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Isolation and Antifungal and Antioomycete Activities of Staurosporine from *Streptomyces roseoflavus* Strain LS-A24

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The actinomycete strain LS-A24 active against some plant fungal and oomycete pathogens was isolated from a soil sample of the Sunghwan Lake in Korea. The cell wall composition and spore shape of strain LS-A24 were LL-diaminopimelic acid and spiral type, respectively. On the basis of the physiological and biochemical characteristics and 16S ribosomal DNA sequence analysis, strain LS-A24 was identical to *Streptomyces roseoflavus*. An antifungal and antioomycete antibiotic was isolated from LS-A24 using various chromatographic procedures. The molecular formular of the antibiotic was determined to be $C_{28}H_{26}N_4O_3$, and on the basis of the NMR data, the antibiotic was confirmed to be staurosporine, 2,3,10,11,12,13-hexahydro-10*R*-methoxy-9*S*-methyl-11*R*-methylamino-9*S*,13*R*-epoxy-1H,9H-diindolo[1,2,3-gh:3′,2′,1′-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one. Staurosporine completely inhibited the mycelial growth of *Colletotrichum orbiculare*, *Phytophthora capsici*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Cladosporium cucumerinum* with minimum inhibitory concentration (MIC) values of 1−50 μg/mL for MICs. Staurosporine also was active against *Saccharomyces cerevisiae*, *Bacillus subtilis* ssp. *subtilis*, and *Xanthomonas vesicatoria*. Staurosporine and the commercial fungicide metalaxyl inhibited the development of *Phytophthora* blight on pepper plants. However, the control efficacy of staurosporine against the *Phytophthora* disease was somewhat less than that of metalaxyl. This is the first study to isolate staurosporine from *S. roseoflavus* and demonstrate its in vitro and in vivo antioomycete activity against *P. capsici*.

KEYWORDS: ***Streptomyces roseoflavus*; staurosporine; antifungal activity; antioomycete activity; *Phytophthora* blight**

INTRODUCTION

In modern pest management, there is a continuous need for new crop protection chemicals, because of the emergence of pesticide-resistant fungi, adverse effects of residual chemicals on environmental ecosystems, high application rates with many older compounds, and increasing regulatory requirements (*1*, *2*). The goal of the search for natural products is to identify novel, bioactive metabolites as new commercial products per se or as novel lead structures for chemical synthesis. The discovery of new natural products with novel modes of action is also valuable for effective control of plant diseases (*3*−*6*).

Microbial metabolites have sophisticated and widely varying structures. The complexity of their stuctures is often associated with highly specific modes of action, which may reflect a greater selectivity on application. A microbe-derived pesticide would be likely to show a high rate of degradation in the biosphere, thus leading to low residue levels. They are degraded usually within a month, or even a few days, when exposed to soil. Thus, microbial metabolites are thought to have several merits as pesticides (*7*, *8*). Fungicides of microbial origin introduced into practical application in fields during the last three decades include blasticidin S (*6*), polyoxin (*7*), kasugamyin (*11*), validamycin A (*12*), mildiomycin (*13*), pyrrolnitrin (*14*), fenpiclonil (*15*), oudemansin A (*16*), and strobilurin (*17*). There is a concern, however, that their application in the environment may cause natural resistance, rendering these antibiotics useless. Nevertheless, the search for useful antifungal products and analogues is very successful for the control of pathogenic fungi in agriculture. Recently, various antifungal compounds such as the manumycin type antibiotics (*18*) rhamnolipid B (*19*), phenazine-1-carboxylic acid (*20*), aerugine (*21*), and thiobutacin (*22*) were found to have potent antifungal activity for the control of plant fungal diseases.

In our previous study (*23*), we isolated a number of actinomycete strains from soils of various regions in Korea.

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During the screening procedure for actinomycete strains producing potent antifungal metabolites active against some plant pathogenic fungi, the actinomycete strain LS-A24 that showed a high level of antifungal activity was identified as *Streptomyces roseoflavus*. In the present study, a compound was isolated from the culture broth and its in vitro antimicrobial activity was evaluated against various plant pathogenic fungi and other microorganisms. The in vivo control efficacy against *Phytophthora* blight of pepper plants was evaluated and compared with that of the commercial fungicide metalaxyl under greenhouse conditions.

