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Article

Identification of 12-Methyltetradecanoic Acid from Endophytic *Senotrophomonas maltophilia* as Inhibitor of Appressorium Formation of *Magnaporthe oryzae*

Yul-Taek Jeon[1§], Eun-Mi Jun[1§], Ki-Bong Oh[1], Pham Quang Thu[2]*, and Soo-Un Kim[1]*

[1]*Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea*

[2]*Forest Science Institute of Vietnam, Dong Ngac, Tu Liem, Hanoi, Vietnam*

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The rice blast fungus *Magnaporthe oryzae* is an extremely effective plant pathogen that produces specialized infectious structures known as appressoria. Four hundred thirty eight endophytic bacterial strains were isolated from trees growing in Vietnam and screened for antifungal activity. A strain of a Gram-negative bacterium, *Stenotrophomonas maltophilia* was found to have an activity that inhibits appressorium formation of *M. oryzae*. Activity-guided fractionation of the bacterial extract yielded a hydrophobic active fraction, which was purified through MPLC and PTLC, and analyzed by GC-MS. Three kinds of fatty acid, 13-methyltetradecanoic acid, 12-methyltetradecanoic acid (12-MTA), and palmitic acid were identified from the active fraction, among which, only 12-MTA had the activity. It specifically inhibited the appressorium formation with IC_{50} value of 83.5 μM. The action mechanism and agrochemical use of 12-MTA should be further studied.

Key words: branched-fatty acid, endophytic bacteria, GC/MS, *Magnaporthe oryzae*, 12-methyltetradecanoic acid, *Stenotrophomonas maltophilia*

Rice, *Oryza sativa*, is the primary food source for more than half of the world's population, and this continuing growth of population will demand 40% more rice production by 2030. Satisfying the demand is challenged by shrinking cropland, decreasing water supply, and labor shortage [Khush, 1999; 2005]. *Magnaporthe oryzae*, was recently defined as a new species, separate from *M. grisea*, based on multilocus genealogy and mating experiments [Couch and Kohn, 2002]. It is the causal fungus of rice blast, the most devastating disease of rice throughout the rice-growing areas of the world. In Korea, among about one million hectares of rice cultivation acreage, leaf blast occurs in seventy thousand hectares and panicle blast in fourteen thousand hectares annually on average [RDA, 2009]. *M. oryzae* forms appressorium, a specialized infection structure, as do many other plant pathogen, to adhere to the leaf surface and then penetrate into the host tissue by high turgor pressure [Bourett and Howard, 1990]. For the initiation of infection, the differentiation and maturation of appressorium are crucial steps [Gilbert *et al.*, 1996]. Thus, disturbance of these steps is suggested as a target for the development of fungicides against *M. oryzae*.

Antifungal metabolites produced by microorganism have been studied not only to control human fungal infections, but also to use the metabolites directly as potent agrochemicals with high selectivity against plant pathogens. In addition, these metabolites could provide lead structures for the development of new chemical fungicides [Lange *et al.*, 1993]. One important source recently receiving attention is endophytes, microorganisms that reside in the tissues of living plants. The source of endophytes is enormous: it is generally regarded that each individual plant of approximately 300,000 higher plant species that exist on earth is host to one or more endophytes [Strobel *et al.*, 2004]. They are relatively understudied compared to the soil microorganisms as a

[§]YTJ and EMJ contributed equally to this work.

*Corresponding authors
Phone: +82-2-880-4642; Fax: +82-2-873-3112
Email: soounkim@snu.ac.kr (Soo-Un Kim)
Phone: +84-4-836-2376; Fax: +84-4-3838-9722
E-mail: phamquangthu@fpt.vn (Pham QT)

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source of novel natural products. Only a handful of these plants have ever been completely studied for their endophytic biology. Discovery of paclitaxel-producing microorganism from Pacific yew tree is a fine example [Stierle *et al.*, 1993].

The aim of the present study is to find antibiotics that inhibit appressorium formation of *M. oryzae* from endophytic bacteria; 438 different endophytic bacterial strains were isolated from 24 tree samples from Vietnam, and one of the strains was found to be active against appressorium formation. The strain was identified, and the culture broth was analyzed to identify the active compound.

