substances (data not shown). The antifungal activity of the six strains

Table 1. Cultural and physiological characteristics of strain PM-1.

Morphological characteristics	
Shape	Rods
Gram stain	Positive
Cell dimension (μm)	1.2–1.4 × 2.9–3.2
Motility	+
Flagellum	>1
Endospore formation	+
Culture characteristics	
Anaerobic growth	+
Growth at :	
5°C	-
40°C	+
50°C	+
Biochemical characteristics	
Oxidase activity	+
Catalase activity	+
Urease activity	-
Voges-Proskauer test	-
Citrate utilization	-
Indol production	-
Acid from:	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
Gas from glucose	+
Hydrolysis of:	
Casein	-
Gelatin	+
Starch	+
Carbon sources for growth:	
D-Arabinose	+
β-Methylxyloside	+
Galactose	+
Rhammose	+
N-Acetyl glucosamine	-
L-Fucose	+
Xylitol	-

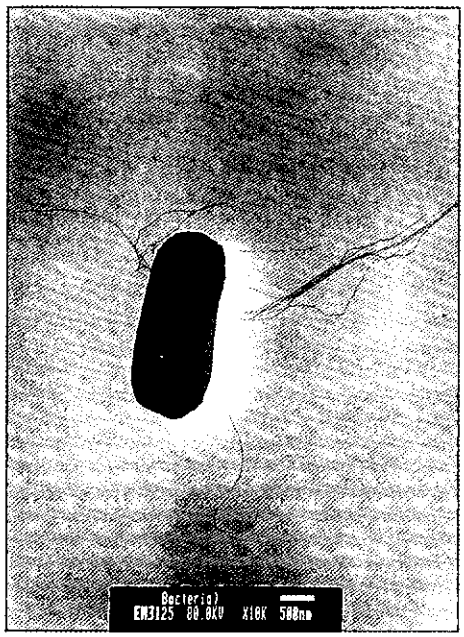

Fig. 1. Transmission electron micrograph of strain PM-1.

was further examined and the strain PM-1 was finally selected as the most potent producer.

Characterization and Identification of Strain PM-1

Table 1 summarizes morphological and biochemical characteristics of the strain PM-1. The strain PM-1 was facultative aerobic, motile, gram positive, endo-spore-forming, and rod shaped, and had a multi polar flagella (Fig. 1). It was catalast and oxidase positive and also oxidation-fermentation positive. Voges-Proskauer, indole, and urease tests were negative (Table 1). Based on these results and MIDI analyses (data not shown), it was identified as a strain of *Paenibacillus macerans*.

Antifungal Activity of *P. macerans* PM-1

The antifungal activity of the strain PM-1 was investigated against twenty two strains of *P. oryzae* races (Table 2). The growth of most strains was significantly inhibited. But the strain PM-1 was less inhibitory to the growth of KI 101: 90-12, KJ 201: J87-53 and KJ 201: J87-44 than others.

Production of Antifungal Substances

Culture condition for the production of antifungal substances of the strain PM-1 was studied and evaluated. The optimum temperature and pH were 20°C and pH 10.0, respectively (data not shown). The extracellular antifungal activity was monitored during the cell growth of the strain PM-1. As shown in Fig. 2, the antifungal activity of the culture appeared after 15 h of cultivation (mid-term of log phase) and reached a maximum level in 24–27 h (stationary phase). But thereafter, the activity drastically decreased.

Table 2. Growth inhibition of pathogenic races of *P. oryzae* by *P. macerans* PM-1 on PDA.