MATERIALS AND METHODS

Identification of Actinomycete Strain LS-A24. Actinomycete strain LS-A24 active against various plant pathogenic fungi was isolated from a soil sample of the Sunghwan Lake in Sunghwan, Korea (*23*). The microorganism was grown at 28 ± 1 °C on yeast−malt extract agar (4 g of yeast extract, 10 g of malt extract, 4 g of glucose, 18 g of agar, and 1 L of H_2O). For long-term maintenance, strain LS-A24 was preserved in yeast−malt extract broth containing 15% glycerol at −70 °C. The spores and mycelia of the strain LS-A24 were observed by scanning electron microscope (*24*). Diaminopimelic acid isomers were analyzed by the method of Becker et al. (*25*). Cultural and physiological characterizations of the strain LS-A24 were carried out by the method of the International *Streptomyces* Project (ISP) recommended by Shirling and Gottlieb (*24*). Physiological and biochemical characteristics of strain LS-A24 were also evaluated according to the methods of Williams et al. (*25*, *26*) and Bergey's Manual of Systematic Bacteriology (*27*). To examine the color of the spore mass of the strain LS-A24 on the starch/mineral salts agar medium [10 g of soluble starch, 1 g of NaCl, 2 g of $(NH_4)_2SO_4$, 2 g of $CaCO_3$, 1 g of K_2HPO_4, 20 g of agar, and 1 L of H_2O], spore masses were matched against the seven color wheels (*28*). For the determination of 16S ribosomal DNA (rDNA) sequences of the strain LS-A24, genomic DNA was isolated using a method of Pospiech and Neumann (*29*). Isolated genomic DNA (ca. 200 ng) was used for polymerase chain reaction (PCR) amplification of the 16S rRNA gene using universal primers fD1 (5′-AGAGT TTGAT CCTGG CTCAG-3′) and rP2 (5′-ACGGC TACCT TGTTA CGACTT-3′). The 16S rRNA gene was amplified by PCR in a reaction mixure containing 10× Taq polymerase buffer, primer fD1 and rP2 (each at a concentration of 0.125 μM), a mixture of deoxynucleoside triphosphates (each at a concentration of 0.125 μM), and 2.5 mM $MgCl_2$. The final volume of the PCR mixture was adjusted to 20 μL by adding distilled H_2O. Taq polymerase (0.4 U/μL) (Roche) was then added to the reaction solution. Thermal cycling was performed with GeneAmp PCR system 2400 (Perkin-Elmer, Boston, MA). The sample was subjected to an initial denaturing step consisting of 4 min at 95 °C. The thermal profile used was 35 cycles consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 58 °C, and 2 min of extension at 72 °C. A final extension step consisting of 8 min at 72 °C was included. Amplified DNA was detected by agarose gel electrophoresis and visualized by UV fluorescence after ethidium bromide staining. Amplified 16S rDNA was purified from 0.8% agarose gel using the method of Wu et al. (*30*). Purified rDNA was ligated into the pCR2.1-TOPO T vector (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instruction. Ligated plasmid was then transformed into *Escherichia coli* TOP10 cell (Invitrogen Co.) by electroporation (E = 12.5 kV/cm in 0.2 cm cuvettes). The transformed competent cells were smeared on LB−ampicillin agar treated with IPTG and X-gal. Transformants were selected on the basis of the results of the blue-white screening procedure (*31*). Plasmids containing 16S rDNA were isolated using Wizard plus SV Minipreps DNA Purification system (Promega Co., Madison, WI). These DNA genes were sequenced on AB1310 DNA sequencer (Applied Biosystems) using Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) with M13 forward and reverse and gene specific primers according to the manufacture's instruction. DNA sequence analysis was performed using BLAST network services at the NCBI and DNASTAR software program (*32*). The 16S rDNA sequence of the strain LS-A24 was aligned with reference sequences obtained from the database of GenBank (NCBI, Bethesda, Md.) using a clustal method (DNASTAR Inc., Madison, WI). Phylogenetic analyses were performed according to the neighbor-joining method (*33*) using Paup* version 4b10 (*34*).

