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CHARACTERIZATION OF ANTIFUNGAL METABOLITES PRODUCED BY *Penicillium* SPECIES ISOLATED FROM SEEDS OF *Picea glehnii*

K. YAMAJI,[1,2] Y. FUKUSHI,[1,2] Y. HASHIDOKO,[1,2]
T. YOSHIDA,[3] and S. TAHARA[1,2,*]

[1]*Department of Applied Bioscience*
Faculty of Agriculture, Hokkaido University
Kita-ku, Sapporo 060-8589, Japan
[2]*CREST, Japan Science and Technology Corporation*
Honmachi 4-1-8, Kawaguchi 332-0012, Japan
[3]*Department of Food and Nutrition*
Hokkaido Bunkyou Junior College
Kogane-cho 196, Eniwa 061-1408, Japan

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Abstract—We screened the microorganisms that are present on the surface of *Picea glehnii* seeds and produced antifungal compounds against *Pythium vexans*, a fungus that causes damping-off. Four isolates of *Penicillium* species that produced patulin, citrinin, palitantin, and arthrographol, respectively, were identified from 149 different microorganisms screened. This study is the first step in an examination of the ecological interaction between host conifers and fungi located on the surface of their seeds.

Key Words—*Picea glehnii*, conifer seedling, root protection, *Pythium vexans*, associated fungi, *Penicillium* spp., patulin, citrinin, palitantin, arthrographol.

INTRODUCTION

Picea glehnii (Fr. Schm.) Masters is one of the most important needle-leafed trees in Hokkaido, Japan, especially in the northern and eastern portions of the serpentine district, in volcanic sand and gravel areas, and in bogs (Tatewaki, 1958). Conifer seedlings are often threatened in the early stages of growth by damping-off fungi, including *Pythium*, *Fusarium* and *Rhizoctonia* species. This disease sometimes also causes a serious problem in tree nurseries (Ito, 1955).

*To whom correspondence should be addressed.

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Once the roots of seedlings are associated with ectomycorrhizal fungi, host plant defenses are enhanced against the fungi that cause damping-off. Many researchers have investigated the defensive mechanisms associated with ectomycorrhizal fungi. The production of antifungal compounds by either the mycosymbiont or the host plant, a barrier effect of the fungal sheath around roots, and nutrient competition between the ectomycorrhizal and pathogenic fungi have all been discussed as possible mechanisms (Zak, 1964; Marx, 1972). A series of experiments that inoculated seedlings with ectomycorrhizal fungi showed that it took a long time to form ectomycorrhizae around the roots of seedlings under nursery conditions. For example, in Kasuya's (1995) experiment, ectomycorrhizae formation was still incomplete 15 months after 2-month-old seedlings of *Pi. glehnii* grown in sterilized soil were inoculated with the ectomycorrhizal fungi, *Pisolitus tinctorius* and *Scleroderma flavidum*. Therefore, it is unlikely that the fungal sheath forms a physical barrier around roots, so protecting young seedlings from pathogenic attacks.

Other defense systems are reported to function in roots inoculated with ectomycorrhizal fungi prior to ectomycorrhiza formation (Stack and Sinclair, 1975; Chakravarty and Unestam, 1987). When sterilized *Pinus resinosa* seedlings growing in test tubes containing a nutrient solution were inoculated with *Paxillus involutus*, followed by an inoculation with *Fusarium oxysporum* 24 hr later, the mortality of the seedlings was significantly reduced, compared with the control, after two weeks (Duchesne and Peterson, 1988; Duchesne et al., 1988). Furthermore, Duchesne et al. (1989) isolated an antifungal compound from a filtrate of the mycosymbiont and identified it as oxalic acid.

It is believed that plants have evolved many chemical defenses against phytopathogens (Ingham, 1973). Therefore, it is possible that several defense systems operate in *Pi. glehnii* seedlings in association with ectomycorrhizae formation, including the production of antifungal compounds by either the mycosymbiont or host plant. We postulated that germinating seeds and seedlings of *Pi. glehnii* might be protected from pathogens in the soil by epiphytic microorganisms that attach to the seed coat and produce antimicrobial compounds. To test this idea, we screened the microorganisms on the surface of seeds to look for those able to produce antimicrobial compounds against the damping-off fungus, *Pythium vexans*. In this paper, we present the results of the screening test. The antifungal compounds produced by the fungi are identified and their roles in the rhizospheric ecosystem are discussed briefly.