Materials and Methods

Microbial screening. The twigs with diameter of approximately 1 cm were collected from 24 trees in the rain forest of Vietnam and stored at 4°C until bacteria were isolated. The samples were surface-sterilized by spraying 70% ethanol and briefly flamed. The twigs were cut with sterilized razor blade into three parts: bark, cambium, and inner wood. Three pieces of tissue from each part were placed in 1 mL of sterile phosphate-buffered saline (PBS; NaCl 8.5 g, KH_2PO_4 6.8 g, NaOH 1.16 g per litter, pH 7) in a test tube. After shaking for 4 h at 28 and 250 rpm or overnight at 4°C, the bacterial suspension in PBS was streaked onto King's B agar plate (protease peptone 20 g, K_2HPO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, agar 15 g per L) and nutrient glucose agar plate (nutrient broth 8 g, glucose 2.5 g, agar 15 g per L) and incubated for 48 h at 28°C. Every single colony was picked and separately streaked on the same type of agar medium, and then incubated at 28°C for 1 week. The isolated strains were stored at −80°C in 80% glycerol.

An agar block of 1 cm×1 cm was taken from each culture plate, and the block was extracted by shaking in 1.5 mL methanol overnight at 28°C. The methanol extract was evaporated in a vacuum centrifugal concentrator and diluted to the original volume before screening.

Inhibition assay against *M. oryzae*. *M. oryzae* strain KJ201 [Park *et al.*, 2003] was grown on an oatmeal agar (Difco, Franklin Lakes, NJ) at 25°C under continuous fluorescent light. Conidia were harvested from 10 to 14 days old cultures in sterile distilled water and filtered through four layers of Miracloth (Calbiochem, La Jolla, CA). Thirty-six microliters of conidial suspension (3×10^4 conidia per mL) and 4 μL of methanolic solution of analyte were placed on a cover glass in a moistened plastic box. After 24 h of incubation at 25°C [Oh and Lee, 2000], inhibitory effects of analytes on conidial germination and appressorium formation were determined. The number of conidia that formed appressorium at the end of germ tube and those that did not were counted using a microscope (×100; CK40-F100; Oylmpus, Tokyo, Japan) in triplicate samples.

To determine effect of 12-MTA on hyphal growth of *M. oryzae* in liquid medium, conidial suspension of *M. oryzae* was prepared as described earlier. CM broth (yeast extract 6 g, casamino acid 6 g, sucrose 10 g per L) was inoculated with the conidial suspension and incubated at 22°C for 36 h with shaking. 12-MTA (60 μg/mL) was then added to the culture and incubated for additional 6 h. Mycelia were observed under a light microscope.

Mass production of *S. maltophilia*. Isolated bacterium was streaked on YMA (yeast extract 3 g, malt extract 3 g, peptone 5 g, dextrose 10 g, agar 15 g per liter) and incubated at 28°C, from which a single colony was picked to inoculate 5 mL of YM broth medium. The broth was scaled up to 100 mL, transferred to a 15-L Nalgene culture vessel with ports (Rochester, NY) containing 10 L of YMB (yeast extract 3 g, malt extract 3 g, peptone 5 g, dextrose 10 g per L), and incubated at 28°C with aeration at 1 L/min for 5 days. This culture medium was used for subsequent isolation of antifungal compounds.

Isolation and purification of compound suppressing appressorium formation. Equal volume of acetone was added to 15 L culture and kept overnight at 4°C. The volume of the mixture was reduced to 4 L on a rotary evaporator and then centrifuged to collect the supernatant, which was then extracted successively with three solvents: methylene chloride, ethyl acetate, and butanol. Each extract was evaporated and weighed before bioassay and purification.

Methylene chloride extract (530 mg) that exhibited activity against appressorium formation of *M. oryzae* was separated by medium pressure liquid chromatography (MPLC) on a Flash 40M silica column (40 mm×150 mm; Biotage, Charlottesville, VA) with solvent composed of 2% MeOH in chloroform at a flow rate of 15 mL/min. The active fractions were collected and further separated on Si gel preparative thin-layer chromatography (PTLC, 1 mm thickness, Merck, Darrnstadt, Germany) with 20% ethyl acetate in chloroform as the solvent.

Gas chromatography/electron impact mass spectrometry analysis. The active fraction from PTLC was methylated with diazomethane and analyzed by GC/MS consisting of HP 5890 series II system gas chromatograph (Hewlett Packard, Germany) and AX505WA mass spectrometer (JEOL). GC was operated using a DB 5 capillary column (30 m×0.25 mm×0.25 μm; Agilent, Santa Clara, CA) with He as the carrier gas. The injection volume was 1 μL, and the injection was in split-less mode. Separation condition was as follows: the inlet and

detector temperatures were 200 and 250°C, respectively, and the temperature program included an initial 2 min hold at 100 to 250°C (5°C/min) and a final raise (10°C/min) to a post-run temperature of 290°C for 5 min. Mass spectra were obtained at an ionizing energy of 70 eV.

