Phytotoxic Components Produced by Pathogenic *Fusarium* against Morning Glory

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A pathogenic isolate of *Fusarium*, *F. oxysporum* f. sp. *batatas* O-17 (PF), causes wilt disease in leaf etiolation in sweet potato (*Ipomoea batatas*) and morning glory (*Ipomoea tricolor*). Extracts from PF cultures were screened for phytotoxic components using a growth inhibition assay with morning glory seedlings. The extracts were fractionated using differential solvent extraction and two active compounds, ergosterol and fusalanipyrone, were isolated from the less-polar fraction. Growth inhibition of morning glory seedlings showed a sigmoidal dose-response relationship, with fusalanipyrone exhibiting a two order of magnitude higher EC_{50} value than ergosterol (18 nM and 1.6 μM, respectively). Both compounds showed lower growth inhibition activity towards lettuce seedlings (*Lactuca sativa*). This study provides information on the phytotoxic components of PF and discusses the mechanism behind PFf-induced phytotoxicity.

Key words: Pathogenic *Fusarium*, Fusalanipyrone, Morning Glory

Introduction

Wilt disease caused by pathogenic *Fusarium* is often found in many plants (Komada, 1976; Baker *et al.*, 1978; Nakamura, 1981; Ogawa, 1988). It has been shown that pathogen-produced toxins play important roles in interactions between plants and pathogens (Savard *et al.*, 1990; Abbas *et al.*, 1992; Knogge, 1996). In the case of *Fusarium* wilt of sweet potato, the disease symptoms (leaf etiolation and wilting) are induced by treatment with cell-free filtrate cultures of the pathogen, *Fusarium oxysporum* f. sp. *batatas* O-17 (pathogenic *Fusarium*: PF) (Ogawa, 1988). This observation strongly implies contribution of toxic component(s) produced by PF to the disease symptoms, which has not yet been described.

We have reported a facile model using morning glory (*Ipomoea tricolor*) to study the interactions between *Fusarium* spp. and plants (Shimizu *et al.*, 2000, 2005), in which PF also exhibited pathogenicity against morning glory and caused leaf etiolation and wilting. In this study, the phytotoxic compounds produced by PF were explored using morning glory seedlings as test plants.

Materials and Methods

Chemicals

All chemicals were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemicals (Osaka, Japan) unless otherwise specified.

Seedling assay

The seeds of morning glory (*Ipomoea tricolor* cv. Heavenly Blue, purchased from Sakata Seed, Yokohama, Japan) were placed on a filter paper in a glass dish (16 cm I. D.) with 10 ml of sterilized water. The seedlings were incubated for 24 h at 25 °C under continuous lighting were used as the test plant (0.8–1.0 cm length). Test fractions or compounds were assayed as described below. The portion of the fractions or the compounds dissolved in ethyl acetate was dropped on the filter paper. After air-drying the filter paper to remove the solvent, 10 ml of sterilized water with 0.05% (v/v) Tween 20 was poured into the dish. The seedlings were put on the filter paper and incubated for 3 d at 25 °C under continuous lighting, after which the length of the seedlings was measured. Lettuce seedlings (*Lactuca sativa* cv. President, purchased from Takayama Seed, Kyoto, Japan)

were incubated and subjected to the same assay described above.

Culturing, extraction and silica gel column chromatography

A pathogenic isolate of *Fusarium*, *F. oxysporum* f. sp. *batatas* O-17 (PF) was cultured on potato/sucrose/agar medium (90 mm I. D., 300 plates) at 25 °C under darkness for 2 weeks.

The culture plates were soaked in 6 l of acetone over night, and then the acetone extract was condensed under reduced pressure. The aqueous residue was partitioned against the same volume (~ 300 ml) of *n*-hexane, toluene and ethyl acetate, respectively. The organic layers were concentrated under reduced pressure. Each fraction was diluted with ethyl acetate to the intended volume and stored at 4 °C until use. Of the fractions partitioned by organic solvents, the highest activity was found in the *n*-hexane fraction, which gave 830 mg of a yellowish viscous oil after removal of the solvent. The oil from the *n*-hexane fraction was subjected to chromatography on a flash silica gel column (20 g; silica gel 60, Merck) eluted in 300 ml steps of *n*-hexane/ethyl acetate mixtures (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, and 0:1) followed by 300 ml of methanol.