Production and Isolation of the Antibiotic. Strain LS-A24 was precultured in 500 mL of yeast−malt extract broth in a 1 L Erlenmeyer flask on a rotary shaker at 150 rpm for 3 days at 28 °C. A 5 mL aliquot of the culture broth was inoculated into 500 mL of glycerol peptone broth (20 g of glycerol, 10 g of polypeptone, 5 g of beef extract, and 1 L of H_2O) (*35*) in a 1 L Erlenmeyer flask, which was optimal for production of antibiotic substances. The inoculated flasks were incubated for 12 days at 28 °C on a rotary shaker at 150 rpm. The total culture broth (100 L) was centrifuged at 4000*g* for 15 min. The culture filtrate was loaded on a 600 × 100 column packed with Diaion HP-20 resin (Mitsubishi Chemicals, Tokyo, Japan). The column was eluted with a stepwise gradient of methanol and water (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0, v/v). The antifungal activities of all fractions were examined against *Colletotrichum orbiculare*, *Phytophthora capsici*, and *Rhizoctonia solani* using a paper disk assay method. The fungi used in this study were provided by the department of plant pathology, National Institute of Agricultural Science and Technology, in Korea. The antifungal fractions were pooled, concentrated by a rotary evaporator, dissolved in water, and then extracted with ethyl acetate. The organic layer was concentrated in vacuo and loaded in a 200 mm × 100 mm open column packed with Dichroprep RP-18, 40−63 μm, C_{18} resin (Merck, Darmstadt, Germany). The column was eluted with a stepwise gradient of methanol and water (0:100, 20:80, 40:60, 60:40, 80:20, and 100:0, v/v). Each fraction (3 L) was concentrated in vacuo to a small volume. The active fractions from C_{18} column chromatography were combined and purified by preparative thin-layer chromatography (TLC) on 20 cm × 20 cm, 2 mm, $60F_{254}$ silica gel plates (Merck). The TLC plates were developed with chloroform−methanol (9:1, v/v) and then visualized under UV light at 254 nm. The antifungal band was scraped off, extracted with methanol, and filtered through Whatman paper #1. The concentrated methanolic extract was further purified by a 950 cm × 26 cm Sephadex LH-20 column chromatography (Pharmacia, Uppsala, Sweden). The column was eluted with methanol at a flow rate of 0.15 mL/min. The 2 mL fractions were collected using a fraction collector. The antifungal eluates from the gel filtration were purified by preparative high-performance liquid chromatography (HPLC) (Gilson, Middleton, WI) with a 300 mm × 7.8 mm i.d., 7 μm, Symmerty Prep C_{18} reverse phase column (Waters, Milford, MA). The chromatography was performed using a linear gradient, starting from 30% acetonitrile in water to 100% acetonitrile for 40 min at a flow rate of 2 mL/min. Detection was done at 250 nm by model 118 UV/vis detector (Gilson). Finally, the pure antibiotic (40 mg) was obtained as a yellow powder at a retention time of 27.37 min at 250 nm.

Structure Elucidation of the Antibiotic. The UV spectrum of the antibiotic was recorded on a Beckman DU 650 spectrometer (Beckman instruments, Inc., Fullerton, CA). ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, and distortionless enhancement by polarization transfer spectra were recorded on a Bruker AMX 500 NMR spectrometer (Bruker, Rheinstetten, Germany) using $CDCl_3$ with TMS as an internal standard. Two-dimensional NMR spectra, such as $^1H-^1H$ correlation spectroscopy, heteronuclear single quantum coherence, and heteronuclear correlations via multiple bond connectivities (HMBC), also were measured using the Bruker AMX 500 NMR spectrometer. The fast atom bombardment (FAB) mass spectrum was measured on a JEOL JMS-700 MS mass spectrometer (JEOL, Tokyo, Japan).

Detection of in Vitro Antimicrobial Activity. The bioassays for the minimum inhibitory concentration (MIC) to investigate the antimicrobial spectrum of the purified antibiotic were performed using plant pathogenic oomycetes and fungi, yeast, and bacteria (*36*). One milliliter of sterile potato dextrose broth (PDB) was pipetted into each well of a Cell Wells 24 well microtiter dish (Corning Glass Works, Corning, NY). Antibiotics dissolved in methanol were added into the microwells to be a range of 0−100 μg/mL. The concentration of methanol did not exceed 0.3% of the volume of bioassay mixture. Nutrient broth was used for bacteria and yeast. Zoospore suspensions of *P. capsici*, mycelial suspensions of *R. solani*, spore suspensions of *Alternaria mali*, *C.*

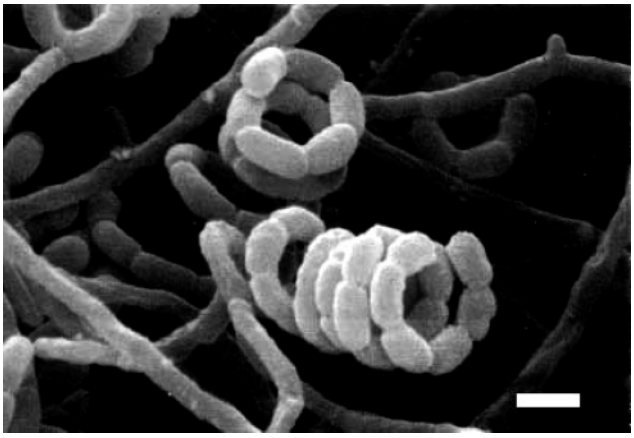

Figure 1. Scanning electron micrograph of spore morphology of actinomycete strain LS-A24 incubated for 7 days in inorganic salt starch agar. Bar = 1 μm.

