Biotechnology Letters **26:** 695–698, 2004.

New antifungal activity of penicillic acid against *Phytophthora* species

S.W. Kang[1] & S.W. Kim[2,*]
[1]*Graduate School of Biotechnology and* [2]*Department of Chemical and Biological Engineering, Korea University, 1, Anam-dong, Sungbuk-ku, Seoul 136-701, Korea*
[*]*Author for correspondence (Fax: +82-2-926-6102; E-mail: kimsw@korea.ac.kr)*

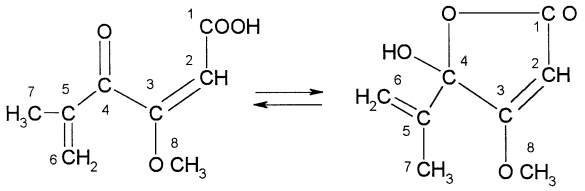
Received 5 December 2003; Revisions requested 23 December 2003; Revisions received 23 February 2004; Accepted 24 February 2004

Key words: Aspergillus sclerotiorum, morphological abnormality, penicillic acid, *Phytophthora* species

Abstract

Penicillic acid was isolated from a culture filtrate of *Aspergillus sclerotiorum*. It had a high *in vitro* antifungal activity against *Phytophthora* spp., which has not been previously reported. MICs of penicillic acid were from 1 to 25 μg ml^{-1} against *Phytophthora* spp. Penicillic acid induced abnormal branch formation, apical branching, and swelling in *P. capsici*, in *P. cactorum* mycelia contained irregular branching and small spherical swelling at apices, in *P. cambivora* there was irregular branching and swelling, and in *P. drechsleri* there was irregular multiple spherical swelling at or near hyphal apices.

Introduction



I

Penicillic acid (**I**) is a secondary metabolite of *Penicillium puberulum* Bainier. It possesses antibacterial activity against both Gram-positive and Gram-negative bacteria, and has a high herbicide activity against various field weeds (Madhyastha *et al.* 1994, Michito *et al.* 1996). It also inhibits the germination of corn seed, affects the germination of fungal spores, and affects the overall turnover of the metabolites in *Zea mays* (Keromnes & Thouvenot 1985, Deploey *et al.* 1996, Ahmad & Eqbal 2002).

In the course of screening for new antifungal agents against phytopathogenic fungi, *Aspergillus sclerotiorum* CGF was found to produce penicillic acid. This particular antifungal activity of penicillic acid has not been previously reported. In this paper, we suggest that because of this activity, penicillic acid has a potential for the biocontrol of *Phytophthora* disease.

Materials and methods

Fungal strains

Aspergillus sclerotiorum CGF was isolated from domestic soil sample and identified (S.W. Kang & S.W. Kim, unpublished work). *Phytophthora cactorum*, *P. cambivora*, *P. capsici*, *P. drechsleri*, *P. infestans* and *P. nicotianae* were from our laboratory stock and were used as the test microorganisms for bioassay.

Production and isolation of penicillic acid

Aspergillus sclerotiorum CGF was cultured on potato/dextrose/agar (PDA, 4 g potato infusion, 20 g dextrose, 15 g agar per liter of distilled water) plate at 28 °C. Potato/dextrose broth (PDB, 4 g potato starch, 20 g dextrose per liter of distilled water), 100 ml in a 500 ml Erlenmeyer flask, was inoculated with 10 agar pieces (5 × 5 mm) from a 12-d plate-culture and incubated at 28 °C for 11 d with shaking at 200 rpm. The culture was filtered through GF/C filter paper and the filtrate was adjusted to pH 4 and extracted with

ethyl acetate. Ethyl acetate extract was concentrated in *vacuo* and successively purified by silica gel column chromatography eluted with ethyl acetate/hexane (1:2, v/v) and then by silica gel column chromatography using chloroform/methanol (93:7, v/v). Active fractions were combined and concentrated in *vacuo*. Crystals were obtained by repeated recrystallization from ethyl acetate/hexane.

Spectral measurement

IR spectrum was measured using a KBr disc. NMR spectra were recorded on AMX500 NMR spectrometer (Bruker). ^{1}H and ^{13}C NMR spectra were measured in deuterated chloroform ($CDCl_3$) at room temperature. FAB-MS was recorded on JMS-700 mass spectrometer (JEOL).