METHODS AND MATERIALS

General

Potato–dextrose broth was purchased from Difco and potato–dextrose agar (PDA) medium with 2% agar was prepared in the usual way.

Spectroscopic analyses of the isolated compounds were conducted using the following apparatus. ^{1}H and ^{13}C NMR spectra were recorded on Jeol EX 270 and Bruker AMX500 spectrometers, respectively. 2D NMR (H-H COSY, HMQC, HMBC, and NOESY) to elucidate carbon sequences of the antibiotics were recorded on the Bruker AMX500. The solvent used was $CDCl_3$ and chemical shifts are reported relative to the TMS (^{1}H) and solvent peaks (δ = 77.0 ppm; ^{13}C). EI-MS and EI-HR-MS spectra to determine their molecular weights and formulas were recorded on the Jeol DX 500 spectrometer and FD-MS on a Jeol JMS-SX102A. UV and IR spectra to give information about conjugation systems and functional groups of the compounds were recorded on Hitachi model U-3210 and model 285 spectrometers, respectively. The melting points were determined on a Yanako MP-30 micro-melting point apparatus and are uncorrected. The optical rotations for asymmetric compounds were recorded on a Jasco DIP-370.

Screening Test of Microorganisms Producing Antifungal Compounds

Seeds. Seeds of *Pi. glehnii* were collected at Shirataki, Shiranuka, Oketo, and Teshio in Hokkaido. The seeds were harvested at the first two locations in 1995, from Oketo in 1989, and from Teshio in 1985.

Screening Test. The agar-on-paper method (Shimazu, 1986) was used for the screening. A piece of sterilized filter paper (8.5 cm ID) was put on each agar plate (10 ml of 2% agar in a 9-cm Petri dish) and 2% PDA (10 ml) was layered on top of it. Then, two seeds were put in each plate and incubated at 25°C for 10 days. There were 50 replications per habitat. The filter paper was then transferred to another Petri dish together with the upper layer and stored at room temperature. PDA medium (10 ml) impregnated with mycelial fragments of *Py. vexans* was layered over the remaining lower agar layer, and the plates were incubated at 25°C for 24 hr. When sufficient amounts of antifungal compounds were produced by the fungi and diffused to the lower layer, inhibitory zones appeared in the upper layer containing test mycelia. When this happened, the microorganisms producing the antifungal compounds were isolated from the appropriate colony by the streak culture method.

Identification of Isolated Fungi. Fourteen isolates from a total of 149 examined were selected for further experiments, based on the antifungal potential of the ethyl-acetate-soluble metabolites that they produced against *Py. vexans*. Four of the 14 isolated fungal strains showed remarkable antifungal activity against *Py. vexans*. These fungi were identified by referring to the descriptions of Ramirez (1982); PGS-T5 from the Teshio seeds was identified as *Penicillium cyareum* (Bainier et Sartory) Biourge; PGS-O7 from the Oketo seeds was identified as *Pe. damasceum* Baghdadi; and PGS-S4 and S16 from the Shiranuka seeds were both identified as *Pe. implicatum* Biourge.

Paper Disk Method. The paper disk method was used to evaluate the anti-

fungal activity of the fungal metabolites. The metabolites in each fungal culture were partitioned between ethyl acetate and water. Concentrated ethyl acetate extracts and water layers (each 10 μl) equivalent to 62.5 and 125 μl of each culture filtrate were aseptically charged on paper disks (8 mm ID). Mycelial disks (8 mm ID) of *Py. vexans* freshly grown on 2% PDA were inoculated at the center of plates of the same medium and paper disks, which had previously been dried in vacuo to remove the solvent, were put on the plates 1.5 cm from the edge of the mycelial disks. When the test fungus was *C. herbarum*, plates of 2% PDA impregnated with the spores were used. The plates were incubated at 25°C for 36 hr. The growth inhibition was measured as the width (millimeters) of the inhibitory zone from the edge of the paper disk. Every test was triplicated and the results were averaged.