Time course of metabolites production. Cells were harvested by centrifugation and washed with distilled water. Subsequently, 40 mg (wet mass) of the cells were placed in a test tube for saponification. One milliliter of methanolic NaOH (NaOH 45 g, MeOH 150 mL, ddH_2O 150 mL) was added and vortexed for 5-10 s. The sample was heated in boiling water (100°C) for 5 min, vortexed, and then reheated at 100°C for 30 min. After cooling to ambient temperature, the sample was added with 2 mL methanolic HCl (6 N HCl 925 mL, MeOH 275 mL) and warmed for 10 min at 80°C. At the end of heating, the tube was cooled rapidly in an ice-cold water bath, followed by the addition of 1.25 mL hexane, and tumbled gently for 10 min. After the aqueous phase was discarded, 3 mL of 0.25 M NaOH was added, and the tube was tumbled again for 5 min. The hexane layer was collected for analysis. One microliter of the hexane layer was injected onto a GC-2010 (Shimadzu, Japan) gas chromatograph equipped with a DB5 capillary column (30 m×0.25 μm×0.25 mm; Agilent, Santa Clara, CA). The inlet and detector temperatures were 200 and 300°C, respectively. The temperature program included an initial 5 min hold at 100°C, followed by a ramp to 250°C (5°C/min), and finally a post-run temperature of 290°C (10°C/min) for 10 min.

Chemical complementation of appressorium formation. To reveal possible sites of inhibition on the appressorium formation, chemical complementation tests were performed by the addition of known effector chemicals for appressorium formation of *M. oryzae* to the bioassay mixture. cAMP, 1,16-hexadecanediol, and 3-isobutyl-1-methylxanthine (IBMX) were added at final concentrations of 10 mM, 1 μM, and 2.5 mM, respectively [Oh and Lee, 2000].

Antimicrobial activity assay. The following microorganisms, obtained from the stock culture collection at the American Type Culture Collection (ATCC, Manassas, VA) and the Institute for Fermentation (Osaka, Japan), were used in the present study: *Staphylococcus epidermis* ATCC 12228, *S. aureus* ATCC 64389, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumeniae* ATCC 10031, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* ATCC 3851, *Candida albicans* ATCC 10231, *Aspergilus flavus* ATCC 28539, *Aspergilus furnigatus* HIC 6094, and *Penicillium citrium* IFO 6952. *Phytophthora capsici* and *Phythium ultimum* used in the present study were field strains. The antibacterial activity was determined by the two-fold microtiter broth dilution method [Kim and Oh, 2002]. Activities of the compound were compared with that of the antibacterial antibiotic ampicillin (Sigma). Antifungal activity test on yeast cell-like fungus was carried out by the macrobroth dilution method M27-T proposed by the National Committee on Clinical Laboratory Standards (NCCLS) [NCCLS, 1992]. The antifungal spectrum against filamentous fungi was determined by the macrobroth dilution method of Association of Official Analytical Chemist (AOAC) [1995]. Activities of the compound were compared with the antifungal antibiotic amphotericin B or metalaxyl (Sigma). The minimum inhibitory concentration (MIC) was taken as the concentration at which no growth was observed.

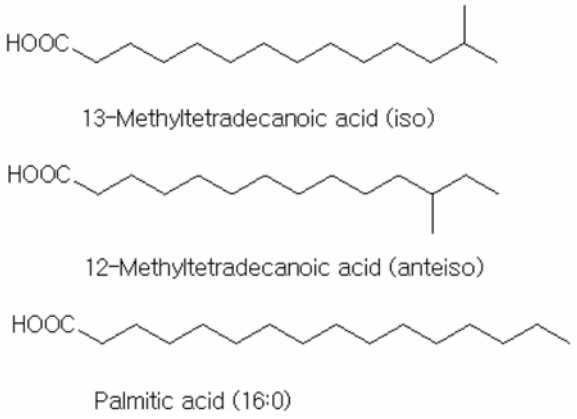

Fig. 1. Structures of three compounds from active fraction.