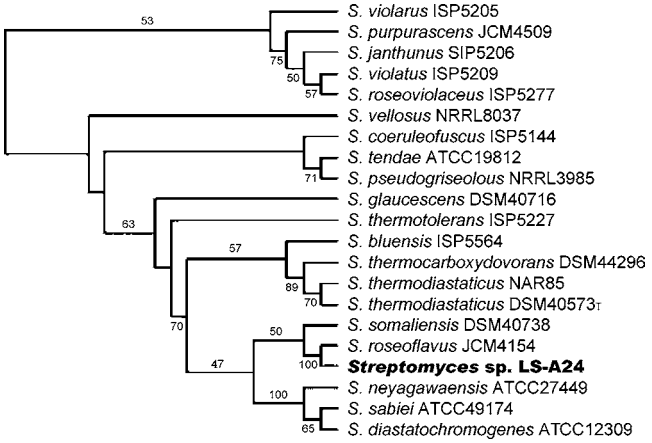

Figure 2. Phylogenetic localization of *Streptomyces* sp. LS-A24 based on 16S rDNA sequences of 30 *Streptomyces* strains. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the phylogenetic tree indicate the number of substitution events.

orbiculare, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Sclerotinia sclerotiorum* (*37*), *Didymella bryoniae*, *Fusarium oxysporum* f.sp. *lycopersici*, *Magnaporthe grisea*, and *Cylindrocarpon destructans*, and cell suspensions of *Candida albicans* and *Saccharomyces cerevisiae*, *Bacillus subtilis* ssp. *subtilis*, *Pactobacterium carotovorum*, *Ralstonia solanacearum*, and *Xanthomonas vesicatoria* were used as the inocula. The inoculum suspension (10^6-10^7 cell/10 μL) was added to each microwell. The MICs of antibiotics that completely inhibited mycelial and bacterial growth were evaluated after incubation for 4−7 days.

Evaluation of in Vivo Antioomycete Activity. The antioomycete activity of the antibiotic was evaluated against *Phytophthora* blight of pepper (*Capsicum annuum*). Pepper plants (cv. Hanbyul) were raised in a growth room at 28 ± 2 °C under 80 μmol photons/m^2/s illumination (fluorescent lamps) for 16 h each day. When the pepper plants were at the first branch stage, stems of the plants were sprayed with each of the antibiotics and the commercial fungicide metalaxyl. The antibiotics and metalaxyl dissolved in methanol were diluted with 0.05% Tween 20 to give the concentrations of 1, 10, 50, 100, and 500 μg/mL. The plants treated with each solution were wounded by making 1 cm slits approximately 1 cm above the soil surface. The zoospore suspension of *P. capsici* was prepared as previously described (*38*). The sterile cotton dipped in zoospore suspension (10^5 zoospores/mL) was placed on the wounded sites of stem. The inoculated sites were covered with plastic tape to maintain moisture. Disease severity of the *Phytophthora* blight was assessed daily after inoculation based on a scale 0−5: 0, no visible disease symptoms; 1, leaves slightly wilted with brownish lesions beginning to appear in stem; 2, 30−50% of entire plant diseased; 3, 51−70% of entire plant diseased; 4, 71−90% of entire plant diseased; and 5, plant dead.