Identification of ergosterol

Silica gel TLC [expanded by *n*-hexane/ethyl acetate, 3:1; visualized by spraying with 5% (v/v) H_2SO_4 ethanol solution before heating at 110 °C] showed that the active fractions from the *n*-hexane fraction eluted by the *n*-hexane/ethyl acetate mixture (5:5–0:1) contained a single compound. Concentration of this fraction resulted in a white residue, which gave 64 mg of colorless powder after recrystallization from an *n*-hexane/ethyl acetate mixture (10:1, approximately). This crystallized compound was analyzed by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra recorded on a Bruker AC-300 instrument and cochromatography with the standard sample (Nacalai Tesque).

HPLC purification and identification of fusalanipyrone

The mixture of the active fractions eluted by the mixed solvent of *n*-hexane/ethyl acetate, 9:1–6:4 were concentrated under reduced pressure and applied to a Sep-Pak C18 cartridge (Waters) equilibrated with water. The cartridge was eluted with methanol (5 ml), and the eluate was subjected to further purification by reversed-phase HPLC on a ODS column, Cosmosil 5C18-AR column (250 × 10 mm I. D., Nacalai Tesque) eluted with the methanol/water isocratic solvent system (7:3 at flow rate of 3 ml/min; 25 °C column temperature; peak detection by UV 254 nm). The active fraction collected was concentrated under reduced pressure, resulting in a clear viscous oil. This material was analyzed by ^1H and ^{13}C NMR spectra. The molecular weight was determined on a Perkin-Elmer Sciex API 165 instrument operating in the electrospray ionization mode (ESI-MS, positive mode, 70 eV, methanol solution of the sample was introduced by a continuous flow at 5 μl/min). An IR spectrum was collected in chloroform solution.

Results

Identification of the active components

Treatment with PF bud-cell suspension at 10^8 bud-cells/ml exhibited inhibition of the seedling growth, albinism of the hypocotyls, etiolation of the cotyledons and browning of the apical part of the radicle, resulting in death of the seedlings after approximately one week (data not shown). The acetone extract of the PF culture exhibited inhibitory activity to growth of the morning glory seedlings (Fig. 1). The seedlings treated with the acetone extract for 3 d showed growth inhibition,

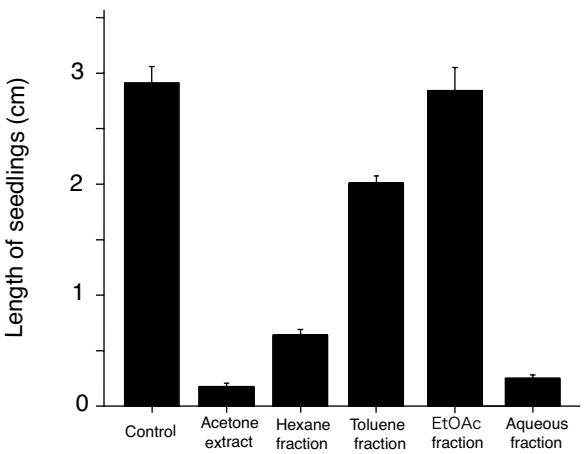
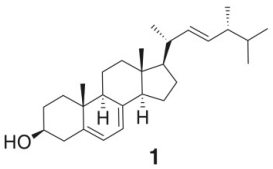


Fig. 1. Inhibition of the fractions from PF extract against growth of morning glory seedlings. The length of the seedlings is shown with standard error (n = 3). The concentration of each fraction was equivalent to the extract from five culture plates of PF in 10 ml of aqueous 0.05% Tween 20. Seedlings were treated for 3 d.