Bioassay for minimum inhibitory concentrations (MICs)

A bioassay for the minimum inhibitory concentrations (MICs) of penicillic acid against *Phytophthora* spp. was performed by two methods.

Method I was a paper disc diffusion method on an overlaid agar plate. *Phytophthora* spp. were cultured on oatmeal agar (Difco, USA) at 24 °C for 7 d. Five agar pieces (0.5 × 0.5 mm) of fungi were inoculated in PDB and incubated at 24 °C for 5 d on a rotary shaker at 150 rpm. Culture broth was homogenized at 10 000 rpm at room temperature for 2 min and a homogenized broth was added to the molten potato/dextrose/agar and then 5 ml of seeded agar was overlaid on the PDA plate (87 × 15 mm). Paper discs loaded with the penicillic acid in methanol were dried in air and placed in the center of the overlaid agar plates and incubated at 24 °C for 5 d. The amounts of penicillic acid used were 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μg/disc. The lowest concentrations of penicillic acid where inhibition of fungi was observed were designated as minimum inhibitory concentrations (MICs).

Method II was as follows: molten PDA, homogenized culture broth and penicillic acid or Metalaxyl (1, 5, 10, 25, 50 μg ml^{-1}) in distilled water were placed in petri dishes (60 × 15 mm) and fungi were grown for 5 d. The lowest concentrations of antifungal agent where no growth of fungi was observed were evaluated as the MIC values.

Table 1 Minimum inhibitory concentrations of penicillic acid (PA) and Metalaxyl (MX) against *Phytophthora* spp.

Species	Bioassay I	Bioassay II	
	PA (μg/disc)	PA (μg ml^{-1})	MX (μg ml^{-1})
P. cactorum	15	10	<1[a]
P. cambivora	10	10	>50[b]
P. capsici	35	25	1
P. drechsleri	15	10	>50
P. infestans	<5	<1	>50
P. nicotianae	5	5	<1

[a] < Represents that the growth of microorganism was inhibited at the lowest concentration tested.
[b] > Represents that the growth of microorganism was not inhibited at the highest concentration tested.

Observation of morphological changes of Phytophthora *spp.*

A small agar piece of each *Phytophthora* strain was placed on the slide glass having the strip of PDA and incubated at 24 °C. After 2 d, a paper disc (50 μg/disc) soaked with penicillic acid in methanol was deposited at 3 cm apart from the growth of agar piece. Morphological changes of the *Phytophthora* spp. were observed under the light microscope.

Results and discussion

The antifungal activity of *Aspergillus sclerotiorum* CGF culture filtrate was evaluated. Growth of *Phytophthora* spp., amongst various phytopathogenic fungi that were tested, was inhibited. The growth of *Phytophthora* spp. was greatly inhibited, with the diameter of inhibition zone ranging from 41 to 68 mm, using the paper disc diffusion method. The inhibition of the germination of *P. capsici* zoospore has also been observed (S.W. Kang & S.W. Kim, unpublished work).

The unknown compound was isolated from culture filtrates and successively purified by using silica gel column chromatography followed by crystallization to give white needles from ethyl acetate-hexane. The FAB-MS spectrum showed a molecular ion peak at m/z 171.1 ($M+H^+$). The purified compound showed identical IR spectra as authentic penicillic acid; OH at 3278 cm^{-1}, C=O at 1742 cm^{-1} and C=C at 1641 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.75 (3H, s, $-CH_3$), 3.9 (3H, s, $-OCH_3$), 5.13 (H, s, $=CH-$), 5.17 and 5.47 (2H, s, $=CH_2$), 5.74 (H, s, $-OH$). ^{13}C NMR ($CDCl_3$) δ 17.54 (C-7), 60.14 (C-8), 89.54 (C-2),

