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Antifungal Activity of Valinomycin, a Peptide Antibiotic Produced by *Streptomyces* sp. Strain M10 Antagonistic to *Botrytis cinerea*

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A strain of *Streptomyces* sp. (M10) antagonistic to *Botrytis cinerea* was isolated from orchard soil obtained from Jeju Island, Korea. An antifungal substance (CN1) was purified from the culture extracts of the strain, and then identified as valinomycin through extensive spectroscopic analyses. Valinomycin showed potent *in vitro* antifungal activity against *Botrytis cinerea* and also *in vivo* control efficacy against *Botrytis* blight development in cucumber plants. Overall, the disease control efficacy of valinomycin was similar to that of vinclozolin, a commercial fungicide. This study provides the first report on the disease control efficacy of valinomycin against *Botrytis* blight.

Keywords: Valinomycin, peptide antibiotics, *Botrytis cinerea*, plant disease control

Botrytis diseases such as *Botrytis* blight or Gray-mold rot, caused by *Botrytis cinerea*, affect most vegetable and fruit crops [2]. The symptoms of *Botrytis* diseases are very diverse depending on the host and plant part infected. Since the diseases are favored by cool, moist, and poorly ventilated conditions, they are the most problematic diseases of greenhouse-grown crops, especially in the spring and fall seasons. Fungicides are commonly used to manage the disease, but their effectiveness is reduced because of the rapid evolution of fungicide resistance in *B. cinerea* [27]. As a result, there is an increasing need for new alternative antifungal agents different from those currently in use in terms of mode of action and chemical properties [5, 10, 29]. Moreover, the growing concerns of the public regarding safer foods compel the research community to develop safer fungicides and environmentally friendly disease control methods [9, 23].

During the screening for antagonistic microorganisms against *Botrytis cinerea*, we isolated *Streptomyces* sp. strain M10 from orchard soils collected on Jeju Island, Korea. In this study, the antifungal substance produced by strain M10 was purified using various chromatographic procedures and its structure was determined by analyzing various spectral data. The *in vitro* antifungal activity of the antibiotic substance was examined against various plant pathogenic fungi, and its *in vivo* control efficacy against *Botrytis* disease on cucumber plants was evaluated under a growth room condition.

Morphological, physiological, and biochemical characteristics of the antagonistic bacteria strain M10 were examined using the methods of Waksman [26], Shirling and Gottlieb [24], Schaal [20], and Williams *et al.* [28]. Chemotaxonomic evaluations indicated that the cell wall of strain M10 contained LL-DAP lacking diagnostic sugars, suggesting that it is a cell-wall type I. The strain produced a gray spore mass on ISP2 medium with a yellow-brown substrate mycelia mass. The M10 strain produced irregularly oriented and branched aerial mycelium. The spore chain was rectiflexible and consisted of 15–30 spores. The spores were cylindrical with a 4.0–5.5 μm diameter and 5.0–8.5 μm length. Observations by electron microscopy revealed the spores having a smooth surface. Based on the type of cell wall components and morphological characteristics examined by scanning electron microscopy, the strain M10 was confirmed to belong to the genus *Streptomyces*. A partial 16S rDNA sequence of 1,465 nucleotides was determined to be strain M10 (GenBank database under the accession number EF034030). The strain M10 had high percentage nucleotide similarity of the 16S rDNA sequences (99.88%) with *Streptomyces albolongus* NBRC13465 and *S. tsusimaensis* ATCC15141. The phylogenetic tree was constructed based on the percent difference in the genetic relationships between the allied strains of Streptomycetes (Fig. 1). Among the allied strains, *S. griseolus* (99.14%), *S. fulvissimus* (96.55%), and *S. tsusimaensis* have been reported as producers of the antibiotic compound valinomycin [4, 8, 15].