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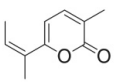

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Fig. 2. Phytotoxic compounds isolated from PF extract: ergosterol (**1**) and fusalanipyrone (**2**).

albinism of the hypocotyls, and thickening of the radicle (data not shown).

Of the partitioned fractions, the aqueous and *n*-hexane fractions were active. We focused on the *n*-hexane fraction. The compound isolated from the *n*-hexane fractions and eluted by a *n*-hexane/ethyl acetate mixture (5:5–0:1) was identified to be ergosterol (**1**, Fig. 2). Identification was verified by cochromatography with the authentic standard and comparison of NMR spectra between the isolated compound and the standard (data not shown).

HPLC purification of the active fractions eluted by a mixed solvent of *n*-hexane/ethyl acetate (9:1–6:4) gave the active compound (8.8 mg, colorless, viscous oil, retention time: 5.5 min). The molecular weight of this compound was determined to be 164 g/mol based upon ESI-MS analysis, in which a pseudomolecular ion m/z = 165 $[M+H]^+$ was observed. ^1H and ^{13}C NMR spectra showed 12 protons and 10 carbon atoms (Table I). An absorption band at 1700 cm^{-1}, characteristic of a carbonyl group, was found in the IR spectra. In accordance with the data in the previous reports (Abraham and Arfmann, 1988), we concluded that the compound is fusalanipyrone (**2**, Fig. 2). No other fractions besides that containing fusalanipyrone exhibited inhibitory activity in the active fractions eluted by the mixed solvent of *n*-hexane/ethyl acetate (9:1–6:4).

Table I. Chemical shifts (ppm) of fusalanipyrone in chloroform-*d*.

^{13}C (75 MHz)	^1H (300 MHz)
163.5	
159.8	
139.9	7.12 dq (J = 6.9, 1.2)[a]
128.0	6.61 q (J = 7.5)
126.8	
122.9	
100.4	6.05 d (J = 6.9)
16.5	2.08 s (J = 1.2)
14.0	1.83 d (J = 7.5)
12.0	1.85 s

[a] Coupling constant (Hz).

Inhibitory activity against morning glory seedlings

The seedling growth showed a sigmoidal response to the logarithmic value of the concentration of fusalanipyrone and ergosterol (Fig. 3). At higher concentrations, growth of the seedlings during 3 d of the testing term was completely inhibited. Treatment with 25 μM of ergosterol exhibited significant inhibition, with seedlings growing to ~ 1.0 cm length compared to 3.1 cm for control. Ergosterol exhibited no inhibition at treatment levels of 2.5 μM and the length of the seedlings was 2.9 cm. On the other hand, fusalanipyrone clearly inhibited the growth of the seedlings at a concentration range of 0.06–60 μM, in which seedling

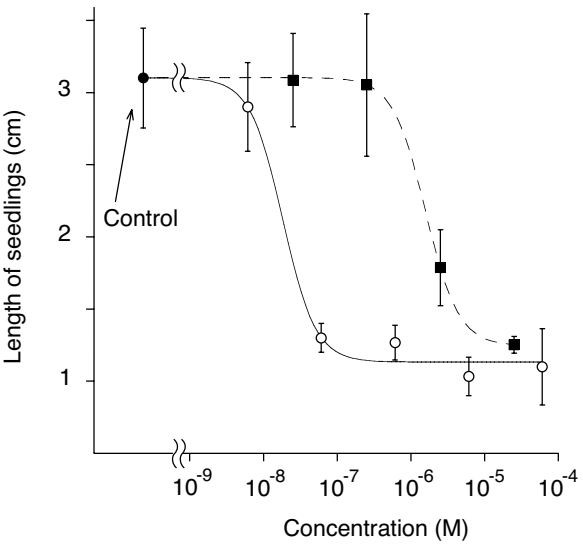

Fig. 3. Concentration-response relationship of ergosterol and fusalanipyrone and ergosterol in inhibition of seedling growth of morning glory. Length of the seedlings treated with control (solid circle), fusalanipyrone (blank circles with a solid line) and ergosterol (solid squares with a dotted line) are shown with standard error (n = 3). The horizontal abscissa is drawn on a logarithmic scale of concentration (M) of the components treated for 3 d. Sigmoidal regression curves were drawn by KaleidaGraph ver.3.6 (Synergy Software). EC_{50} value was (1.6 ± 0.11) μM for ergosterol and (18 ± 6.0) nM for fusalanipyrone, respectively.