Fungi. Pythium vexans (Oomycetes), a damping-off fungus isolated from the roots of *Pi. glehnii* seedlings in the nursery of Hokkaido University (Kasuya, 1995), was used as a representative test fungus. *Cladosporium herbarum* AHU 9262 (Hyphomycetes) was also used to detect antifungal compounds (Homans and Fuchs, 1970).

TLC Bioautography Assay. This assay was used to determine the activity of compounds soluble in ethyl acetate against *Py. vexans* and *C. herbarum*. Extracts equivalent to 125 and 250 μl of the filtrate were charged on TLC plates (0.25 mm, Kieselgel 60 F_{254}, Merck) and developed in ethyl acetate. The resulting TLC plates were put on PDA plates impregnated with mycelial fragments of *Py. vexans*. The plates were incubated at 25°C for 24 hr. With *C. herbarum*, a spore suspension was sprayed over the developed TLC plates, which were then incubated at 25°C under humid conditions for three days (Homans and Fuchs, 1970). The observed inhibitory zones were correlated with the spots seen on the TLC plates under UV light.

Identification of Antifungal Metabolites Produced by Isolated Fungi

Preparation and Identification of Antifungal Metabolites. Each mycelial disk (8 mm ID) cut from the margin of young colonies of *Pe. cyareum* PGS-T5, *Pe. damasceum* PGS-O7, *Pe. implicatum* PGS-S4, or *Pe. implicatum* S16 grown on PDA, was inoculated into a 300-ml Erlenmeyer flask containing 100 ml of potato dextrose medium and incubated at 25°C for 10 days under stationary culture conditions. Each culture filtrate was extracted with 30 ml of ethyl acetate three times. The combined extracts were washed with saline, dried over Na_2SO_4, and evaporated to give an oily residue, which was diluted to 3 ml with ethyl acetate. The antifungal activity of the ethyl acetate extracts and water-soluble compounds was determined. The ethyl acetate extract from the culture filtrate of *Pe. cyareum* PGS-T5 was charged on TLC plates (0.25 mm, Kieselgel 60 F_{254} Merck) and developed in 50% hexane–ethyl acetate twice to elute

compound **1** (patulin, 3.4 mg from 2.7 ml of culture filtrate, an amorphous powder, R_f = 0.59 in EtOAc). EI-HR-MS determined that the molecular formula of compound **1** was $C_4H_7O_4$ (found 154.0258; calcd. 154.0266). It was identified as patulin on the basis of its ^1H and ^{13}C NMR, UV data, and melting point, which were all in full agreement with reported data (Okeke et al., 1993; Paterson and Kemmelmeier, 1990).

The culture filtrates of *Pe. damasceum* PGS-O7 and the two strains of *Pe. implicatum* (PGS-S4 and S16) yielded oily compounds soluble in ethyl acetate. The residuals were crystallized from ethanol, to yield compounds **2** (citrinin, 10 mg from 340 ml of culture filtrate, yellow needles, R_f = 0.10 in EtOAc), and **3** (palitantin, 7.5 mg from 220 ml of culture filtrate, colorless needles, R_f = 0.30 in EtOAc), respectively. Compound **2** from *Pe. damasceum* PGS-O7 has the molecular formula $C_{13}H_{14}O_5$ (EI-HR-MS found 250.0863; calcd. 250.0841) and was identified as citrinin by comparing the spectroscopic results (^1H and ^{13}C NMR, UV spectrometry, melting point and $[\alpha]_D$) with reported values (Betina, 1984; Colombo et al., 1980; Paterson and Kemmelmeier, 1990). Compound **3** from *Pe. implicatum* PGS-S4 and S16 has the molecular formula $C_{14}H_{22}O_4$ (EI-HR-MS found 254.1499; calcd. 254.1519) and was identified as palitantin by analyses of its ^1H and ^{13}C NMR, UV spectra, melting point, and $[\alpha]_D$, which fully agreed with published data (Mierisova et al., 1996; Hanessian et al., 1989; Paterson and Kemmelmeier, 1990).