Statistical Analysis. Statistical analysis was performed with the general linear mode procedure, and treatment means were separated by the least significant difference test at $P \leq 0.05$ with the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Identification of Actinomycete Strain LS-A24. The stereochemical form of the diaminopimelic acid in cell wall hydrolysates of strain LS-A24 was the L-form (data not shown). The strain LS-A24 was confirmed to belong to the group of cell wall type I, which includes the genera *Arachinia*, *Pemelobacter*, *Nocardioides*, *Intrasporangium*, *Kineospora*, *Streptomyces*, and *Streptoverticillium* (*27*). The spore surface ornamentation of the actinomycete strain LS-A24 was smooth. The spore chain of the spiral type also was observed under the scanning electron microscope (**Figure 1**). On the basis of the type of cell wall components and morphological characteristics examined by scanning electron microscopy, strain LS-A24 was confirmed to belong to the genus *Streptomyces*. Cultural characteristics of the strain LS-A24 were observed on various ISP media described by Shiring and Gottlieb (*24*). The color of vegetative mycelia was light brown to orange on ISP2 agar (data not shown). Aerial mycelia were pinkish white on ISP4 agar. Strain LS-A24 grew well on ISP3, ISP4, and ISP6 media. The reverse side colors were light brown to orange on ISP2, brownish orange on ISP3, light yellow on ISP4, and creamy on ISP5, ISP6, and ISP7 media. Strain LS-A24 did not produce any significant pigments in the tested media. The color of aerial mass was pinkish white on ISP4 medium. Melanin pigment was not produced on ISP6 or ISP7 media. Strain LS-A24 showed antibiosis against *B. subtilis* ssp. *subtilis* NCIMB 3610 and *Aspergillus niger* ATCC 26550. Positive results were found in pectin hydrolysis and nitrate reduction. Strain LS-A24 was able to degrade elastin, arbutin, starch, tyrosine, and esculin. No growth occurred in media containing each of 10% NaCl, 0.01% NaN_3, 0.1% phenol, and 0.01% thallous acetate. L-Valine and L-histidine were utilized as nitrogen sources. Strain LS-A24 was able to utilize xylose and arabinose as carbon sources but not sucrose, *meso*-inositol, mannitol, *r*-rhamnose, raffinose, D-melezitose, adonitol, D-melibiose, dextran, xylitol, and fructose (data not shown).

A nearly complete 16S rDNA sequence of 1487 nucleotides was determined for strain LS-A24 (EMBL/GenBank database under the accession number AF474177). Strain LS-A24 had a high percentage nucleotide similarity of the 16S rDNA sequences with *S. roseoflavus* JCM 4154 (99%) and *Streptomyces somaliensis* DSM 40738 (98%). The phylogenetic tree was constructed based on the percent difference in the genetic relationships between the allied strains of *Streptomycetes* (**Figure 2**). The physiological and biochemical characteristics and the 16S rDNA sequence analysis confirmed that the strain LS-A24 was identical to *S. roseoflavus*.

Structure Elucidation of the Antibiotic. The antibiotic in methanol showed UV absorption maxima at 240, 292, 334, 357, and 372 nm (data not shown). The molecular formula of the antibiotic was determined to be $C_{28}H_{26}N_4O_3$ $[M + H]^+$, *m*/*z* 467) by FAB mass analysis in positive mode, which showed peaks at *m*/*z* 467 $[M + H]^+$ and *m*/*z* 489 $[M + Na]^+$. On the basis of the spectral data and detailed HMBC interpretation, the structure of the antibiotic was confirmed to be identical to staurosporine (**Figure 3**), which was consistent with previous reports (*39*, *40*).

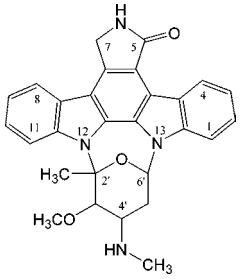

Figure 3. Structure of staurosporine isolated from *S. roseoflavus* strain LS-A24.

Table 1. MICs of Staurosporine from *S. roseoflavus* Strain LS-A24 against Various Microorganisms Including Plant Pathogenic Fungi and Oomycetes

test microorganism	MIC (μg/mL)[a]
P. capsici	1
R. solani	1
A. mali	>100[b]
C orbiculare	1
B. cinerea	50
C. cucumerinum	1
S. sclerotiorum	>100
D. bryoniae	10
F. oxysporum f.sp. *lycopersici*	50
M. grisea	>100
C. destructans	>100
C. albicans	>100
S. cerevisiae	1
B. subtilis ssp. *subtilis*	10
P. carotovorum	>100
R. solanacearum	>100
X. vesicatoria	50

[a] The lowest concentration that completely inhibits the growth of microorganisms was examined after incubation for 4–7 days. [b] The value >100 indicates that the growth of microorganisms was not inhibited at the concentration of 100 μg/mL.