Results and Discussion

Identification of bacterial strain suppressing appressorium formation. One active strain was isolated from the bark of an *Acacia* hybrid. Its 16S rRNA was amplified and sequenced (GenBank accession no. HM143858). Blast search in Ribosomal Database (http://rdp.cme.msu.edu/seqmatch) revealed that the strain was most close to *Stenotrophomonas maltophilia* strain LMG 11002 [Denton and Kerr, 1998] with 99% identity and 99.5% homology. The strain was thus designated as *Stenotrophomonas maltophilia* AhsB4.

Identification and activity of active compound. Only the methylene chloride fraction had the activity against appressorium formation. Thus, the methylene chloride extract was further separated by MPLC and PTLC to yield about 50 mg of the active fraction. Inspection of the ^{1}H NMR spectrum of the active fraction indicated that it was a mixture of fatty acids. Analysis by GC/EIMS revealed the fraction was composed of three kinds of fatty acids: palmitic, 13-methytetraldecanoic (iso, 13-MTA),

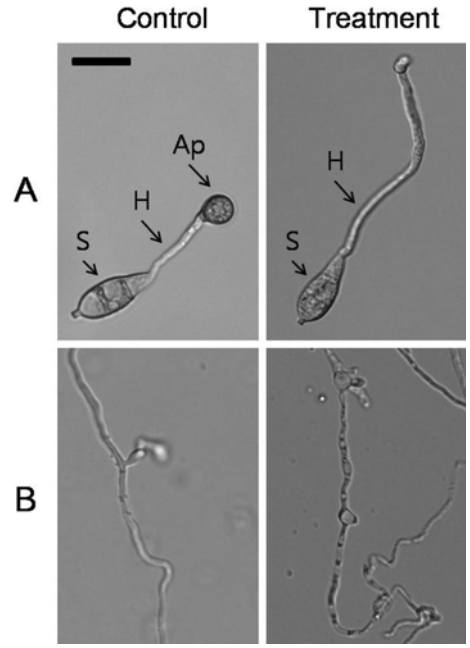

Fig. 2. Effects of 12-MTA on *M. oryzae*. Conidia of *M. oryzae* on hydrophobic surface (A), and mycelia growth in CM broth (B). 12-MTA was treated at 248 μM for the right panels. S, spore; H, hypha; Ap, appressoruim. Scale bar=20 μm.

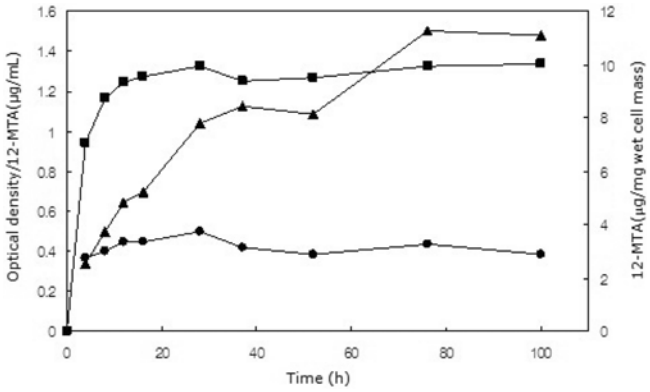

Fig. 3. Time course of 12-MTA production by *S. maltophilia* AhsB4. ■: growth (optical density); ▲: production of 12-MTA (μg/mL medium); ●: production of 12-MTA (μg/mg wet cell mass).

and 12-methytetraldecanoic (anteiso, 12-MTA) acids (Fig. S1-S3; Supplementary materials). Their Rt values and fragmentation pattern were identical to the authentic compounds purchased from Sigma (data not shown). These authentic samples were bioassayed for the activity. The result clearly indicated that the active principle in the fraction was 12-MTA with IC_{50} of 83.5 μM. 12-MTA completely inhibited the appressorium formation at 826 μM (Fig. 2A), and also had limited activity against spore germination with IC_{50} as high as 781 μM. Interestingly, treatment of the compound to the actively growing mycelia of *M. oryzae* had no effect on the elongation of mycelium, though swelling of mycelium at a certain point was observed (Fig. 2B). Therefore, it was evident that 12-MTA specifically inhibited appressorium formation with limited activity against germination of spores. 13-MTA had relatively low activity against appressorium formation with IC_{50} at 1.6 mM. In the present study, palmitic acid was shown to inhibit the appressorium formation by 18% at 200 ppm. 12-MTA did not have any activity against several human fungal and bacterial pathogens (Table S1, Supplementary materials).

