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Biological Characterization of Periconicins, Bioactive Secondary Metabolites, Produced by *Periconia* sp. OBW-15

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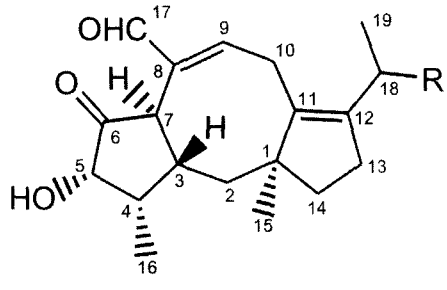
Abstract Periconicin A and B, two new fusicoccane diterpenes originally isolated from the cultures of endophytic fungus *Periconia* sp. OBW-15, were tested by several biological assays. Periconicin A was consistently more active than periconicin B. In an antifungal activity assay, periconicin A showed potent inhibitory activity against the agents of human mycoses, including *Candida albicans*, *Trichophyton mentagrophytes*, and *T. rubrum*, with minimum inhibitory concentration (MIC) in the range of 3.12–6.25 μg/ml. In a plant growth regulatory activity assay, periconicins inhibited hypocotyl elongation and root growth of *Brassica campestris* L. and *Raphanus sativus* L. At concentrations below 1 μg/ml, however, both compounds accelerated root growth by 110–135%. From these results, it is apparent that a methyl group positioned in a cyclopentane ring may play an important role in plant and fungal growth inhibitory activity.

Key words: *Periconia* sp., endophyte, periconicin, antifungal activity, plant growth regulatory activity

Fusicoccanes are significant biosynthetic plant growth regulators [14]. The fusicoccane-type compounds, which are based on a dicyclopenta[a,d]cyclooctane ring system, have often been described among di- and sesterterpenes of fungal origin (fusicoccin, ophiobolin, cotylenin) [2, 6, 17, 22]. Such substances have been found in fungi, lower and higher plants, and insects [14]. Fusicoccanes bear structural resemblance to gibberellins [15]. Fusicoccin, the first member of this group reported from the phytopathogenic fungus *Fusicoccum amygdali* Del. [3], was found to stimulate plant growth through elongation mechanism, promotion of opening of leaf stomata, acceleration of seed germination, and induction of root formation [14].

Endophytic fungi are prodigious producers of biologically active natural products [4, 8, 9, 12, 13, 18, 19]. Since more than 1.5×10^6 endophytic fungi are now thought to live inside the estimated 270,000 species of vascular plants, the prospects for additional discoveries of fungal metabolites are bright [5, 7, 10]. Periconicins A and B, new fusicoccane diterpenes, were originally isolated from the cultures of endophytic fungus *Periconia* sp. OBW-15 [11], which was collected from small branches of *Taxus cuspidata*, by using the growth inhibition of various bacteria as a bioassay to guide the isolation process. These compounds have the same carbon skeleton as the fusicoccins, cotylenins, and ophiobolins [2, 7, 14, 17]. It is intriguing to note that periconicins have the *trans* relative stereochemistry between the C-1 methyl group and C-3 hydrogen (Fig. 1), while all other fusicoccanes have the *cis* relationship. Periconicins



R= CH_3, periconicin A

R= CH_2OH, periconicin B

Fig. 1. Chemical structures of periconicins A and B.

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exhibited significant antibacterial activity against Gram-positive and Gram-negative bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* [11]. However, a more in-depth investigation of their biological properties seems to be desirable. Herein, we report a wider biological evaluation of the antifungal activities of periconicins A and B toward filamentous fungi and yeasts, as well as their potential as plant growth regulators.

