Antifungal Compounds Induced in the Dual Culture with *Phytolacca americana* Callus and *Botrytis fabae*

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In order to investigate new metabolites which are only induced in a plant callus infected by a pathogenic fungus, dual cultures with combinations of 10 species of fungi and 6 plant cell lines from different species were established. Among the combinations tested, the methanolic extract of a dual culture consisting of a plant cell line, *Phytolacca americana* and a fungus, *Botrytis fabae* showed a marked antifungal activity to *Cladosporium herbarum*. The main active constituent of this extract was identified to be phytolaccoside B (Pls B) by the spectroscopic analyses.

Introduction

Plants react to a variety of stimuli and newly induce numerous metabolites in the tissues. These metabolites play an important role in the defense systems (Purkayastha, 1976). At the initial stage of fungal infection to plants, the spores germinate, and the resulting hyphae penetrate the cuticle layer getting over physical and chemical barriers. When pathogens attack, hydrolases as well as oxidases in the plant tissues are activated, and such enzymes convert cell components into new compounds in the lesions (Kobayashi *et al.*, 1994). As invading plant tissues, pathogens often accumulate a variety of compounds in the lesions as their own metabolites (phytotoxins) or degradation products originated from plant cell components. In the viewpoint of survey for new metabolites, the compounds specifically found in the lesions could be promising candidates with a variety of biological activities. The production of novel metabolites could be attained if pathogenic infections to incompatible races are established. In general, fungal pathogens in the field strictly select the host plants and have a narrow range of host-specificity (Kohmoto *et al.*, 1991). Quantity and quality of cell components as well as structural features of the tissue may be closely related to infectivity. Whereas dedifferentiated tissues no more sustain such physical and chemical barriers, pathogens readily establish infection. Therefore, we inoculated fungal pathogens onto calli and set up dual cultures. We chose several pathogens and plant calli, and tried to establish the dual cultures for which an arbitrary combinations with fungi and calli was made. The dual culture was maintained for a certain period after the growth of each individual culture. Among the several dual cultures, the methanolic extracts from the dual culture consisting of a plant cell line, *P. americana* and a plant pathogen, *B. fabae* exhibited a pronounced antifungal activity. This paper deals with the production of antifungal compounds by the dual culture method and the elucidation of their chemical structures together with the antifungal activities.

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Results and Discussion

Ten species of fungal pathogens (Table I) and 6 plant cell lines initiated from different species (Table II) were separately grown. The fungal cultures grown in a potato-malt extract-sucrose medium was inoculated on the callus in the logarithmic growth phase, and the culture was maintained for a week. The methanolic extracts of the dual cultures were subjected to the antifungal assay, respectively. Among the combinations tested, the

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Table I. Fungi inoculated on calli.

Botrytis fabae PF 10*
Glomerella cingulata PF 13
Rhizoctonia solani PF 52
Rhizoctonia solani PF 61
Rhizoctonia solani PF 70
Rhizoctonia solani PF 159
Cercospora beticola PF 218
Alternaria brassicicola PF 227
Verticirium dahliae PF 229
Altenaria alternate PF 261

* PF 10, phytopathogenic fungus No. 10 (of our stocks).

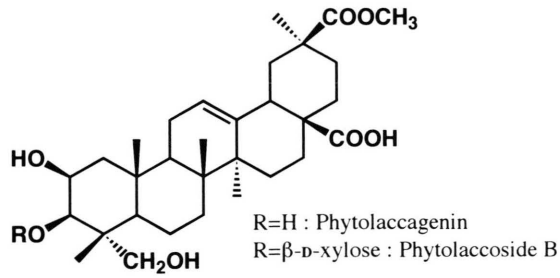

Fig. 1. Structures of phytolaccoside B (**1**) and phytolaccagenin (**2**).

Table II. Medium requirements for establishment of callus tissue cultures.