SGGP agar medium (10 g glucose, 4 g peptone, 4 g yeast extract, 4 g casamino acids, 2 g glycine, 0.5 g $MgSO_4$·

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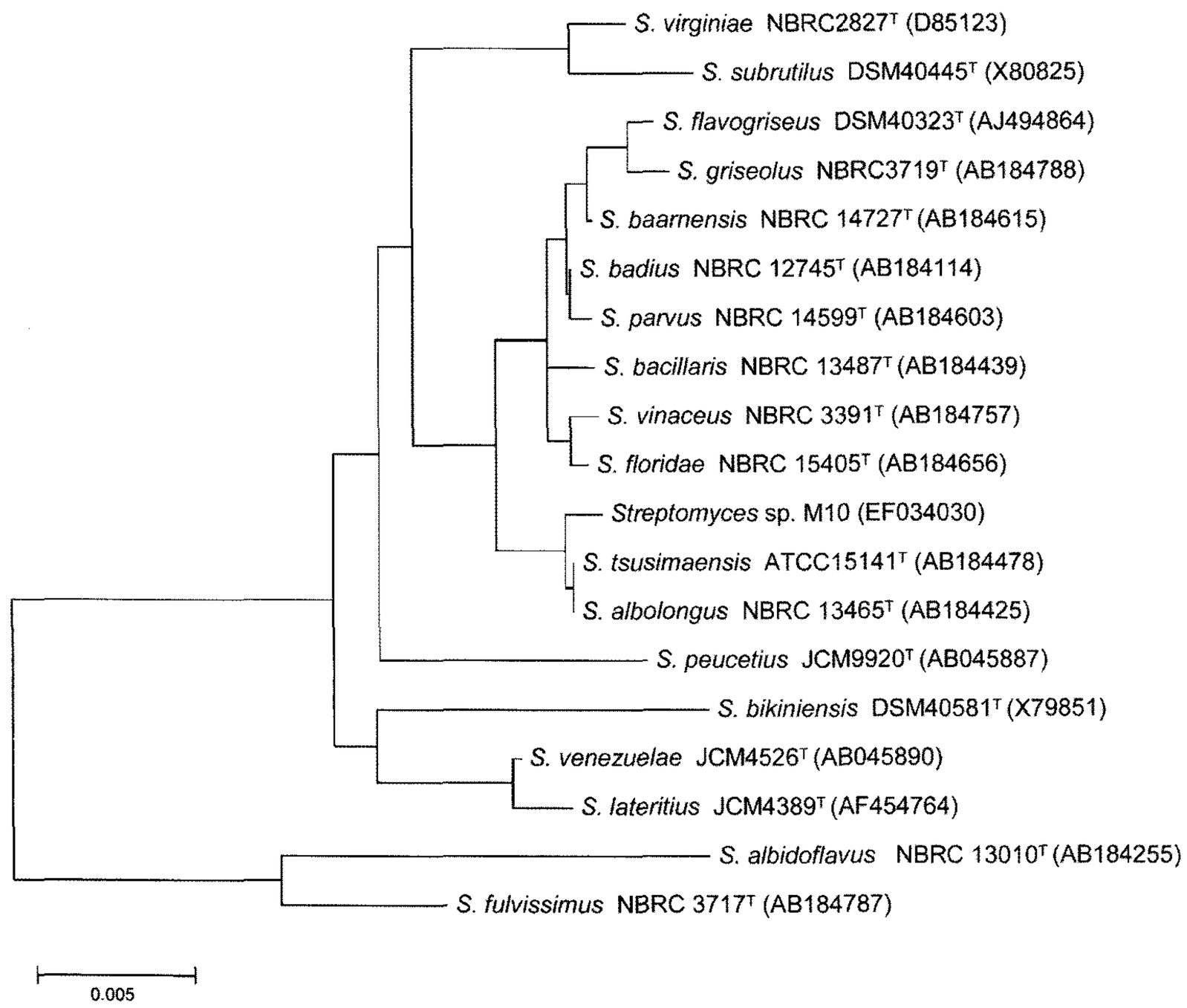

Fig. 1. Phylogenetic localization of *Streptomyces* sp. M10 based on 16S rDNA sequences.
The length of each pair of branches represents the distance between sequence pairs.