The concentrated EtOAc solutes remaining after compound **3** was precipitated from the *Pe. implicatum* PGS-S4 culture filtrate were applied to a silica gel column (Wako gel 60, 19 g) and eluted with ethyl acetate. Fractions 9–13 (2 ml each) containing compound **4** were combined, concentrated further, and subjected to reverse phase column chromatography (26 g of Cosmosil 75C_{18}-OPN) eluted with MeOH–H_2O (1 : 1). Fractions 28–46 (2 ml each) from the second chromatography step were combined and concentrated to yield compound **4** as an amorphous powder, 2 mg from 760 ml of the culture filtrate, R_f = 0.67 in EtOAc, with the molecular formula $C_{13}H_{14}O_5$ (EI-HR-MS, found 218.0933; calcd. 218.0943). The ^1H and ^{13}C NMR, UV spectra, melting point, and $[\alpha]_D$ of compound **4** from *Pe. implicatum* PGS-S4 fully agreed with the published data for arthrographol (Pfefferle et al., 1990).

RESULTS AND DISCUSSION

Of the 149 microorganisms isolated from the surface of *Pi. glehnii* seeds, four showing high antifungal activity against *Py. vexans* were selected by the paper disk method. These four isolated fungi were all identified as *Penicillium* species: *Pe. cyareum* PGS-T5, *Pe. damasceum* PGS-O7, and *Pe. implicatum* PGS-S4 and S16. Ethyl acetate extracts equal to 62.5 and 125 μl of the

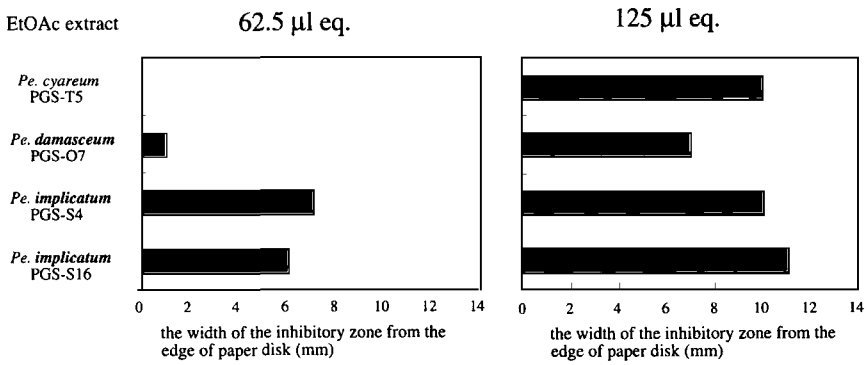


FIG. 1. Antifungal activity of ethyl acetate extracts from culture filtrates of isolated fungi against *Pythium vexans*. The ethyl acetate extracts equal to 62.5 and 125 μl of culture filtrates of *Pe. cyareum* PGS-T5, *Pe. damasceum* PGS-O7, and *Pe. implicatum* PGS-S4 and S16 showed antifungal activity against *Py. vexans*. Ethyl acetate extract equal to 62.5 μl of the culture filtrate of *Pe. cyareum* PGS-T5 did not show antifungal activity, but retardation of mycelial growth around paper disks was observed.

culture filtrates of these strains showed antifungal activity against *Py. vexans* (Figure 1). The activities of *Pe. implicatum* PGS-S4 and S16 were especially remarkable. Although the water-soluble constituents in the culture filtrate of *Pe. cyareum* PGS-T5 also exhibited weak antifungal activity, this activity was due to the incomplete extraction of antifungal components with ethyl acetate (data not shown). The water-soluble metabolites from the other three fungi were inactive.

The antifungal metabolites in the culture filtrates were examined with TLC bioautography using *Py. vexans* and *C. herbarum* as the test fungi. The antifungal zones appearing with each fungus showed essentially the same patterns. Figure 2 shows a typical result with *C. herbarum*. Under guidance of the TLC bioautography, the antifungal compounds in the filtrates were isolated and purified, primarily by chromatographic methods, and were identified as patulin (**1**), citrinin (**2**), palitantin (**3**), and arthrographol (= asperfuran, **4**) by spectroscopic analyses (*see* Methods and Materials). Compound **4** isolated from the filtrate of *Pe. implicatum* PGS-S4 selectively inhibited the growth of *C. herbarum*. The structures of the four antifungal compounds are shown in Figure 3.