Staurosporine, an alkaloidal antibiotic, was first isolated in 1977 from *Streptomyces staurosporeus* (*39*), which was transferred to *Saccarothrix aerocolonigene* ssp. *staurosporeus* (*41*). Other actinomycetes that produce staurosporine include *Streptomyces actuosus* (*42*) and *Streptomyces* species strain M-193 (*40*). *S. roseoflavus* produces antibacterial flavomycin (*43*), antiparasitic flavomycoin (*44*), and antifungal mycelin. However, this is the first report to demonstrate that staurosporine is produced by *S. roseoflavus*. Because the first discovery of staurosporine, the absolute structure elucidation of the antibiotic remained unclear for a long time (*45*). The relative structure of staurosporine was elucidated by an X-ray crystallographic study of its methanol solvate (*46*), and absolute configuration by X-ray analysis was accomplished by Funato et al. (*47*). Many derivatives of staurosporine also have important biological activities. Among them, the aglycone moiety of staurosporine was demonstrated to be important for biological activity (*48*).

In Vitro and in Vivo Antifungal and Antioomycete Activities of Staurosporine. The antimicrobial activity of staurosporine was evaluated by inhibition assay of growth of microorganisms on microtiter dishes (**Table 1**). Staurosporine did not inhibit the growth of *A. mali*, *C. destructans*, *M. grisea*, and *S. sclerotiorum* at the concentration of 100 μg/mL but showed activity against *C. orbiculare*, *P. capsici*, *R. solani*, and *C. cucumerinum*. The growth of *B. subtilis* ssp. *subtilis* and *X. vesicatoria* and *S. cerevisiae* was completely inhibited by staurosporine at the concentration of 1−50 μg/mL.

In vivo efficacy of staurosporine for the control of *Phytophthora* blight in pepper plants was evaluated under greenhouse conditions (**Figure 4**). Staurosporine and the commercial fungicide metalaxyl gradually inhibited the development of *Phytophthora* blight, as the treated concentrations of both compounds increased. The control efficacy of staurosporine against *P. capsici* infection was less than that of metalaxyl on pepper plants. The pepper plants treated with 100 μg/mL of metalaxyl did not show any disease symptoms. In contrast, treatment with 500 μg/mL of staurosporine was very effective in inhibiting the *Phytophthora* disease. Staurosporine did not show any phytotoxicity on pepper plants at a concentration of 500 μg/mL.

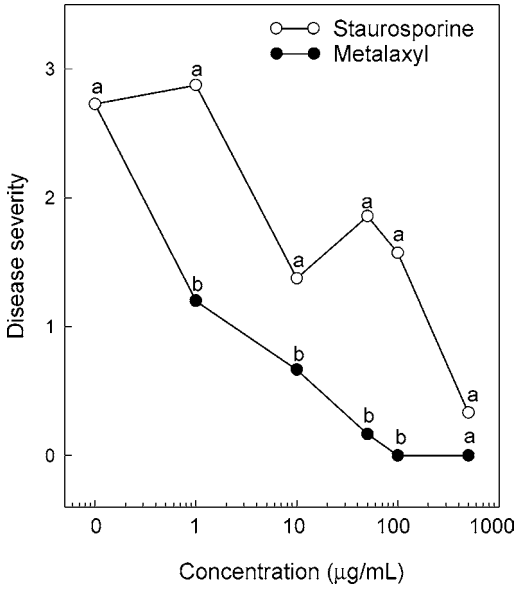

Figure 4. Inhibitory effects of staurosporine and the commercial fungicide metalaxyl at different concentrations on the disease development in pepper plants inoculated with *P. capsici* at the first branch stage. Disease severity was rated 5 days after inoculation based on a 0–5 scale, where 0 = no visible symptom and 5 = plant dead. Means at each concentration followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

Staurosporine is very well-known to be a potent platelet aggregation inhibitor (*40*) and a potent inhibitor of phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C) (*49*). Staurosporine inhibits other serine/threonine protein kinases and also protein tyrosine kinase (*50*, *51*). On the other hand, the antibiotic was earlier reported to show strong hypotensive activity as well as antimicrobial activity against fungi and yeast (*39*). In addition, staurosporine is useful for the inhibition of insect pests including cockroaches, beetles, lice, and ticks (*52*). Staurosporine was found to have a molluscicidal activity to *Oncomelania* snails and their eggs (*53*) and inhibit aldosterone biosynthesis in mice (*54*). However, there is little information about in vivo antioomycete activity of staurosporine against plant disease. To our knowledge, this is the first study to isolate staurosporine from *S. roseoflavus* and demonstrate its in vitro and in vivo efficacy against *Phytophthora* disease on pepper plants.

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