Mother plants	Medium	Hormones (μM)			
Ageratum conyzoides	MS	NAA	1.0	kinetin	1.0*
Annona squamosa	MS	NAA	2.7	BA	2.2
Crassocephalum crepidioides	MS	NAA	10.7	BA	2.2
Nicotiana tabacum	MS	NAA	4.3	BA	2.2
Petunia hybrida	LS	2,4-D	1.5	BA	0.1
Phytolacca americana	LS	2,4-D	4.5		

NAA, naphthalene acetic acid; BA, benzyl adenine; 2,4-D, 2,4-dichlorophenoxyacetic acid. Each medium was containing 3.0% sucrose and 0.2% GELRITE (SAN-EI, Osaka).
* 0.3 mM gibberellins was added.

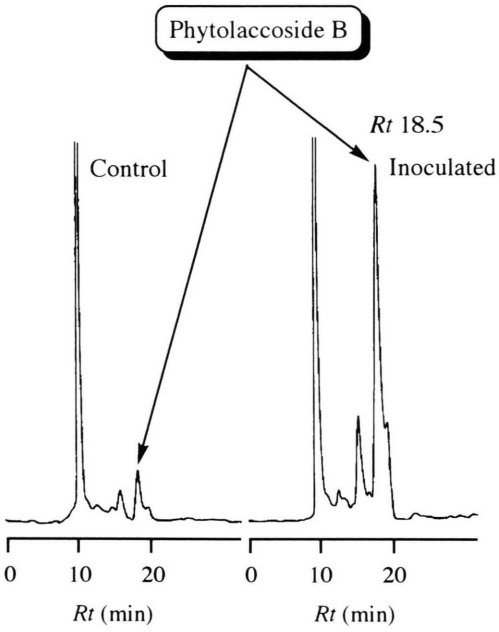

Fig. 2. HPLC profiles of the methanolic extract of *P. americana* callus inoculated or non-inoculated with *B. fabae*.

methanolic extract of *P. americana* callus inoculated with *B. fabae* inhibited the spore germination of *C. herbarum* at a minimum inhibitory concentration (MIC) of 125 μg/ml. This activity was observed in the methanolic extract of the dual culture but not observed in that of the individual cultures (MIC, >1000 μg/ml). Therefore, this evidence suggested that some antifungal substances were produced in the callus after inoculation of the fungus. The ethyl acetate phase (3.1 g) from the methanolic extract of the dual culture (1.6 kg) showed a strong antifungal activity (MIC, 30 μg/ml) and afforded two new spots on silica gel TLC by a spray reagent, vanillin-sulfuric acid. Repeated column chromatography, guided by the antifungal test, enabled us to isolate compounds, **1** (64 mg) and **2** (20 mg). Compounds **1** and **2** showed MICs of 15.6 and 250 μg/ml against *C. herbarum*, respectively. Compound **1** was characterized as a blue spot on silica gel TLC (*Rf* = 0.41; benzene–acetone–methanol, 7:2:2) when heated after spraying with vanillin–sulfuric acid, and also showed positive coloration against diphenylamine and $AgNO_3$ solutions. The ^{1}H-NMR spectrum showed an anomeric proton signal at δ 5.05 (d, *J* = 7.6 Hz), an olefinic proton signal at δ 5.60 (br. s), a methoxyl proton signal at δ 3.65 (s), five tertiary methyl protons at δ 1.04 (s), 1.19 (s), 1.26 (s), 1.32 (s), 1.54 (s). These findings suggested that this compound should be a steroidal saponin. In the LC-MS experiment a molecular ion peak at *m/z* 665 $[M+H]^+$ and a characteristic fragment at *m/z* 515 $[M+H\text{-}150]^+$ also support that this compound had

a triterpene skeleton with one pentose unit. The spectral data in IR: 1708 cm^{-1}, ^{1}H-NMR: δ 3.65 (3H, s), and ^{13}C-NMR: δ 179.1 and δ 51.0 also suggested the presence of a methoxycarbonyl group. Acid hydrolysis of **1** with 1 N HCl afforded quantitatively a triterpene and a sugar. The resulting sugar was treated with 2-aminopyridine to give a PA-derivative, which was identified to be PA-xylose by comparing with an authentic sample of PA-xylose in the HPLC experiment. These results as well as the literature survey let us identify **1** to be Pls B, isolated from *Phytolacca* plants (Woo *et al.*, 1978; Haraguchi *et al.*, 1988).