Patulin (**1**), the major active compound in *Pe. cyareum* PGS-T5, has been isolated from other *Penicillium* and *Aspergillus* species and is both a potent antibiotic and a carcinogenic and phytotoxic agent (Anslow et al., 1943; Betina, 1984, 1989; Katzman et al., 1944; Nickell and Finlay, 1954; Wang, 1948). Citrinin (**2**) from *Pe. damasceum* PGS-O7 has also been reported in several *Penicillium* and *Aspergillus* species and is a fungitoxic and phytotoxic agent (Betina, 1984, 1989; Robinson and Park, 1966; White and Truelove, 1972; Damodaran

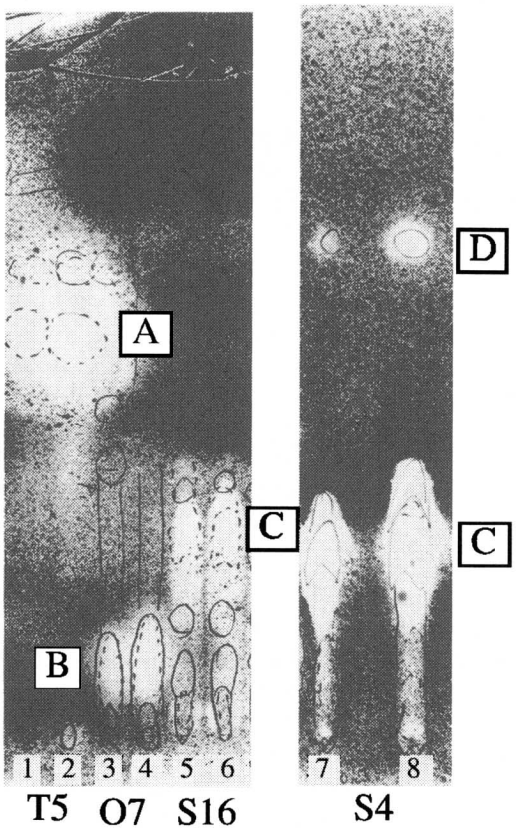
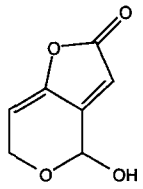
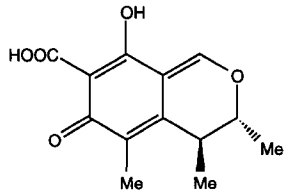


FIG. 2. TLC bioautogram of ethyl acetate solutes from culture filtrates of the positive strains. Ethyl acetate extracts equal to 125 (lanes 1, 3, 5, and 7) and 250 μl (lanes 2, 4, 6, and 8) of culture filtrates on Merck Kieselgel 60 F_{254} plates (0.25 mm) were developed in ethyl acetate, and the chromatograms were then sprayed with a spore suspension of *C. herbarum*. The inhibitory zones labeled with capital letters result from the identified compounds: zone A, patulin (**1**); B, citrinin (**2**); C, palitantin (**3**) and D, arthrographol (**4**).

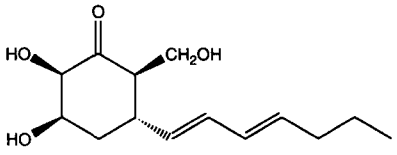
et al., 1975). These fungitoxins are well-known pollutants of stock foods following fungal contamination. On the other hand, there are no reports of phytotoxic activity by palitantin (**3**), although it is known as an antifungal metabolite in some *Penicillium* species (Birkinshaw and Raistrick, 1936; Curtis et al., 1951; Bracken et al., 1954). Arthrographol is a rare polyketide, which has been isolated as an antifungal metabolite from *Arthrographis pinicola* (Ayer and Nozawa, 1990) and *Aspergillus oryzae* (Pfefferle et al., 1990). There is no description of



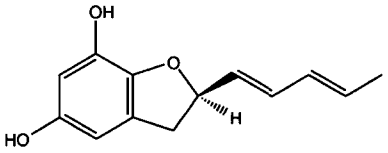
1: patulin (*Pe. cyareum* PGS-T5)



2: citrinin (*Pe. damasceum* PGS-O7)



3: palitantin (*Pe. implicatum* PGS-S16)



4: arthrographol (*Pe. implicatum* PGS-S4)

FIG. 3. Structures of isolated antifungal compounds.

its phytotoxic activity. This is the first time that arthrographol has been isolated from the culture filtrate of a *Penicillium* species.