P. oryzae isolates	Inhibition rate[a]
KI 101: 5-21	+++
KI 101: 6 Jin-02	++
KI 101: 90-12	+
KI 201: J91-29	++
KI 201: 86-111-4	+++
KI 301: 89-110	++
KI 301: 90-111	++
KI 301: J92-21	+++
KI 401: 90-107	+++
KI 401: 4-41	+++
KJ 101: 89-16	+++
KJ 201: 88-62	+++
KJ 201: 4 Nongback-08	++
KJ 201: 92-53	+++
KJ 201: J87-54	+
KJ 201: 90-102	+++
KJ 301: 93-39	+++
KJ 301: 92-55	++
KJ 301: 7 Jin-08	++
KJ 301: 7 Nack-04	++
KJ 401: 89-04	+++
KJ 401: J89-44	+

[a]+++: strong (5–10 mm), ++: moderate (11–15 mm), +: weak (<15 mm) inhibition of mycelial growth.

Localization and Thermostability of Antifungal Substances

The antifungal substances were localized in both intracellular and extracellular fraction (Table 3). However, the antifungal

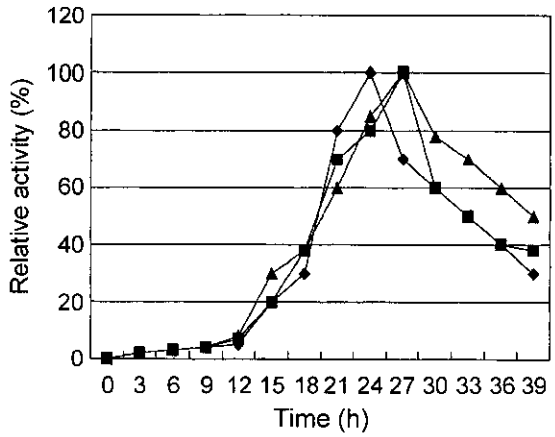

Fig. 2. Time courses of the antifungal activity of *P. macerans* PM-1 against pathogenic races of *P. oryzae*.
■, KI 101: 5-21; ◆, KJ 201: 88-62; ▲, KJ 301: 93-39. One milliliter of suspensions were sampled at designated time intervals and centrifuged at 12,000 rpm. Aliquot (50 µl) of culture supernatant was applied to the paper disc that was placed in PDA cultures inoculated with mycelial discs 3 days before. Relative activity was calculated by the ratio of the diameter of inhibition zone per given plate incubated at 28°C for 5 days.

Table 3. Mycelial growth of *P. oryzae* races on PDA supplemented with or without culture filtrates and cell extracts of *P. macerans* PM-1.

P. oryzae isolates	Control	Cell extract[a]	Supplemented with culture filtrate[b]		
			Not heated	Heated 80°C/20 min	Heated 121°C/15 min
KI 101: 5-21	21[c]	12	7	9	10
KI 201: 86-111-4	23	8	5	7	8
KJ 101: 89-16	21	11	7	8	10
KJ 201: 88-62	22	10	5	7	18
KJ 301: 93-39	20	6	3	4	5

[a]Cell extract was prepared by sonicating bacterial cells for 30 min followed by centrifugation at 8,000 rpm for 20 min at 4°C prior to filtering through 0.22 μm membrane filter.
[b]Culture filtrate was supplemented in the PDA at 10%.
[c]Antifungal activity was measured as diameter of mycelial growth (mm) at given treatment.

activity of extracellular fraction was higher than that of the intracellular fraction. As for thermostability of extracellular antifungal substances, 85.7–94.1% of the activity remained after an incubation at 80°C for 20 min, and 78.5–88.2% of the activity after an incubation at 121°C for 15 min, except 23.5% of the activity toward *P. oryzae* KJ 201: 88–62 (Table 3).