Periconia sp. OBW-15 [11] was grown on a PDA plate as an inoculum for 7 days at 25°C. Ten pieces of 5×5 mm blocks of the well-grown culture were inoculated into 500 ml of liquid S-7 medium [18] in a 3-l narrow-mouth prescription bottle. The fungus was grown by stationary culture at 25°C for 21 days. The ethylacetate (EtOAc) extracts, which were active in antibacterial assays, of large-scale (15 l) fermentations grown in S-7 medium were prepared, according to the modified Kupchan scheme [20], and subjected to open-column chromatography over silica gel with solvent A (7:6:1 $CHCl_3$:Hexane:MeOH). Chromatographic fractions with antimicrobial activities were pooled to yield two different fractions, each containing periconicins A and B, respectively. The active fractions were then subjected to preparative thin-layer chromatography (TLC) on a Merck (RP-18 F254S) 1 mm ODS silica gel plate and developed in solvent B (2:1:1 CH_3CN:H_2O:MeOH). After the separation in solvent B, the area of the plate containing active compound was carefully removed by scraping off the silica at the appropriate R_f regions (periconicin A, 0.32; periconicin B, 0.67; solvent B) and exhaustively extracted with acetonitrile. After solvent evaporation, white solid residues were crystallized from EtOAc to afford periconicins A and B.

The antifungal activity test for periconicins A and B on yeast cells was carried out by the macrobroth dilution method M27-P, proposed by the National Committee for Clinical Laboratory Standards (NCCLS) [16]. Briefly, 0.3 ml of serial two-fold dilutions of the test compounds in dimethyl sulfoxide (DMSO) was mixed with 2.7 ml of RPMI 1640 broth (0.165 M MOPS buffer at pH 7.0) (Sigma, St. Louis, MO, U.S.A.), containing fresh inoculum of 10^3 cells/ml. The assay tubes were incubated at 35°C for 3 days, the range of concentrations tested being 0.1 μg/ml to 100 μg/ml. The antifungal spectrum against filamentous fungi was determined by the macrobroth dilution method of Association of Official Analytical Chemists (AOAC) [1]. YM (1.0% glucose, 0.5% polypeptone, 0.3% yeast extract, and 0.3% malt extract) broth (0.165 M MOPS buffer at pH 7.0) was used as the antifungal assay medium. A spore suspension was collected with 0.1% Tween-80 solution from potato dextrose agar plates that had been incubated at 28°C for 2 weeks. Spores were washed three times with sterile distilled water and resuspended in YM broth to obtain an initial inoculum size of 10^5 spores/ml. Activities of the two compounds were compared with the antifungal antibiotic nystatin. The minimum inhibitory concentration (MIC) was taken as the concentration at which no growth was observed after incubation for 4 days at 28°C.

The plant growth regulatory activity of periconicins A and B was investigated by using two kinds of plants. Seeds of *Brassica campestris* L. ssp. *napus* Hook. fil. et Anders var. *pekinensis* Makino and *Raphanus sativus* L. var. *acanthiformis* Makino were sterilized with 10% sodium hypochloride (v/v) for 10 min, washed with 70% ethanol, and rinsed with four changes of sterile distilled water. Sterile seeds were plated onto 1% agar solid medium, containing 0.1% Murashige-Skoog (MS) medium (Dainippon Pharmaceutical, Tokyo) in the culture vessel ($1^W \times 1^D \times 5^H$ cm, glass) supplemented with or without periconicins. All

Table 1. Antifungal activity of periconicins A and B.

Microorganism[b]	MIC (μg/ml)[a]		
	Periconicin A	Periconicin B	Nystatin
Alternaria alternata ATCC 58868	>200	>200	3.12
Aspergillus flavus ATCC 28539	50	>200	3.12
Aspergillus fumigatus HIC 6094	100	>200	25
Aspergillus niger ATCC 9643	50	>200	3.12
Candida albicans ATCC 10231	6.25	>200	3.12
Candida albicans IFO 1594	6.25	>200	3.12
Cladosporium cladosporioides IFO 6348	50	>200	12.5
Fusarium oxysporum HIC 5651	200	>200	50
Fusarium solani HIC 5670	200	>200	50
Penicillium citrinum IFO 6952	200	>200	50
Trichophyton rubrum IFO 6204	3.12	>200	0.78
Trichophyton mentagrophytes IFO 40769	6.25	>200	1.56

[a]MIC was defined as the lowest concentration of compound that completely inhibited the growth of the organism, compared with a control plate containing no compound.