Compound **2** was also characterized as a blue spot on silica gel TLC (*Rf* = 0.57; benzene–acetone–methanol, 7:2:2) when heated after spraying with the same coloration agent mentioned above. The ^{1}H-NMR spectrum of **2** was identical to that of the triterpene, obtained by the acid hydrolysis of **1**. Therefore, **2** was determined to be phytolaccagenin, the agricone of Pls B.

Callus holds the potentiality to respond to biological stimuli, and consequently induces enzymes responsible for the secondary metabolite production. Oxidative and hydrolytic enzymes activated in the infected tissues convert cell components into oxidized or hydrolyzed compounds which play an important role in the defense system of the margin of the lesion. Such antimicrobial compounds are classified as post-inhibitins, one of preanticipants (Ingham, 1972 and 1973). An antifungal constituent, Pls B, and its analogue with a longer glycoside chain have been isolated from *Phytolacca* plants as molluscicidal saponins (Thilborg *et al.*, 1993) or antiinflammatory saponins and so on (Yi, 1991, and 1992). However, none of the triterpene glycosides (saponins) so far found in *P. americana* tissue culture system have been reported to possess significant antifungal properties. Only a trace amount of Pls B was detected in the fresh callus. The amount of Pls B in the dual culture system was 6 times as high as that of the control (the long-term cultured callus). The fact that the infected callus produced Pls B supports the possibility that a prototype of Pls B could be present in the normal callus, and this compound should be released in response to fungal invasion. This may suggest that Pls B could play an important role in the defense system of the mother plant in the field as does the proteinous antiviral constituent of this plant (Irvin *et al.*, 1980; Barbieri *et al.*, 1982). So, we inoculated this fungus onto young leaves of *Phytolacca* plant grown in a green house. However, infection was not established. This suggests that the *Phytolacca* plant and the *Botrytis* fungus are incompatible. When the fungal mat of *B. fabae* was autoclaved and applied to the callus, no significant accumulation of Pls B was seen, indicating that the viability of the fungus was essentially required for producing the antifungal compounds. These findings suggest that the dual culture method we adopted is useful to produce novel metabolites in the tissue culture systems. A number of random combinations with different pathogens and calli can be made in a simple method, and the dual cultures are easily established since calli no more sustain effective defense systems found in the mother plants, and in some combinations we have detected new metabolites and the results will be soon reported.

Experimental

Spectral analyses

^{1}H- and ^{13}C-NMR spectra were recorded with a Varian VXR-500 instrument. Mass spectra were recorded with a Sciex API-III instrument. IR and UV spectra were recorded with a Nicolet 710 FT-IR and a Shimadzu UV-3000 spectrophotometer, respectively. Optical rotation was measured with a Jasco DIP-360.

Plant material

Plant cell lines were established as follows. Young plant tissues were sterilized with 2% sodium hypochlolide solution for 5 min, and then rinsed three times with sterilized water. The plant tissues were cut into small sections (*ca.* 5×5 mm), which were placed on the MS or LS medium. The callus clumps induced sporadically from the plant tissue sections were dissected and transferred onto the fresh medium. Repeated transplantation to a new medium resulted in establishment of the individual cell lines with marked growth rate (growth increment in fr. weight by two times in 4 days).

Inoculation of fungal pathogens on plant culture

Small aggregates of the callus were transplanted in culture bottles containing the fresh medium and

incubated at 25 °C. Fungal pathogens were grown in 10 ml of a potato-malt extract-sucrose medium with shaking at 27 °C for 7 days. 100 µl of the resulting cultures was inoculated on plant calli grown for 14 days after transplanted. Fungal pathogens developed on the calli, and fungal mat covered each callus surface within 7–10 days. At this point of harvest the callus still survived.