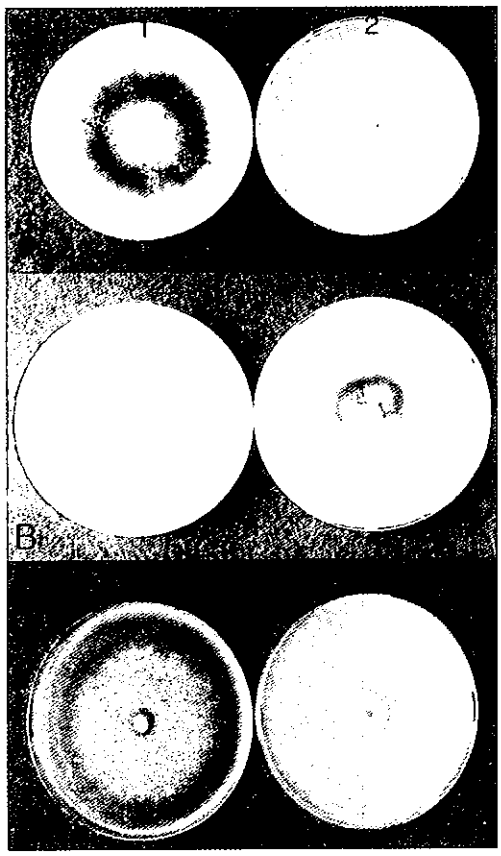

Fig. 3. Growth inhibition of *P. oryzae* by the culture *P. macerans* PM-1.
A, KI 101: 5–21; B, KJ 201: 88–62; C, KJ 301: 93–39. *P. oryzae* isolates were grown at 28°C for 7 days on PDA untreated control (1) or PDA supplemented with 10% supernatant (2) of *P. macerans* PM-1 culture.

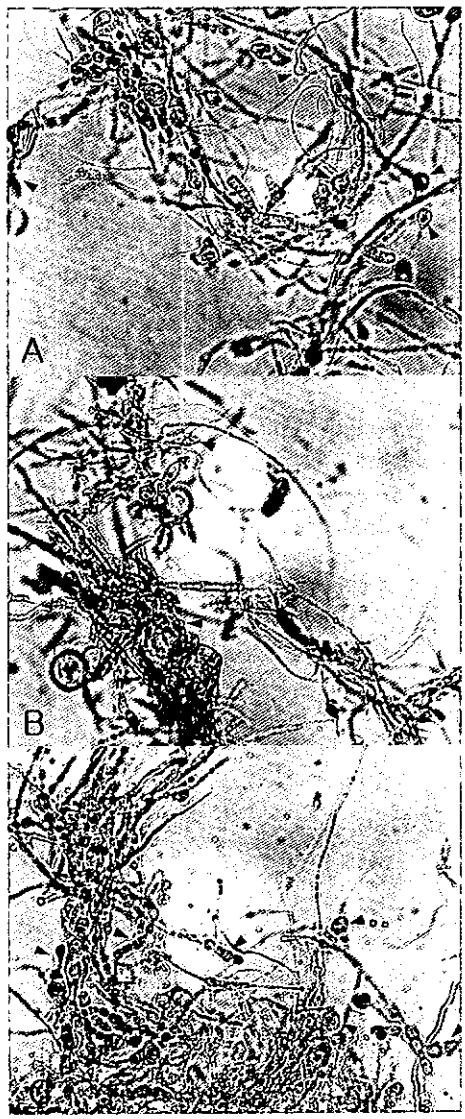

Fig. 4. Light microscopy of mycelial growth affected by culture filtrate of *P. macerans* PM-1.
A, KI 101: 5–21; B, KJ 201: 88–62; C, KJ 301: 93–39. Arrowheads indicate the swellings of mycelia. Isolates of *P. oryzae* were grown at 28°C for 7 days on PDA supplemented with 10% culture supernatant. Portions of colonies of 1 cm^2 carrying mycelial tip were cut and examined under light microscope (Carl Zeiss, Axiovert 135M, ×400).

Table 4. Effect of culture supernatant of *P. macerans* PM-1 on conidial germination of *P. oryzae*.