H_2O, 1.3 g K_2HPO_4, 1 l distilled water) was used for the large-scale production of the antifungal metabolites of *Streptomyces* sp. strain M10. After incubation at 28°C for two days, the culture (3 kg) was extracted with methanol (6 l). The antifungal activity of the strain against *B. cinerea* was observed in the methanol extract. The methanol extracts were loaded onto a column filled with Diaion HP-20 resins (Mitsubishi Chemical Co., Tokyo, Japan) and eluted with stepwise gradients of methanol-water. The active fractions (80–100% methanol, v/v) were pooled and further chromatographed on a silica gel column (silica gel 60, 23–660 μm mesh; Merck) with stepwise gradients of chloroform-methanol. The fractions eluted with chloroform-methanol (70:30–100:0, v/v) retained antifungal activities. The active fractions were chromatographed on a Sephadex LH-20 (Uppsala Sweden, Amersham) column (C26/100, Pharmacia). The active fractions were pooled and further purified by semipreparative HPLC (Gilson, Middleton, WI, U.S.A.) equipped with a C18 reversed-phase column (ODS-H80, 1×25 cm, 4 μm; YMC Co., Kyoto, Japan) using an elution system of linear gradients from 70% to 95% methanol in distilled water amended with 0.1% formic acid for 20 min followed by isocratic elution for 30 min (flow rate: 2 ml/min). The antifungal compound was eluted at a retention time of 48.7 min. The pure antibiotic CN1 (11.5 mg) was yielded as white powder after the evaporation to dryness.

Spectroscopic analyses were performed to determine the structure of the antibiotic CN1. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX 500 MHz NMR spectrometer (Billerica, MA, U.S.A.). A complete analysis of a combination of 1D and 2D NMR spectra suggested that compound CN1 contains one α-hydroxyisovaleryl unit (Hiv), a lactoyl group (Lac), and

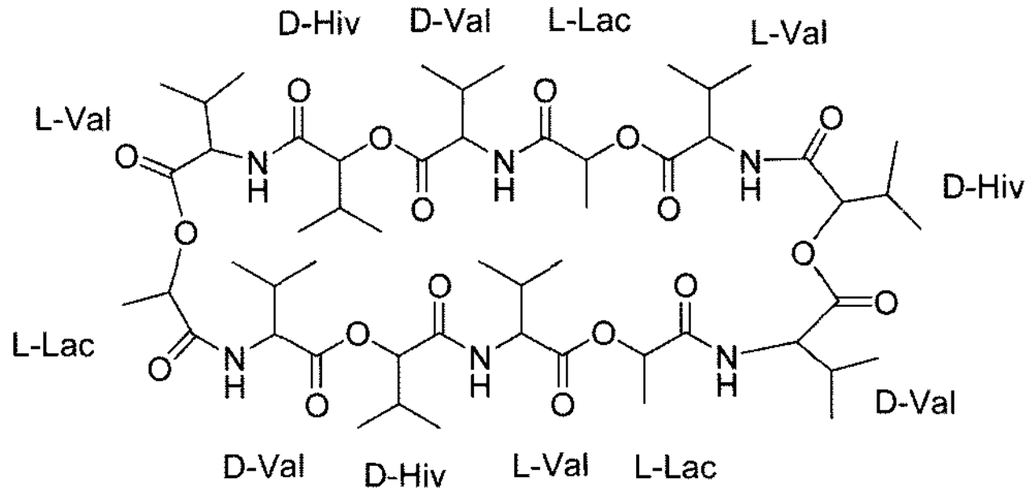

Fig. 2. Structure of the antibiotic valinomycin (CN1) isolated from *Streptomyces* sp. strain M10.

Table 1. Minimum inhibitory concentrations (MICs) of the antibiotic valinomycin (CN1) isolated from the *Streptomyces* strain M10 against various microorganisms.