length was 1.1–1.3 cm. The values of median effective concentration (EC_{50}) calculated by KaleidaGraph ver.3.6 (Synergy Software) using sigmoidal regression curves were (1.6 ± 0.11) μM and (18 ± 6.0) nM for ergosterol and fusalanipyrone, respectively.

Albinism was found in the hypocotyls of morning glory seedlings treated with fusalanipyrone from 0.6–60 μM (data not shown).

Inhibitory activity against lettuce seedlings

Inhibitory activities against lettuce seedlings were examined at 270 μM of ergosterol and 89 μM of fusalanipyrone in aqueous 0.05% Tween 20, equivalent to the concentration present in the extract from five culture plates in an assaying dish. Ergosterol exhibited no inhibition against lettuce seedlings at 270 μM, the seedlings grew to 2.9 cm, whereas the control grew to 3.1 cm. Fusalanipyrone showed a slight inhibition at 89 μM, resulting in 2.6 cm long seedlings (Table II).

Table II. Inhibitory activity of ergosterol and fusalanipyrone against the seedling of lettuce. Ergosterol and fusalanipyrone were applied in water with 0.05% Tween 20, equivalent to the amount extract from five culture plates. Length of the seedlings measured after a 3-d treatment is shown with standard error (n = 3).

	Length [cm]
Control	3.1 ± 0.1
Ergosterol (270 μM)	2.9 ± 0.1
Fusalanipyrone (89 μM)	2.6 ± 0.1

Discussion

We have explored toxic components produced by PF against morning glory seedlings. The extract from PF exhibited inhibitory activity, which was partitioned to aqueous and *n*-hexane fractions. Inhibitory activity of the aqueous fraction after partitioning might be responsible for lytic enzymes, such as polygalacturonase (Pietro and Roncero, 1998; García-Maceira *et al.*, 2001; Pietro *et al.*, 2001). In this study, we focused on the less-polar components.

The fraction partitioned by *n*-hexane contained two active components, ergosterol and fusalanipyrone. Ergosterol is often found in fungus and thought to be a membrane lipid. Ergosterol is often exploited as a biomass marker of fungus for detection in various sources (Kadakal and Artik, 2004; Mille-Lindblom *et al.*, 2004), in which the phytotoxicity of ergosterol has not been often focused. There are some reports that ergosterol elicits plant hypersensitive reaction (Kasparovsky *et al.*, 2004). Ergosterol might also play a role during the interaction as a phytotoxic component against morning glory. Considering the fact that ergosterol is found ubiquitously in fungus, it could be said that the role of ergosterol during the interaction between fungus *Fusarium* and morning glory-plants might be rather assisting other phytotoxic components than a causal factor of pathogenicity.

Fusalanipyrone, an α-pyrone derivative, exhibited high inhibitory activity against the seedlings of morning glory. The EC_{50} value of fusalanipyrone against morning glory seedlings is two orders higher than that of ergosterol. A fusalanipyrone derivative, gibepyrone, *E*-isomer in the side chain, is reported to exhibit moderate antibacterial activity (Barrero *et al.*, 1993). It has also reported that α-pyrone derivatives from fungus exhibit low phytotoxic activity against the other plants (Venkatasubbaiah *et al.*, 1991). Compared with those activities, the inhibitory activity of fusalanipyrone against morning glory seedlings is significantly high. In contrast to morning glory seedlings, fusalanipyrone did not exhibit high activity against lettuce seedlings. Fusalanipyrone is likely to play important roles during the interaction between PF and morning glory as a virulent factor. The relationship between pathogenicity and the level of production of these compounds will require future studies before the exact mechanism can be elucidated. Albinism of the seedlings indicates disturbance of the pigment metabolism, which is one of the candidates for further research to explain the mode of action of fusalanipyrone against morning glory.