P. oryzae isolates	Conidial germination (% ±SD)	
	PM-1 supernatant	Control
KI 101: 5-21	18.4±2.1	82.7±1.5
KI 201: 86-111-4	15.3±1.0	79.5±2.1
KJ 101: 89-16	15.0±0.6	65.3±1.5
KJ 201: 88-62	14.7±2.6	67.0±3.1
KJ 301: 93-39	31.2±1.0	76.3±3.2

Culture supernatant was filtered through 0.22 μm membrane filter and the aliquots of 40 μl of culture filtrate were mixed with 10 μl of conidial suspension of 2.5×10^5 per ml. From this mixture aliquots of 30 μl were transferred to hole slide glass in triplicates on the moistened filter paper supported with two glass rods in a petri-dish. The petri-dish was incubated at 28°C for 24 h. Conidial germination was examined under a light microscope at ×100 magnification.

Inhibition of Mycelial Growth of *P. oryzae*

The culture filtrate (10%, v/v) of the strain PM-1 significantly inhibited the mycelial growth of *P. oryzae* races (Fig. 3). Moreover, the inhibition of the mycelial growth of *P. oryzae* KJ 301: 93-39 was shown extraordinary. After an incubation at 28°C for 7 days under the same condition, the morphological changes of the mycelia were observed under a light microscope. Mycelia were partially elongated, agglutinated and swollen (Fig. 4). Also, the septa was separated and the cell walls of the mycelia lysed, resulting in the release of the cytosolic materials into the external media.

Inhibition of Conidial Germination of *P. oryzae*

The culture filtrate of the strain PM-1 was mixed with the conidial suspension (2.5×10^5/ml) of *P. oryzae* races and the inhibitory activity of the filtrate on the conidial germination of the races was investigated (Table 4). The conidial germination was strongly inhibited by 2.4 to 5.2-fold compared with control (sterilized water). In particular, the most inhibitory effect was observed in *P. oryzae* KI 201: 86-111-4 (15.3%: 79.5%). As for the conidial morphology, the culture caused the races to inhibit both the conidial germination and the formation of appressoria (Fig. 5). The mycelial lysis was also found.

DISCUSSION

An antagonistic bacterial strain to rice blast fungus, *P. oryzae*, was isolated and designated as PM-1. The strain PM-1 was identified as *Paenibacillus macerans*. Generally, antagonistic microorganisms such as fluorescent *Pseudomonas*, *Bacillus* sp. and *Actinomycetes* have been shown to have broad antifungal spectra against soil pathogens [3, 17]. Also, the strain PM-1 exhibited a high antifungal activity against twenty strains of *P. oryzae* races.

The optimum culture condition for the production of antifungal substances of the strain PM-1 in PD broth was 20°C and pH 10.0. The antifungal activity of the culture reached a maximum level after 24-27 h of incubation (stationary phase). The antifungal activity of culture supernatant was higher than that of the cell extract, suggesting that the