Microorganism	MIC (μg/ml)[a]
Alternaria mali	>256
Alternaria solani	>256
Botrytis cinerea	4
Cladisporium cucumerinum	>256
Colletotrichum gloeosporioides	256
Colletotrichum orbiculare	>256
Cylindrocarpon destructans	>256
Didymella bryoniae	>256
Fusarium moniliforme	>256
Fusarium oxysporum f.sp. *cucumerinum*	>256
Fusarium oxysporum f.sp. *lycopersici*	>256
Fusarium moniliforme	>256
Magnaporthe grisea	4
Phytophthora capsici	>256
Rhizoctonia solani	256
Candida albicans	32
Saccharomyces cerevisiae	>256
Bacillus megaterium	>256
Ralstonia solanacearum	>256
Xanthomonas campestris pv. *vesicatoria*	>256

[a]The lowest concentration of the antibiotic valinomycin (CN1) required for complete inhibition of microbial growth.

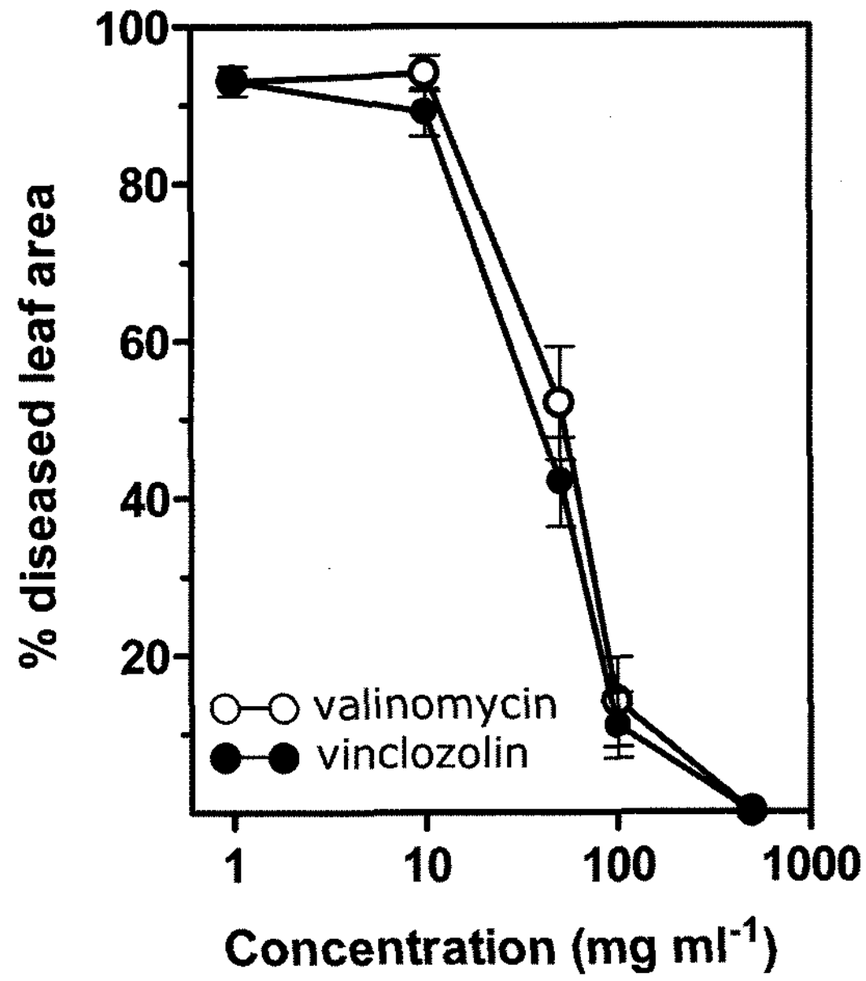

Fig. 3. *In vivo* efficacies of valinomycin (CN1) and vinclozolin for the control of *Botrytis* blight in cucumber plants, caused by *Botrytis cinerea*.
Each compound was applied on the plants one day before fungal inoculation. Disease development was rated five days after the inoculation. Vertical bars represent the standard deviations.

two valines (Val) with the sequence Hiv-Val-Lac-Val, which was confirmed by HMBC experiment. The ESI mass data of compound CN1 ($[M+H]^+$ 1111.8, $[M-H]^-$ 1109.4) indicated the presence of C_3 symmetry, a triple of Hiv-Val-Lac-Val. Through comparison with the NMR and mass spectrum data of authentic valinomycin, the structure of this cyclodepsipeptide CN1 was identified as valinomycin (Fig. 2).