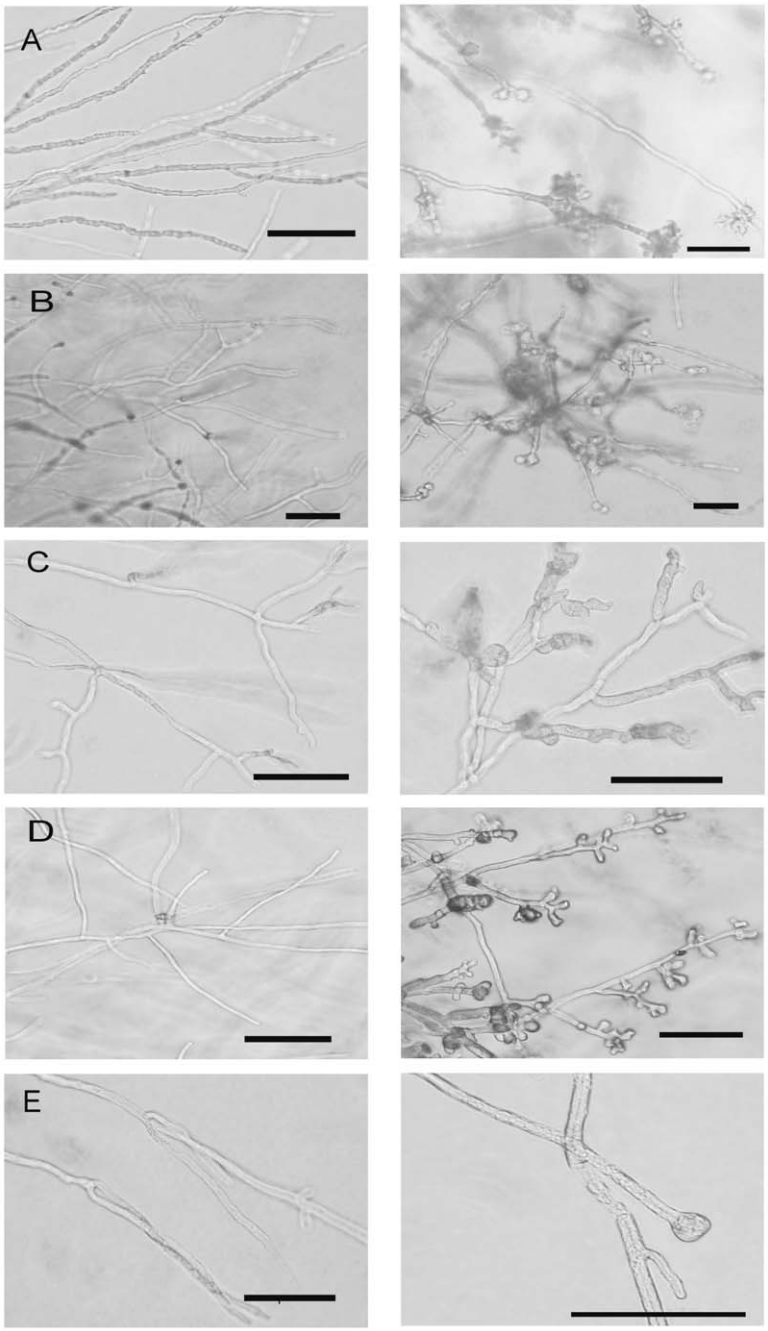


Fig. 1. Fungal morphological abnormalities induced by penicillic acid against *Phytophthora* spp. (A) *P. drechsleri*, (B) *P. cactorum*, (C) *P. cambivora*, (D) *P. capsici*, (E) *P. infestans*. Left: control, right: treatment with penicillic acid. Bar represents 20 μm.

103.56 (C-4), 116.74 (C-6), 139.66 (C-5), 172.08 (C-1), 179.67 (C-3). IR, ^{1}H and ^{13}C NMR data revealed that it was identical to penicillic acid. These results agree with reported data on mass spectrometry, nuclear magnetic resonance and infrared spectra of penicillic acid (Yeh *et al.* 1978, Pohland *et al.* 1982).

The antifungal activity of penicillic acid was evaluated by measuring mycelial growth inhibition using the paper disc diffusion method (I). Minimum inhib-

itory concentrations (MICs) of penicillic acid against *Phytophthora* spp. were from 5 to 35 μg/disc. Penicillic acid inhibited the growth of *P. infestans* at 5 μg/disc (Table 1).