[b]ATCC: American Type Culture Collection; IFO: Institute for Fermentation, Osaka, Japan; HIC: National Institute of Health Sciences, Tokyo, Japan.

of the test solutions, unless otherwise mentioned, contained 1% (v/v) DMSO. Culture vessels were kept vertically in a controlled-environment growth chamber (25°C) with 16 h light (3,000 lux) and 8 h dark for 3 days. The lengths of the hypocotyls and roots of the seedlings after treatment were measured and compared with those of untreated controls.

The antifungal activities of periconicins A and B were tested on various species of phytopathogenic fungi, and medically important filamentous fungi and yeasts (Table 1). A widely varied susceptibilities to the periconicin A was observed, therefore, its activities seemed to be species-specific. Among the microorganisms that were examined, the agents of human mycoses (*Candida* and *Trichophyton*) were the most sensitive to periconicin A: compared to nystatin, periconicin A exhibited potent growth inhibitory activity against *Candida albicans*, *Trichophyton mentagrophytes*, and *T. rubrum* with minimum inhibitory concentration (MIC) in the range of 3.12-6.25 μg/ml (Table 1). Interestingly, however, periconicin B was not active against all the microorganisms, even at the highest concentration tested.

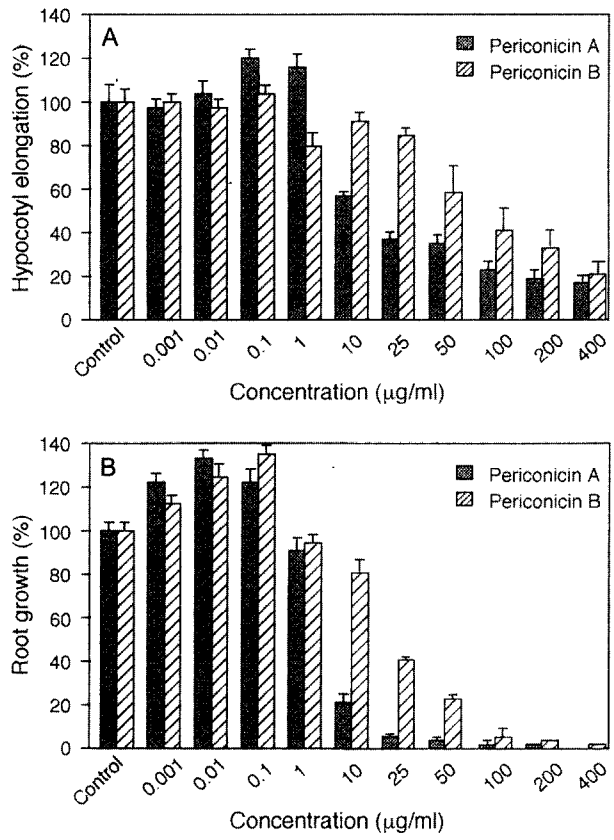

Fig. 2. Effect of periconicins on the hypocotyl elongation (A) and root growth (B) of *Brassica campestris* L. seedlings.
Growth (length in hypocotyls or root) in the negative control experiments (final 1% DMSO) after 3 days was taken as 100%. Each value represents mean±S.D. (N=15) from three separate experiments.