The antifungal activity of the antibiotic CN1 identified as valinomycin was evaluated by an *in vitro* growth inhibition assay on microtiter dishes (Cell Wells; Corning Glass Works, Corning, NY, U.S.A.) using a method modified from Nair *et al.* [14] (Table 1). Valinomycin inhibited the growth of *Rhizoctonia solani* and *Colletotrichum gloeosporioides* at the concentration of 256 μg/ml. The growth of *Candida albicans* was inhibited at 32 μg/ml. The antibiotic valinomycin showed a high level of inhibitory activity against *B. cinerea* and *M. grisea*. Germination of the spores was completely restricted at a concentration of 4 μg/ml. The mycelial growth of the fungi was also inhibited by valinomycin. The EC_{50}s of valinomycin against the mycelial growth of *B. cinerea* and *M. grisea* were 5.2 and 4.3 μg/ml, respectively.

The *in vivo* control efficacy of valinomycin for controlling *Botrytis* blight in cucumber plants was evaluated under a growth room condition in comparison with the commercial fungicide vinclozolin (Fig. 3). As the concentration of valinomycin increased, the *Botrytis* disease was markedly suppressed in the cucumber plants. Valinomycin started to show protective activity against the *B. cinerea* infection at the concentration of 50 μg/ml. No disease symptoms were found on the cucumber leaves treated with 500 μg/ml of valinomycin. Vinclozolin also showed potent disease control capability. The cucumber plants treated with 500 μg/ml of vinclozolin were completely protected from the infection of *B. cinerea*, which was comparable to the disease control efficacy of valinomycin.

Valinomycin has a cyclododecadepsipeptide structure that contains a three-repeat sequence of a tetradepsipeptide basic unit, D-α-hydroxyisovaleryl-D-valyl-L-lactoyl-L-valyl, to form a symmetric 36-membered ring molecule [1, 3, 11]. The structure behaves as an ionophore that specifically modulates potassium ion transport across biological membranes [7]. Nigericin, monensins, and gramicidins are well-known ionophore antibiotics that demonstrate diverse biological activities [18]. With a known activity as a potassium ionophore, valinomycin has been used as an insecticidal-nematocidal agent (W. Patterson, U.S. Patent no. 3,520,973, 1970) and as a key component of K^+ measurement instruments in the biomedical device industry [16]. Another ionophore antibiotic, gramicidin S, is a cyclic peptide antibiotic like valinomycin and also known to be a membrane-active substance [22]. The antifungal activity of gramicidin S against plant pathogenic fungi and its potential as a fungicide have been extensively studied [13]. In contrast to gramicidin S, however, few data are available to demonstrate the antifungal activities

of valinomycin against plant pathogenic fungi or its potential as an antifungal agent for plant disease control. Other cyclic peptide antibiotics such as montanastatin [17], cereulide [25], PF1022A [19], and enniatin A, B [21] are also known to have closely similar chemical structures to valinomycin. Although these antibiotics have been reported to have various biological activities, including antibacterial, antifungal, anthelmintic, and anticancer effects, there are no reports on the antifungal activity and disease control effect against *Botrytis cinerea*.

The potent antifungal activity of valinomycin against *B. cinerea* led us to evaluate the control efficacy properties against *Botrytis* disease in cucumber plants. An *in vivo* assay of antibiotic substances on plants is a requirement for the prediction of any potential antifungal potency of the compound as a plant disease control agent. Such a property reflects not only the intrinsic potency of the compound but also the chemical and physical properties needed as an agricultural fungicide, *e.g.*, stability in a farming environment and distribution in the host plants. *In vivo* control efficacy tests have revealed that valinomycin is an effective control agent for *Botrytis* disease in cucumber plants, which is comparable to the commercially available fungicide vinclozolin. Vinclozolin is a dicarboximide fungicide, which is used mainly on vines, fruits, and vegetables worldwide. The mode of action of vinclozolin has been proposed to be associated with membrane lipid peroxidation, which causes cellular leakage on a susceptible fungal strain [6]. Valinomycin has a different mode of action from vinclozolin. As a potassium ionophore, valinomycin causes a depolarization of fungal cytoplasmic membrane, leading to cell death [12]. The distinct antifungal mechanism suggests that these two compounds may have no cross-resistance to each other. Along with the peptide structure of valinomycin, which is expected to be moderately degradable in an agricultural environment, its potent *in vivo* control efficacy gives it useful characteristics as an antifungal agent for the control of *Botrytis* diseases.