The antifungal activities of the isolated metabolites **1**, **2**, and **3** are shown in Figure 4. Compounds **1** and **2** showed marked antifungal activity against both test fungi when measured in terms of the ID_{50} of mycelial growth. Compound **3** also has relatively high activity against *Py. vexans* (ID_{50} for *Py. vexans*: **1**, ca. 65 nmol; **2**, ca. 80 nmol; **3**, ca. 55 nmol). *Py. vexans* was much more susceptible than *C. herbarum* to these metabolites. However, only the ID_{50} for compound **3** was significantly different when *Py. vexans* and *C. herbarum* were compared (ID_{50} for *C. herbarum*: **1**, ca. 65 nmol; **2**, ca. 92 nmol; **3**, ca. 150 nmol).

The seeds of plants are normally covered with many microorganisms, and germinating seeds encounter more microorganisms that inhabit the soil. During growth, many plants come into contact with particular microorganisms, and this often results in either an association or an infection (Watanabe, 1993). Studying the relationship between *Pi. glehnii* seeds and microorganisms is an important first step in learning about the effects of seed epiphytic microorganisms on seed germination and seedling growth.

All of the microorganisms isolated from the surface of *Pi. glehnii* seeds that produced antifungal compounds were *Penicillium* species. A *Penicillium* species has also been isolated from the surface of *Pi. jezoensis* seeds and seedlings that were unhealthy or blighted (Takahashi, 1980). In this case, the *Penicillium* species seemed to be parasitizing *Pi. jezoensis*. Similarly, *Pe. cyareum* PGS-T5 and *Pe. damasceum* PGS-O7, which were isolated from *Pi. glehnii* seeds, are

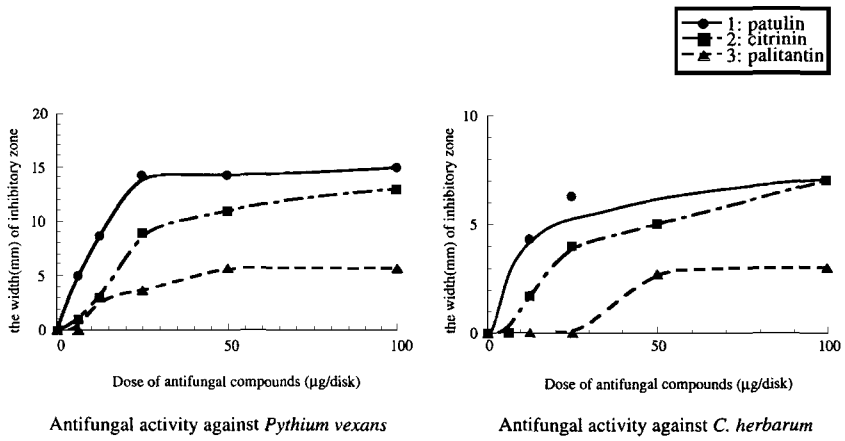


FIG. 4. Antifungal activity of isolated patulin (**1**), citrinin (**2**), and palitantin (**3**). The antifungal activity of each compound was examined using the paper disk method. Five different dosages (6.25, 12.5, 25, 50, 100 μg/disk) of each antifungal compound were tested.

likely to be pathogenic *Penicillium* species, since these strains produced phytotoxins **1** and **2** as the major metabolite, respectively. On the other hand, *Pe. implicatum* PGS-S16 and S4, which produced compound **3**, likely plays a defensive role on the host plant, as **3** does not have phytotoxic activity. A preliminarily examination of the phytotoxicity of all the isolates on lettuce seedlings was made. Further study of these three *Penicillium* species is needed to determine whether they are pathogenic or advantageous microorganisms.