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Abbas H. K., Mirocha C. J., and Shier W. T. (1992), Isolation, identification and biological activity of chlamydosporol from *Fusarium culmorum* HM-8. Mycopathologia **118**, 115–123.

Abraham W.-R. and Arfmann H.-A. (1988), Fusalanipyrone, a monoterpenoid from *Fusarium solani*. Phytochemistry **27**, 3310–3311.

Baker R., Hanchey P., and Dotterar S. D. (1978), Protection of carnation against *Fusarium* stem rot by fungi. Phytopathology **68**, 1495–1501.

Barrero A. F., Oltra J. E., Herrador M. M., Cabrera E., Sanchez J. F., Quílez J. F., Rojas F. J., and Reyes J. F. (1993), Gibepyrones: α-pyrones from *Gibberella fujikuroi*. Tetrahedron **49**, 141–150.

García-Maceira F. I., Pietro A. D., Huertas-González M. D., Ruiz-Roldán M. C., and Roncero M. I. G. (2001), Molecular characterization of an endopolygalacturonase from *Fusarium oxysporum* expressed during early stages of infection. Appl. Envir. Microbiol. **67**, 2191–2196.

Kadakal C. and Artik N. (2004), A new quality parameter in tomato and tomato products: ergosterol. Crit. Rev. Food Sci. Nutr. **45**, 349–351.

Kasparovsky T., Blein J.-P., and Mikesa V. (2004), Ergosterol elicits oxidative burst in tobacco cells *via* phospholipase A2 and protein kinase C signal pathway. Plant Physiol. Biochem. **42**, 429–435.

Knogge W. (1996), Fungal infection of plants. Plant Cell **8**, 1711–1722.

Komada H. (1976), Studies on the evaluation of activity of *Fusarium oxysporum*, fusarium pathogen of vegetable crops, in the soil. Bull. Tokai-Kinki Natl. Agric. Exp. Cent. **29**, 132–269 (in Japanese).

Mille-Lindblom C., von Wachenfeldt E., and Tranvik L. J. (2004), Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. J. Microbiol. Methods **59**, 253–262.

Nakamura H. (1981), *Fusarium* wilt in morning glory (*Ipomoea tricolor* Cav.). Ann. Phytopathol. Soc. Jpn. **47**, 90.

Ogawa K. (1988), Studies on *Fusarium* wilt of sweet potato. Bull. Natl. Agric. Res. Cent. Jpn. (Tsukuba) **10**, 10–45 (in Japanese).

Pietro A. D. and Roncero M. I. G. (1998), Cloning, expression and role in pathogenicity of *pg*1 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **11**, 91–98.

Pietro A. D., Huertas-González M. D., Gutierrez-Corona J. F., Martínez-Cadena G., Méglecz E., and Roncero M. I. G. (2001), Molecular characterization of a subtilase from the vascular wilt fungus *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **14**, 653–662.

Savard M. E., Miller J. D., Salleh B., and Strange R. N. (1990), Chlamydosporol, a new metabolite from *Fusarium chlamydosporum*. Mycopathologia **110**, 177–181.

Shimizu B.-I., Fujimori A., Miyagawa H., Ueno T., Watanabe K., and Ogawa K. (2000), Resistance against *Fusarium* wilt induced by non-pathogenic *Fusarium* in *Ipomoea tricolor*. J. Pestic. Sci. **25**, 365–372.

Shimizu B.-I., Miyagawa H., Ueno T., Sakata K., Watanabe K., and Ogawa K., (2005), Morning glory systemically accumulates scopoletin and scopolin after interaction with *Fusarium oxysporum*. Z. Naturforsch. **60c**, 83–90.

Venkatasubbaiah P., Dyke C. G. V., and Chilton W. S. (1991), Phytotoxins produced by *Pestalotiopsis oenotherae*, a pathogen of evening primrose. Phytochemistry **30**, 1471–1474.