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References

1. Andersson, M. A., R. Mikkola, R. Kroppenstedt, F. A. Rainey, J. Peltola, J. Helin, K. Sivonen, and M. S. Salkinoja-Salonen. 1998. Mitochondrial toxin produced by *Streptomyces griseus* strains isolated from indoor environment is valinomycin. *Appl. Environ. Microbiol.* **64:** 4767–4773.
2. Barnes, S. E. and M. W. Shaw. 2002. Factors affecting symptom production by latent *Botrytis cinerea* in *Primula* x *polyantha*. *Plant Pathol.* **51:** 746–754.
3. Brockmann, H. and G. Schmidt-Kastner. 1955. Valinomycin. I. Über Antibiotica aus Actinomyceten. XXVII. *Chem. Ber.* **88:** 57.
4. Cheng, Y.-Q. 2006. Deciphering the biosynthetic codes for the potent anti-SARS-CoV cyclodepsipeptide valinomycin in *Streptomyces tsusimaensis* ATCC 15141. *ChemBioChem* **7:** 471–477.
5. Choi, G. J., J. Kim, K. S. Jang, H. K. Lim, I. Park, S. Shin, and K. Y. Cho. 2006. *In vivo* antifungal activities of 67 plant fruit extracts against six plant pathogenic fungi. *J. Microbiol. Biotechnol.* **16:** 491–495.
6. Choi, G. J., H. J. Lee, and K. Y. Cho. 1996. Lipid peroxidation and membrane disruption by vinclozolin in dicarboximide-susceptible and -resistant isolates of *Botrytis cinerea*. *Pesticide Biochem. Physiol.* **55:** 29–39.
7. Haynes, D. H., A. Kowalsky, and B. Pressman. 1969. Application of nuclear magnetic resonance to the conformational changes in valinomycin during complexation. *J. Biol. Chem.* **244:** 502–505.
8. Heisey, R. M., J. Huang, S. K. Mishra, J. E. Keller, J. R. Miller, A. R. Putnam, and T. D. J. D'Silva. 1988. Production of valinomycin, an insecticidal antibiotic, by *Streptomyces griseus* var. *flexipertum* var. *nov. J. Agric. Food Chem.* **36:** 1283–1286.
9. Kim, B. S. and B. K. Hwang. 2003. Biofungicides. *In* D. K. Arora, P. D. Bridge, and D. Bhatnager (eds.). *Handbook of Fungal Biotechnology.* Dekker, New York.
10. Kim, C. S., E. K. Lim, K. H. Choi, H. G. Kong, D. W. Kim, S. Lee, B. J. Moon, S. H. Lee, and H. J. Kim. 2007. Biological control of strawberry gray mold caused by *Botrytis cinerea* using *Bacillus licheniformis* N1 formulation. *J. Microbiol. Biotechnol.* **17:** 438–444.
11. Koloditskaia, A. T., I. N. Blinova, G. M. Smirnova, and A. S. Khokhlov. 1975. *Actinomyces cyaneofuscatus*, producer of valinomycin. A study of its pigments and antibiotics. *Izv Akad Nauk SSSR Biol.* **5:** 694–700.
12. Maruyama, M., S. Yamauchi, K. Akiyama, T. Sugahara, T. Kishida, and Y. Koba. 2007. Antibacterial activity of virgatusin-related compound. *Biosci. Biotechnol. Biochem.* **71:** 677–680.
13. Murray, T., F. C. Leighton, and B. Seddon. 1986. Inhibition of fungal spore germination by gramicidin S and its potential use as a biocontrol against fungal plant pathogens. *Lett. Appl. Microbiol.* **3:** 5–7.
14. Nair, M. G., A. Chandra, and D. L. Thorogood. 1994. Gopalamicin, an antifungal macrodiolide produced by soil actinomycetes. *J. Agric. Food Chem.* **42:** 2308–2310.
15. Paananen, A., R. Mikkola, T. Sareneva, S. Matikainen, M. Andersson, I. Julkunen, M. S. Salkinoja-Salonen, and T. Timonen. 2000. Inhibition of human NK cell function by valinomycin, a toxin from *Streptomyces griseus* in indoor air. *Infect. Immun.* **68:** 165–169.
16. Perkins, J. B., S. K. Guterman, C. L. Howitt, V. E. N. Williams, and J. Pero. 1990. *Streptomyces* genes involved in biosynthesis of the peptide antibiotic valinomycin. *J. Bacteriol.* **172:** 3108–3116.
17. Pettit, G. R., R. Tan, N. Melody, J. M. Kielty, R. K. Pettit, D. L. Herald, B. E. Tucker, L. P. Mallavia, D. L. Doubek, and