Production of 12-MTA. Growth of the strain AhsB4 reached plateau in 20 h, and the productivity of 12-MTA lagged by 10 h (Fig. 3). Nevertheless, the specific productivity of 12-MTA remained relatively constant throughout the culture period. Maximal productivity of 12-MTA by the strain was 11 mg per L after 80 h of culture.

Anteiso 12-MTA and iso 13-MTA are normal constituents of the bacterial membrane [Minnikin *et al.*, 1979]. The proportion of anteiso- to iso-fatty acid depends on the culturing temperature favoring higher iso-fatty acid content at higher temperature [Chattopadhyay and Jagannadham, 2003]. A *Pseudomonas* species isolated from Antarctic soil had 90% 12-MTA when grown in a nutrient-rich medium at 5°C. At low environmental temperature, fluidity of the bacterial cell membranes decreases; thus, maintenance of optimum membrane fluidity by increasing the anteiso-fatty acid becomes crucial for survival [Suutari and Laakso, 1994]. Lowering of culture temperature is expected to increase the production of 12-MTA by the isolated strain.

Inhibition sites of 12-MTA. To reveal possible sites of inhibition by 12-MTA on the appressorium formation, chemical complementation tests were performed through the addition of known effectors for appressorium formation of *M. oryzae* including cAMP, 1,16-hexadecanediol, and IBMX. The exogenous addition of cAMP was reported to induce appressorium formation on non-inductive surfaces [Lee and Dean, 1993]. Subsequent studies identified and characterized the genes responsible for the signal transduction pathways involved in the appressorium formation in *M. oryzae*. Targeted disruption of the catalytic subunit gene (*cpkA*) of cAMP-dependent protein kinase and deletion of the adenylate cyclase gene (*MAC1*) effectively inhibited normal appressorium formation and function [Mitchell and Dean, 1995; Choi and Dean, 1997]. The presence of a soluble cutin monomer, 1,16-hexadecanediol, also induces appressorium formation on normally non-inductive surfaces [Gilbert *et al.*, 1996].

This molecule is suggested to initiate multiple signal transduction cascades that bring about terminal differentiation of the germ tube apex into an appressorium. IBMX is also an effector chemical acting through the inhibition of phosphodiesterase, an enzyme responsible for degradation of cAMP in the *M. oryzae* cells [Lee and Dean, 1993]. No significant restoration of appressorium formation was observed by the addition of these effectors (data not shown), indicating either 12-MTA influenced the down-stream of the points regulated by these effectors or acted through a separate pathway.

12-MTA is known to selectively inhibit mammalian 5-lipooxygenase to reduce the formation of 5-HETE, a precursor of leucotrienes and ultimately induce apoptosis, thus acting as an anticancer agent. The reduced 5-HETE production is at least partly responsible for the inhibition of cell proliferation in PC3 [Yang *et al.*, 2003]. 12-MTA is also an inhibitor of angiogenesis [Cole *et al.*, 2007]. It is therefore possible that a lipooxygenase could be involved in the action of 12-MTA against appressorium formation of *M. oryzae*. Eicosanoids including HETEs are known to be produced by fungus and play a role in phase change and differentiation [Noverr *et al.*, 2003]. Therefore, the present work suggests the possible inhibition of a lipooxygenase by 12-MTA in *M. oryzae*. Nevertheless, addition of 5-HETE could not negate the inhibition of appressorium differentiation induced by 12-MTA (data not shown). Further study with lipoxygenase products other than 5-HETE is thus necessary.

In conclusion, to the best of our knowledge, this is the first report on 12-MTA as an antifungal agent to inhibit appressorium formation of *M. oryzae*. Recently, inhibitors of isocitrate lyase such as bromophenols [Lee *et al.*, 2007] and 3-nitopropionate [Kim *et al.*, 2006] are known to suppress appressorium formation of *M. oryzae* with IC_{50} value ranging 2-125 mM and 100 μM, respectively. Since fatty acid in general is regarded as safe to the environment and thus should not pose toxicity problem to plants and animals, 12-MTA could be used as a safe agrochemical to control *M. oryzae*. Another merit of this compound is that it has no chemical functional group easily destroyed by environmental factors such as moisture, temperature, and sun light. The action mechanism of 12-MTA and the agicultural use in controlling *M. oryzae* should be further studied.

Supplemetary materials. Fig. S1. Mass spectra of methyl 13-MTA, methyl 12-MTA, and methyl palmitate eluted from GC; Table S1. Minimum inhibitory concentrations (MICs) of 12-MTA and standard antibiotics against various microorganisms.