A second method, Bioassay II, was used to compare the effectiveness against *Phytophthora* spp of penicillic acid and the commercial fungicide, Metalaxyl. The MICs of penicillic acid were lower than those of Metalaxyl on *P. cambivora*, *P. drechsleri* and *P. infestans*. The inhibition of the growth of *P. infestans*, which causes late blight of potatoes, was at 1 μg ml^{-1}. In the case of Metalaxyl, mycelial growth of *P. infestans*, *P. cambivora* and *P. drescheri* were not inhibited up to 50 μg ml^{-1}. Metalaxyl was more effective against *P. cactorum*, *P. capsici* and *P. nicotianae*. These results indicated that penicillic acid has a potential for the biocontrol of *Phytophthora* infections.

Activities of various antifungal antibiotics can induce morphological abnormalities of fungi including hyphal curing, swelling and branch formation (Gunji *et al.* 1983). Morphological abnormalities of *Phytophthora* spp. were induced by penicillic acid (Figure 1): it caused abnormal branch formation, apical branching and swelling of growing hyphae, especially in *P. capsici*. Yoon & Chol (1998) reported abnormal branching and swelling of *P. capsici*. Mycelia of *P. cactorum* contained irregular branching and small spherical swelling at apices. Irregular branching and swelling in *P. cambivora* and irregular multiple spherical swelling at or near hyphal apices in *P. drechsleri* occurred. Penicillic acid only had a slight effect, spherical swelling at apices, on the morphology of *P. infestans*. We think that this might be related with a relative lower growth of *P. infestans* than other *Phytophthora* spp. (Jee *et al.* 2000).

Conclusions

Penicillic acid was isolated from *A. sclerotiorum* CGF culture filtrate and its structure was confirmed by FAB-MS, IR, ^{1}H and ^{13}C NMR spectral data. MICs of penicillic acid were from 1 to 25 μg ml^{-1} against *Phytophthora* spp. and, when compared with those of a commercial fungicide, Metalaxyl, it shows sufficient potential to be applied to the biocontrol of *Phytophthora* disease. Morphological abnormalities of *Phytophthora* spp., such as the formation of branching and swelling of growing hyphae, were induced by penicillic acid. To verify penicillic acid as a biocontrol agent, further antifungal studies should be performed in an *in vivo* field test.

Acknowledgement

This study was supported by a Korea University Grant.

References

Ahmad A, Eqbal MM (2002) Effect of penicillic acid on some biochemical changes in germinating maize seeds. *GEOBIOS* **29**: 161–163.

Deploey JJ, O'Rear CE, Llewellyn GC (1996) Some fungal toxins can affect the percent germination and germ tube elongation of *Rhizomucor miehei* sporangiospores. *J. Pennsylvania Acad. Sci.* **70**: 80–82.

Gunji S, Arima K, Beppu T (1983) Screening of antifungal antibiotics according to activities inducing morphological abnormalities. *Agric. Biol. Chem.* **47**: 2061–2069.

Jee HJ, Cho WD, Kim CH (2000) *Phytophthora* diseases in Korea. National Institute of Agricultural Science and Technology (NIAST), RDA, Korea. Government Publication Registration Number: 11-1390093-000063-01.

Keromnes J, Thouvenot D (1985) Role of penicillic acid in the phytotoxicity of *Penicillium cyclopium* and *P. canescens* to the germination of corn seeds. *Appl. Environ. Microbiol.* **49**: 660–663.

Madhyastha MS, Marquardt RR, Masi A, Borsa J, Frohlich AA (1994) Comparison of toxicity of different mycotoxins to several species of bacteria and yeasts: use of *Bacillus brevis* in a disc diffusion assay. *J. Food Protect.* **57**: 48–53.

Michito T, Yoichi I, Hisae M (1996) Herbicide. Japan Patent 08-053310.

Pohland AE, Schuller PL, Steyn PS, Van Egmond HP (1982) Physicochemical data for selected mycotoxin. *Pure Appl. Chem.* **54**: 2220–2284.

Yeh CL, Colwell WT, DeGraw JI (1978) A convenient synthesis of penicillic acid. *Tetrahedron Lett.* **42**: 3987–3988.

Yoon SH, Chol C (1998) Tn5 *lac* mediated mutagenesis of *Enterobactor* sp. B54 antagonistic to *Phytophthora capsici*. *Korean J. Appl. Microbiol. Biotechnol.* **26**: 393–399.