J. M. Schmidt. 1999. Antineoplastic agents. Part 409: Isolation and structure of montanastatin from a terrestrial Actinomycete. *Bioorg. Med. Chem.* **7:** 895–899.
18. Pressman, B. 1976. Biological applications of ionophores. *Annu. Rev. Biochem.* **45:** 501–530.
19. Samson-Himmelstjerna, G. V., A. Harder, N. C. Sangster, and G. C. Coles. 2005. Efficacy of two cyclooctadepsipeptides, PF1022A and emodepside, against anthelmintic resistant nematodes in sheep and cattle. *Parasitology* **130:** 343–347.
20. Schaal, K. P. 1985. Identification of clinically significant Actinomycetes and related bacteria using chemical techniques. *In* M. Goodfellow and D. E. Minnikin (eds.). *Chemical Methods in Bacterial Systematics.* Academic Press, London.
21. Seo, Y. S., J. C. Kim, B. S. Kim, Y. W. Lee, and K. Y. Cho. 1996. Isolation and identification of antifungal substances produced by *Fusarium* sp. BYA-1. *Plant Pathol. J.* **12:** 72–79.
22. Sharom, F. J., P. Lu, R. Liu, and X. Yu. 1988. Linear and cyclic peptides as substrates and modulators of P-glycoprotein: Peptide binding and effects on drug transport and accumulation. *Biochem. J.* **333:** 621–630.
23. Shin, D., M. S. Park, S. Jung, M. S. Lee, K. H. Lee, K. S. Bae, and S. B. Kim. 2007. Plant growth-promoting potential of endophytic bacteria isolated from roots of coastal sand dune plants. *J. Microbiol. Biotechnol.* **17:** 1361–1368.
24. Shirling, E. B. and D. Gottlieb. 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* **16:** 313–340.
25. Teplova, V. V., R. Mikkola, A. A. Tonshin, N. L. Saris, and M. S. Salkinoja-Salonen. 2006. The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration. *Toxicol. Appl. Pharmacol.* **210:** 39–46.
26. Waksman, S. A. 1961. *Classification, Identification and Descriptions of Genera and Species.* The Williams & Willkins Co., Baltimore.
27. Wardlow, L. R. and T. M. O'Neill. 1992. Management strategies for controlling pests and diseases in glasshouse crops. *Pestic. Sci.* **36:** 341–347.
28. Williams, S. T., M. E. Saharpe, J. G. Holt, R. G. E. Murray, D. J. Brener, N. R. Krieg, J. W. Mouldar, N. Pfennig, P. H. A. Sneath, and J. T. Staley. 1989. *Bergery's Manual of Systematic Bacteriology*, Vol. 4. The Williams & Wilkins Co., Baltimore.
29. Yun, B., E. M. Kwon, J. Kim, and S. H. Yu. 2007. Antifungal cyclopeptolide from fungal saprophytic antagonist *Ulocladium atrum*. *J. Microbiol. Biotechnol.* **17:** 1217–1220.