References

AOAC (1995) In *Official Methods of Analysis of AOAC International*, Association of Official Analytical Chemists, (16th ed.). AOAC International, Arlington, TX, USA.

Bourett TM and Howard RJ (1990) In vitro development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Can J Bot* **68**, 329-342.

Chattopadhyay MK and Jagannadham MV (2003) A branched chain fatty acid promotes cold adaptation in bacteria. *J Biosci (Bangalore)* **28**, 363-364.

Choi W and Dean RA (1997) The adenylate cyclase gene *MAC1* of *Magnaporthe grisea* controls appressorium formation and other aspects of growth and development. *The Plant Cell Online* **9**, 1973-1983.

Cole N, Hume EBH, Jalbert I, Kumar Vijay A, Krishnan R, and Willcox MDP (2007) Effects of topical administration of 12-methyl tetradecanoic acid (12-MTA) on the development of corneal angiogenesis. *Angiogenesis* **10**, 47-54.

Couch BC and Kohn LM (2002) A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* **94**, 683-693.

Denton M and Kerr KG (1998) Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clin Microbiol Rev* **11**, 57-80.

Gilbert RD, Johnson AM, and Dean RA (1996) Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Physiol Mol Plant Pathol* **48**, 335-346.

Khush GS (1999) Green revolution: preparing for the 21st century. *Genome* **42**, 646-655.

Khush GS (2005) What it will take to feed 5.0 billion rice consumers in 2030. *Plant Mol Biol* **59**, 1-6.

Kim S and Oh KB (2002) Evaluation of antimicrobial activity of farnesoic acid derivatives. *J Microbiol Biotechnol* **12**, 1006-1009.

Kim SY, Park JS, and Oh KB (2006) Effects of isocitrate lyase inhibitors on spore germination and appressorium development in *Magnaporthe grisea*. *J Microbiol Biotechnol* **16**, 1158-1162.

Lange L, Breinholt J, Rasmussen FW, and Nielsen RI (1993) Microbial fungicides-the natural choice. *Pestic Sci* **39**,

Lee HS, Lee TH, Lee JH, Chae CS, Chung SC, Shin DS, Shin J, and Oh KB (2007) Inhibition of the pathogenicity of *Magnaporthe grisea* by bromophenols, isocitrate lyase inhibitors, from the red alga *Odonthalia corymbifera*. *J Agric Food Chem* **55**, 6923-6928.

Lee YH and Dean RA (1993) cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *The Plant Cell Online* **5**, 693-700.

Minnikin DE, Collins MD, and Goodfellow M (1979) Fatty acid and polar lipid composition in the classification of *Cellulomonas, Oerskovia* and related taxa. *J Appl Microbiol* **47**, 87-95.

Mitchell TK and Dean RA (1995) The cAMP-dependent

protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *The Plant Cell Online* **7**, 1869-1878.

NCCLS (1992) In *Proposed Standard*, National Committee for Clinical Laboratory Standards, (Edition number and publisher?) Villanova, PA, USA.

Noverr MC, Erb-Downward JR and Huffnagle GB (2003) Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev* **16**, 517-533.

Oh HS and Lee YH (2000) A target-site-specific screening system for antifungal compounds on appressorium formation in *Magnaporthe grisea*. *Phytopathology* **90**, 1162-1168.

Park SY, Milgroom MG, Han SS, Kang S, and Lee YH (2003) Diversity of pathotypes and DNA fingerprint haplotypes in populations of *Magnaporthe grisea* in Korea over two decades. *Phytopathology* **93**, 1378-1385.

RDA (2009) In *Crop Protection Report for 2008*, Rural Development Administration, Suwon, Korea.

Stierle A, Strobel G, and Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* **260**, 214-214.

Strobel G, Daisy B, Castillo U, and Harper J (2004) Natural products from endophytic microorganisms. *J Nat Prod* **67**, 257-268.

Suutari M and Laakso S (1994) Microbial fatty acids and thermal adaptation. *Crit Rev Microbiol* **20**, 285-328.

Yang P, Collin P, Madden T, Chan D, Sweeney-Gotsch B, McConkey D, and Newman RA (2003) Inhibition of proliferation of PC3 cells by the branched-chain fatty acid, 12-methyltetradecanoic acid, is associated with inhibition of 5-lipoxygenase. *The Prostate* **55**, 281-291.