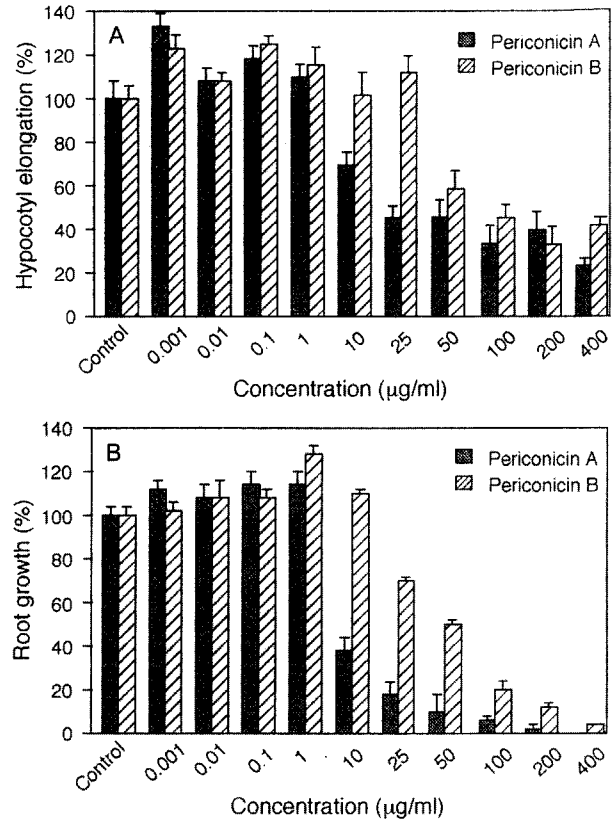

Fig. 3. Effect of periconicins on the hypocotyl elongation (A) and root growth (B) of *Raphanus sativus* L. seedlings.
Growth (length in hypocotyls or root) in the negative control experiments (final 1% DMSO) after 3 days was taken as 100%. Each value represents mean±S.D. (N=15) from three separate experiments.

The plant growth regulatory activity assay showed that periconicin A inhibited hypocotyl elongation and root growth of *Brassica campestris* L. seedlings by 64% and 95% at 25 μg/ml concentration, respectively, and completely inhibited root growth at 400 μg/ml concentration (Fig. 2). The inhibitory activity of periconicin B against hypocotyl elongation and root growth was somewhat less, when compared with periconicin A. Interestingly, both compounds at concentrations below 1 μg/ml accelerated root growth by 110-135%. The effects of periconicins on the hypocotyl elongation and root growth of *R. sativus* L. were also investigated, and similar growth regulatory activities were obtained (Fig. 3). Although periconicin B did not completely inhibit the growth of *Brassica campestris* L. and *R. sativus* L., it showed significantly strong growth inhibitory activity on these plants at the same concentration. The data from the present study indicate that a methyl group at the R position in a cyclopentane ring (Fig. 1) might play an important role in plant and fungal growth inhibitory activity.

In the present experiments, we have observed that plant root growth was very sensitive to periconicins (Figs. 2B

and 3B). At low periconicin concentrations, root growth stimulatory effects were observed. At high concentrations, however, the root elongation was markedly inhibited, and finally stopped. It has been reported that the biological actions of fusicoccanes are diverse. For example, ophiobolins, which have the same carbon skeleton as the periconicins, show a broad spectrum of biological activity against bacteria, fungi, nematodes, and cancer-cell lines [2]. In addition, some ophiobolins could reduce the growth of roots and coleoptiles of wheat seedlings, reduce seed germination, change cell membrane permeability, and stimulate leakage of electrolytes and glucose from roots [2, 21]. Based on these early studies, our present observations could possibly be explained by the fact that the growth stimulation and inhibition of plant root should reflect the disturbance of plasma membrane architecture.

In conclusion, the biological activities of periconicins toward filamentous fungi and yeasts, as well as their potential as plant growth regulators were investigated. These compounds exhibited a broad spectrum of inhibitory activity against bacteria [11], fungi, and plant growth. Our data indicate that periconicins are more potent growth inhibitors toward plants than toward phytopathogenic fungi. At the same concentrations, a higher inhibition was observed on root elongation than on hypocotyl elongation, and it was concluded that these compounds have more than one target site. The potent plant root growth inhibitory activity suggests that periconicins may provide promising lead chemistries that could be optimized by chemical syntheses to provide agriculturally useful compounds.

Acknowledgments

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