Antifungal test (Kobayashi *et al.*, 1993)

A fungus, *C. herbarum* was grown on the slant culture using a potato-malt extract-sucrose agar medium at 27 °C for 7 days to form a well-expanded fungal mat with spores. The spores were suspended in 50 ml of a liquid medium containing glucose 0.2%, yeast extract 0.1%, Na_2HPO_4 $12H_2O$ 0.37% and citric acid monohydrate 0.1%. Spore-suspension containing the test sample were placed in the wells of a 96-well microplate at 27 °C for 24 h. The spore germination was examined under a microscope.

Extraction and purification

The dual culture of *P. americana* callus and *B. fabae* (fresh weight: 1.6 kg) was soaked in methanol (2 l) over night. The methanolic extract was concentrated *in vacuo* to give a brownish oil (12.2 g). The oil was suspended in 200 ml of water, and partitioned with ethyl acetate (300 ml×3). The ethyl acetate phases were combined and concentrated *in vacuo*, and then subjected to a silica gel column (∅ 4.0×30 cm, Wakogel C-100), eluted stepwise with acetone in benzene with increasing polarity. Each fraction was monitored by the antifungal test. The 100% acetone eluate showed a strong activity. This fraction was concentrated, and then loaded on the top of Sephadex LH-20 column (∅ 2.0×40 cm, Pharmacia-LKB, Sweden), eluted with methanol–dist. water (7:3, 30 ml×13 fractions) and finally eluted with 100% methanol. The activity was found in fractions 3–8. The highly active fractions were combined. The active constituents were further purified with an ODS column (∅ 1.0×100 cm, Millipore Preparative C18), eluted in a linear gradient system (methanol–dist. water) to give Pls B (64 mg). Phytolaccagenin (20 mg) was purified from the 100% methanol eluate by preparative TLC.

The properties of Pls B

Pls B. $[\alpha]_D$ (*c* 1.0 MeOH): +80.8°, LC-MS m/z: 1329 $[2M+H]^+$, 682 $[M+NH_4]^+$, 665 $[M+H]^+$, 515 $[M+H\text{-}150]^+$; UV λ_{max} (MeOH) nm (ε): 210 (2850); IR ν_{max} (KBr) cm^{-1}: 3397, 2944, 1708, 756; NMR δC (125 MHz, pyridine-d_5): 14.3, 16.5, 16.7, 17.2, 23.1, 23.2, 25.5, 27.6, 27.7, 30.1, 32.3, 33.8, 36.3, 39.1, 41.5, 41.9, 42.1, 42.6, 43.4, 43.5, 45.4, 47.0, 47.8, 51.0, 64.5, 66.5, 70.2, 70.3, 74.6, 77.8, 82.1, 106.1, 122.6, 143.7, 176.4, 179.1; NMR δH (500 MHz, pyridine-d_5): 1.04 (3H, s), 1.19 (3H, s), 1.26 (3H, s), 1.32 (3H, s), 1.54 (3H, s), 1.68–1.83 (4H, m), 1.92–2.34 (8H, m), 3.26 (1H, brd, J = 13.7 Hz), 3.65 (3H, s), 3.98 (1H, dd, J = 7.6, 8.6 Hz), 4.07 (1H, dd, J = 8.6, 8.7 Hz), 4.14 (1H, ddd, J = 5.3, 8.7 Hz), 4.25 (1H, dd, J = 5.3, 10.7 Hz), 4.28 (1H, br. s), 4.36(1H, d, J = 10.7 Hz), 4.74 (1H, br. s), 5.05 (1H, d, J = 7.6 Hz), 5.60 (1H, br. s).