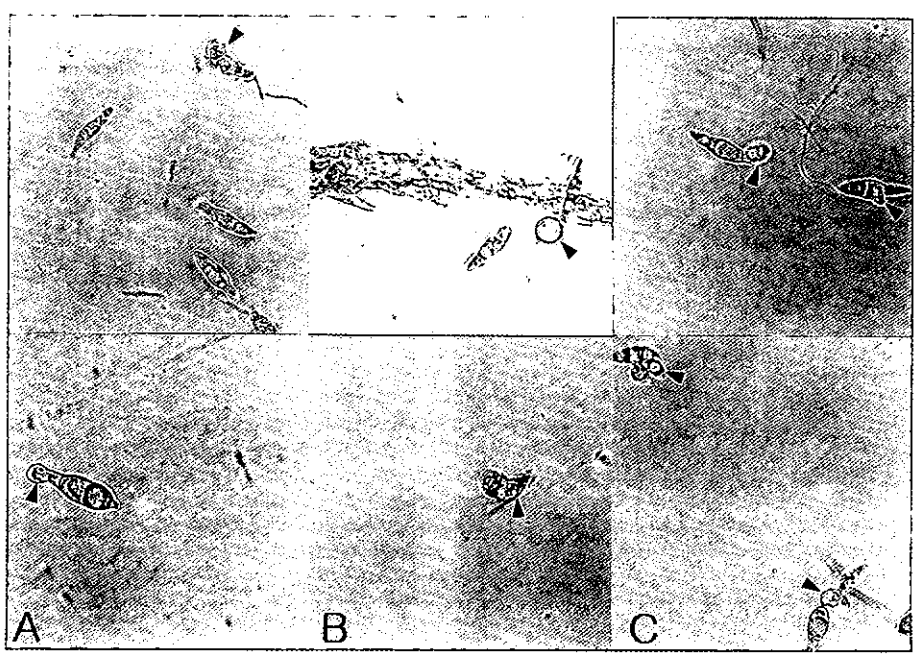

Fig. 5. Inhibition of antifungal substances from *P. macerans* PM-1 on the conidial germination of *P. oryzae*.
Swelling and germination of conidia are indicated with arrowheads; A, KI 101: 5-21; B, KJ 201: 88-62; C, KJ 301: 93-39.

main antifungal substances were secreted into the culture medium. The extracellular antifungal substances were very stable up to 121°C (78.5-88.2%) except in the activity toward *P. oryzae* KJ 201: 88-62 (23.5%). This thermostability was similar to that of antifungal substances from *Bacillus subtilis* YB-70 [10] and *Bacillus* sp. SS279 [11].

The antifungal substances of the strain PM-1 strongly inhibited the mycelial growth of *P. oryzae* and induced morphological changes of the mycelia. These results were very similar to the antifungal mechanisms of polyoxin and polyene related antifungal antibiotics [4], kimorexin A [25] and iturin [7]. A novel antifungal antibiotic cepacidine A was identified by *Pseudomonas cepacia* AF2001 [14]. Medium-chain (C_6-C_{11}) alkenals were evaluated for antifungal activity toward *Saccharomyces cerevisiae* [15]. The secreted antifungal substances of the strain PM-1 was thought to distort the mycelial cell wall or cytoplasmic membrane, resulting in the swelling of mycelia and the inhibition of mycelial growth. Moreover, the culture filtrate of the strain PM-1 significantly inhibited the conidial germination of *P. oryzae* races. Inhibition of the formation of appressoria and mycelial lysis occurred as well.

Therefore, it can be stated that identification and overproduction of the antifungal substances from the strain PM-1 may prove to be valuable for the biocontrol of rice blast disease.

Acknowledgments

This research was supported by a grant from the Korea Research Foundation, 1998.