This was the initial search for new interrelationships between tree seedlings and microorganisms and focused on seed epiphytic fungi and seedlings. We found our results encouraging and are conducting further research. *Pi. glehnii* is a model plant for studying the ecological relationships between coniferous trees and seed epiphytic microorganisms.

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REFERENCES

ANSLOW, W. K., RAISTRICK, H., and SMITH, G. 1943. Anti-fungal substances from moulds. Part I. Patulin (anhydro-3-hydroxymethylene-tetrahydro-1 : 4-pyrone-2-carboxylic acid), a metabolic

product of *Penicillium patulum* Bainier and *Penicillium expansum* (Link) Thom. *J. Soc. Chem. Ind.* 62:236–238.

AYER, W. A., and NOZAWA, K. 1990. Taxonomy and chemistry of a new fungus from bark beetle infested *Pinus contorta* var. *latifolia*. Part 2. Arthrographol, the metabolite inhibitory to *Ophiostoma clavigerum*. *Can. J. Microbiol.* 36:83–85.

BETINA, V. 1984. Mycotoxins—Production, Isolation, Separation and Purification. Elsevier, Amsterdam, p. 217–236, 291–314.

BETINA, V. 1989. Mycotoxins: Chemical, Biological and Environmental Aspects. Elsevier, Amsterdam, pp. 242–269.

BIRKINSHAW, J. H., and RAISTRICK, H. 1936. CXVIII. Studies in the biochemistry of micro-organisms. XLIX. Palitantin, $C_{14}H_{22}O_4$, a hitherto undescribed metabolic product of *Penicillium palitans* Westling. *Biochem. J.* 30:801–808.

BRACKEN, A., POCKER, A., and RAISTRICK, H. 1954. Studies in the biochemistry of micro-organisms. 93. Cyclopenin, a nitrogen-containing metabolic product of *Penicillium cyclopium* Westling. *Biochem. J.* 57:587–595.

CHAKRAVARTY, P., and UNESTAM, T. 1987. Differential influence of ectomycorrhizae on plant growth and disease resistance in *Pinus sylvestris* seedlings. *J. Phytopathol.* 120:104–120.

COLOMBO, L., GENNARI, C., RICCA, G. S., and SCOLASTICO, C. 1980. Biosynthetic origin and revised structure of ascochitine, a phytotoxic fungal metabolite. Incorporation of [1-^{13}C]- and [1,2-$^{13}C_2$]acetates and [Me-^{13}C]methionine. *J. Chem. Soc. Perkin Trans. I* 1980:675–676.

CURTIS, P. J., HEMMING, H. G., and SMITH, W. K. 1951. Frequentin: An antibiotic produced by some strains of *Penicillium frequentans* Westling. *Nature* 167:557–558.

DAMODARAN, C., KATHIRVEL-PANDIAN, S., SEENI, S., SELVAM, R., GANESAN, M. G., and SHANMUGASUNDARAM, S. 1975. Citrinin, a phytotoxin? *Experientia* 31:1415–1417.

DUCHESNE, L. C., and PETERSON, R. L. 1988. Interaction between the ectomycorrhizal fungus *Paxillus involutus* and *Pinus resinosa* induces resistance to *Fusarium oxysporum*. *Can. J. Bot.* 66:558–562.

DUCHESNE, L. C., PETERSON, R. L., and ELLIS, B. E. 1988. Pine root exudate stimulates the synthesis of antifungal compounds by the ectomycorrhizal fungus *Paxillus involutus*. *New Phytol.* 108:471–476.

DUCHESNE, L. C., ELLIS, B. E., and PETERSON, R. L. 1989. Disease suppression by the ectomycorrhizal fungus *Paxillus involutus*: Contribution of oxalic acid. *Can. J. Bot.* 67:2726–2730.

HANESSIAN, S., SAKITO, Y., DHANOA, D., and BAPTISTELLA, L. 1989. Synthesis of (+)-palitantin. *Tetrahedron* 45:6623–6630.