Identification of the sugar moiety

The component sugar of Pls B was analyzed according to the method of J. Suzuki *et. al* (1991). Pls B (2 mg) was hydrolyzed by adding 2 ml of 1 N HCl and heated at 100 °C for 3 hrs. The solution was partitioned with ethyl acetate. Water solution was evaporated and to the residue was added 20 µl of a coupling reagent prepared by mixing 100 µg of 2-aminopyridine, 50 µl of acetic acid, and 60 µl of methanol and then heated at 90 °C for 15 min. The excess reagents were removed with nitrogen gas. Then, 30 µl of a reducing reagent prepared by mixing 5.9 mg of borane-dimethylamine complex and 100 µl of acetic acid was added. The reaction mixture was heated at 90 °C for 30 min. The excess reagents were removed with nitrogen gas. The residue was dissolved in 500 µl of water and analyzed by HPLC. HPLC analysis was carried out on a TSK gel Sugar AXI column (∅ 4.6×150 mm, TOSOH, eluent: 90% of 0.7 M boric acid, adjusted to pH 9 with potassium hydroxide, in acetonitrile, flow rate: 0.3 ml/min, detection: fluorescence of an excitation wavelength of 310 nm and an emission wavelength of 380 nm).

HPLC analysis of Pls B

A part of the methanolic extracts of callus samples (inoculated or non-inoculated with *B. fabae*)

were partitioned with ethyl acetate, and washed with *n*-hexane, respectively. 4 µl of each sample adjusted to 10,000 ppm with HPLC eluent (50% acetonitrile) was subjected to HPLC. HPLC analysis was carried out on a Inertsil ODS column (∅ 4.6×250 mm, GL Sciences, eluent: 50% acetonitrile, flow rate: 0.3 ml/min, detection: RI).

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Barbieri L., Aron G. M. Irvin J. D. and Stirpe F. (1982), Purification and partial characterization of another form of the antiviral protein from the seeds of *Phytolacca americana* L. (pokeweed). Biochem. J. **203**, 55–59.

Haraguchi M., Motidome M. and Gottlieb O. R. (1988), Triterpenoid saponins and flavonol glycosides from *Phytolacca thyrsiflora*. Phytochemistry **27** (7), 2291–2296.

Ingham J. L. (1972), Phytoalexins and other natural products as factors in plant disease resistance. Botan. Rev. **38**, 343–424.

Ingham J. L. (1973), Disease resistance in plants: the concept of pre-infectional and post-infectional resistance. Phytopathol. Z. **78**, 314–335.

Irvin J. D., Kelly. T. and Robertus J. D. (1980), Purification and properties of a second antiviral protein from *Phytolacca americana* which inactivates eukaryotic ribosomes. Arch. Biochem. Biophys. **200**, 418–425.

Kobayashi A., Akiyama K. and Kawazu K. (1993), A pterocarpan, (+)-2-hydroxypisatin from *Pisum sativum*. Phytochemistry **32** (1), 77–78.

Kobayashi A., Koguchi Y. Kanzaki H., Kajiyama S. and Kawazu K. (1994), Production of a new type of bioactive phenolic compound. Biosci. Biotech. Biochem. **58** (1), 133–134.

Kohmoto K. and Otani H. (1991), Host recognition by toxigenic plant pathogens. Experientia **47** (8), 755–764.

Purkayastha R. P. (1976), Induction of phytoalexins in relation to plant disease control. Sci. Cult. **42** (12), 586–590.

Suzuki J., Kondo A., Kato I., Hase S. and Ikenaka T. (1991), Analysis by high-performance anion-exchange chromatography of component sugars as their fluorescent pyridylamino derivatives. Agric. Biol. Chem. **55** (1), 283–284.

Thilborg S. T., Christensen S. B., Cornett C., Olsen C. E. and Lemmich E. (1993), Molluscicidal saponins from *Phytolacca dodecandra*. Phytochemistry **32** (5), 1167–1171.

Woo W. S., Kang S. S., Yamasaki K. and Tanaka O. (1978), Carbon-13 NMR spectra of phytolaccagenin and its glycosides. Arch. Pharm. Res. **1** (1), 21–25.

Yi Y. H. (1992), A triterpenoid saponin from *Phytolacca esculenta*. Phytochemistry **31** (7), 2552–2554.

Yi Y. H. (1991), A triterpenoid and its saponin from *Phytolacca esculenta*. Phytochemistry **30** (12), 4179–4181.