REFERENCES

1. Bell, A. A. and M. H. Wheeler. 1986. Biosynthesis and functions of fungal melanin. *Annu. Rev. Phytopathol.* **24:** 411-451.
2. Bonman, J. M., G. S. Khush, and R. J. Nelson. 1992. Breeding rice for resistance to pests. *Annu. Rev. Phytopathol.* **30:** 507-528.
3. Fravel, D. R. 1988. Role of antibiosis in the biocontrol of plant disease. *Annu. Rev. Phytopathol.* **26:** 75-91.
4. Gunji, S., K. Arima, and T. Beppu. 1983. Screening of antifungal antibiotics according to activities inducing morphological abnormalities. *Agric. Biol. Chem.* **47:** 2061-2069.
5. Isono, K., K. Asahi, and S. Suzuki. 1969. Studies on polyoxins, antifungal antibiotics. VIII. The structure of polyoxins. *J. Am. Chem. Soc.* **91:** 7490-7505.
6. Iwasa, T., E. Higashide, H. Yamamoto, and M. Shibata. 1970. Studies on validamycins, new antibiotics. II. Production and biological properties of validamycins A and B. *J. Antibiotics* **23:** 595-602.
7. Kim, B. S. and K. Y. Cho. 1995. Antifungal effects on plant pathogenic fungi and characteristics of antifungal substances produced by *Bacillus subtilis* SJ-2 isolated from sclerotia of *Rhizoctonia solani*. *Kor. J. Plant Pathol.* **11:** 165-172.
8. Kim, H. K., B. S. Kim, S. S. Moon, and B. K. Hwang. 1998. Purification of antifungal antibiotic NH-B1 from actinomycete NH 50 to plant pathogenic fungi. *Kor. J. Plant Pathol.* **14:** 191-202.
9. Kim, K. Y. and S. D. Kim. 1997. Biological control of *Pyricularia oryzae* blast spot with the antibiotic substances produced by *Bacillus* sp. KL-3. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 396-402.
10. Kim, Y. S., J. K. Son, D. C. Moon, and S. D. Kim. 1997. Isolation and structure determination of antifungal antibiotics from *Bacillus subtilis* YB-70, a powerful biocontrol agent. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 62-67.
11. Kim, S. S., G. J. Joo, J. Y. Uhm, Y. J. Kim, and K. I. Rhee. 1997. Antifungal activity of *Bacillus* sp. SS279 biocontrol of apple white rot fungus, *Botryosphaeria dothidea*. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 527-536.
12. Krieg, N. R. and J. G. Holt. 1984. *Bergey's Manual of Systematic Bacteriology*, vol. **2**. The Williams and Wilkins Co., Baltimore, U.S.A.
13. Lange, L., J. Breinholt, F. W. Rasmussen, and R. I. Nielsen. 1993. Microbial fungicides the natural choice. *Pestic. Sci.* **39:** 155-160.
14. Lee, C.-H., J. W. Suh, and Y. H. Cho. 1999. Immunosuppressive activity of cepacidine A, a novel antifungal antibiotic produced by *Pseudomonas cepacia*. *J. Microbiol. Biotechnol.* **9:** 672-674.
15. Lee, J. R., S. H. Lee, I. Kubo, and S. D. Hong. 1998. Antifungal activity medium-chain (C_6-C_{13}) alkenals against, and their inhibitory effect on the plasma membrane H^+-ATPase of *Saccharomyces cerevisiae*. *J. Microbiol. Biotechnol.* **8:** 197-202.
16. Leung, H. and M. Taga. 1988. *Magnaporthe grisea* (*Pyricularia oryzae*), The blast fungus. pp. 175-188. *In* D. S. Ingram, D. H. Williums., Advances in pathology genetic of plant pathogenic fungi. Academic Press Inc., U.K.
17. Mckeen, C. D., C. C. Reilly, and P. L. Pusey. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. *Phytopathology* **76:** 136-139.
18. Ou, S. H. 1980. Pathogen variability and host resistance in rice blast disease. *Annu. Rev. Phytopathol.* **18:** 167-187.
19. Porter, N. and F. M. Fox. 1993. Diversity of microbial products discovery and application. *Pestic. Sci.* **39:** 161-168.
20. Powel, K. A. and A. R. Jutsum. 1993. Technical and commercial aspects of biocontrol products. *Pestic. Sci.* **37:** 315-321.
21. Staub, T. and D. Sozzi. 1984. Fungicde resistance: A continuing challenge. *Plant Dis.* **68:** 1026-1031.
22. Sy, A. A., L. Albertini, and M. Moletti. 1998. Biological control of rice leaf blast. pp. 521-527. *In* R. S. Zeigler, S. A. Leong, P. S. Teng., Rice blast disease. CAB International, Wallingford, U.K.
23. Takeuchi, S., K. Hirayama, K. Ueda, H. Sasaki, and H. Yonehara. 1958. Blasticidin S, a new antibiotic. *J. Antibiotics* **11:** 1-5.
24. Umezawa, H., Y. Okami, T. Hashimoto, Y. Suhara, M. Hamada, and T. Takeuchi. 1965. A new antibiotic, kasugamycin. *J. Antibiotics* **18:** 101-103.
25. Yeo, W. H., S. K. Kim, S. S. Kim, S. H. Yu, and E. K. Park. 1994. Isolation, physico-chemical properties, and biological activity of new thiopeptide antibiotics, kimorexins. *J. Microbiol. Biotechnol.* **4:** 349-353.