HOMANS, A. L., and FUCHS, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. *J. Chromatogr.* 51:327–329.

INGHAM, J. L. 1973. Disease resistance in higher plants. The concept of pre-infectional and post-infectional resistance. *Phytopath. Z.* 78:314–335.

ITO, K. 1955. Jyubyo-kougi. Chikyu-syuppansya, Tokyo. pp. 19–30 (in Japanese).

KASUYA, M. K. M. 1995. Ecological and physiological studies on ectomycorrhizae of *Picea glehnii* (Fr. Schm.) Masters. Doctoral thesis. Graduate School of Agriculture, Hokkaido University, Sapporo.

KATZMAN, P. A., HAYS, E. E., CAIN, C. K., VAN WYK, J. J., REITHEL, F. J., THAYER, S. A., and DOISY, E. A. 1944. Clavacin, an antibiotic substance from *Aspergillus clavatus*. *J. Biol. Chem.* 154:475–486.

MARX, D. H. 1972. Ectomycorrhizae as biological deterrents to pathogenic root infections. *Annu. Rev. Phytopathol.* 10:429–454.

MIERISOVA, S., PROKSA, B., and LIPTAJ, T. 1996. ^{1}H and ^{13}C NMR data for palitantin and its derivatives. *Magn. Reson. Chem.* 34:414–415.

NICKELL, L. G., and FINLAY, A. C. 1954. Antibiotics and their effects on plant growth. *J. Agric. Food Chem.* 2:178–182.

OKEKE, B., SEIGLE-MURANDI, F., STEIMAN, R., BENOIT-GUYOD, J. L., and KAOUADJI, M. 1993. Identification of mycotoxin-producing fungal strains: A step in the isolation of compounds active against rice fungal diseases. *J. Agric. Food Chem.* 41:1731–1735.

PATERSON, R. R. M., and KEMMELMEIER, C. 1990. Neutral, alkaline and difference ultraviolet spectra of secondary metabolites from *Penicillium* and other fungi, and comparisons to published maxima from gradient high performance liquid chromatography with diode-array detection. *J. Chromatogr.* 511:195–221.

PFEFFERLE, W., ANKE, H., BROSS, M., and STEFFAN, B. 1990. Asperfuran, a novel antifungal metabolite from *Aspergillus oryzae*. *J. Antibiot.* 43:648–654.

RAMIREZ, C. 1982. Manual and atlas of the Penicillia. Elsevier Biomedical, Amsterdam, pp. 235–286.

ROBINSON, P. M., and PARK, D. 1966. Citrinin—a fungistatic antibiotic and narrowing factor. *Nature* 211:883–884.

SHIMAZU, A. 1986. Antibiotics, p. 754, in Yamazato, K., Udagawa, S., Kodama, T. and Shinchi, T. (eds.). Biseibutu-no douteihou. R&D Planning, Tokyo (in Japanese).

STACK, R. W., and SINCLAIR, W. A. 1975. Protection of Douglas-fir seedlings against *Fusarium* root rot by a mycorrhizal fungus in the absence of mycorrhiza formation. *Phytopathology* 65:468–472.

TAKAHASHI, I. 1980. The research for fungi which appeared during natural regeneration of *Picea jezoensis* and *Abies sachalinensis*. *Trans. Meet. Hokkaido Br. Jpn. For. Soc.* 29:125–127 (in Japanese).

TATEWAKI, M. 1958. Forest ecology of the islands of the North Pacific Ocean. *J. Fac. Agric. Hokkaido Univ.* 50:371–486.

WANG, F. H. 1948. The effects of clavacin upon root growth. *Bot. Bull. Acad. Sinica* 2:265–269.

WATANABE, T. 1993. Dojyou-shijyoukin. Softscience-sya. Tokyo, pp. 1–5 (in Japanese).

WHITE, A. G., and TRUELOVE, B. 1972. The effects of aflatoxin B_1, citrinin, and orchratoxin A on amino acid uptake and incorporation by cucumber. *Can. J. Bot.* 50:2659–2664.

ZAK, B. 1964. Role of mycorrhizae in root disease. *Annu. Rev. Phytopathol.* 2:377–392.