Phytotoxins Produced by Germinating Spores of *Bipolaris oryzae*

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ABSTRACT

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Bipolaris oryzae produces ophiobolin A and ophiobolin B during spore germination. Ophiobolin A, 6-epiophiobolin A, anhydroophiobolin A, 6-epianhydroophiobolin A, and ophiobolin I are produced during culture in a nutrient medium. Each compound showed nonselective phytotoxicity to host and nonhost plants in assays for root elongation and induction of leaf chlorosis. Ophiobolin A was most toxic, with an ID_{50} value of 5 μg/ml, followed by 6-epiophiobolin A, with an ID_{50} value of 10 μg/ml for inhibition of root elongation. Others were toxic only at levels of more than 100 μg/ml. At a concentration of approximately 3 μg/ml, ophiobolin A also induced susceptibility of rice leaf tissues to a nonpathogenic isolate of *Alternaria alternata*. However, ophiobolins are not likely to function as disease determinants of *B. oryzae*, because their concentrations in spore-germination fluids were only about one one-thousandth of that required for biological activity. The spore-germination fluids nevertheless exhibited extensive susceptibility-inducing activity and phytotoxicity. These activities were host selective for rice and were detectable soon after spore germination, when ophiobolins were not detectable at effective concentrations.

Bipolaris oryzae (Breda de Haan) Shoemaker (syn. *Helminthosporium oryzae* Breda de Haan, the anamorph of *Cochliobolus miyabeanus* (Ito & Kuribayashi) Drechsler), the causal agent of brown leaf spot disease of rice, is known to produce C_{25}-terpenoid phytotoxins called ophiobolin A (or cochliobolin A) and ophiobolin B (or cochliobolin B) during culture in nutrient media (2,7,15,17). The ophiobolins are phytotoxic and cause inhibition of root- and coleoptile-elongation and leaf-chlorosis on many plants including hosts and nonhosts of *B. oryzae* (8,17).

Ophiobolin A and chemically related compounds also were isolated from other *Cochliobolus* spp. (2–6,12,13,15,20). Sugawara et al (20) reported the production of six ophiobolins during culture by *B. maydis* (Nisikado & Miyake) Shoemaker and *B. sorghicola* (Lefebvre & Sherwin) Alcorn. Among them, 6-epiophiobolin A from *B. maydis*, a pathogen of corn, was selectively toxic to corn bearing Texas-male-sterile cytoplasm, when tested in a dark CO_2 fixation assay. These results suggested that 6-epiophiobolin A may play a critical role in disease development.

Work on ophiobolins, however, had been focused on their chemistry as metabolites of phytopathogenic fungi, rather than on the phytopathological significance of their production. Oku (16) detected the presence of ophiobolin A in ungerminated conidia and in diseased leaf tissues, but did not evaluate its production in spore-germination fluids of *B. oryzae*. From a pathological point of view, however, metabolites released from germinating spores may be more important for the initial events of the infection process than those from culture filtrates or other sources (9–11). In our preliminary experiments (24), nonpathogenic *Alternaria alternata* (Fr.) Keissler caused a large number of disease lesions on rice leaves when the fungus was inoculated together with spore-germination fluids of *B. oryzae*. This implies that the most interesting principles for understanding early events in the pathogenesis of *B. oryzae* may be provided by a careful and extensive analysis of metabolites produced and released from germinating spores of the fungus. The objectives of the present study were to conduct a critical evaluation of the role of ophiobolins in pathogenesis and to search for pathogenicity factor(s) produced by *B. oryzae* by analysis of physiologically active compounds in spore-germination fluids.

MATERIALS AND METHODS

Plants. All plants were grown in a glasshouse during spring, summer, and autumn, and in a phytotron at 25 C during daytime and 20 C during nighttime in winter. Detached young leaves of rice at the 4–6 leaf stage and young leaves of the other plants were used in all experiments.

Culture of the fungus. A pathogenic isolate KU-13 of *B. oryzae*, which is a stock culture in our laboratory, was used throughout the experiments. The fungus was maintained on potato dextrose agar and was cultured in 1-liter Roux bottles, each containing 400 ml of potato dextrose broth, at 28 C. After 2-wk of still culture, culture filtrates were harvested by sequential filtration through two layers of gauze and filter paper. The filtrates were used for detection and isolation of ophiobolins.

Preparation of spores and spore-germination fluids. Conidia were prepared according to a method developed for *Pyricularia oryzae* Cavara (18). The fungus was inoculated in petri dishes containing 30 ml of oatmeal-V8 juice agar (18) and cultured for 5 days at 28 C in the dark. The mycelial surfaces, laden with spores, were rubbed with a brush and floated with distilled water, and then the resultant spore suspensions were filtrated through two layers of gauze to eliminate mycelial debris. The spores were collected on filter paper and thoroughly washed with distilled water. The collected spores were resuspended in distilled water to a final concentration of 5×10^5 spores/ml, and the suspension was uniformly sprinkled onto paper towels (27 × 23 cm, Lion Co., Ltd., Tokyo, Japan) placed in a moist chamber. The chambers were incubated at 24 C for appropriate periods under fluorescent light. Spore germination was checked microscopically, and the spore-germination fluids were harvested by squeezing the towels and filtering the liquid through filter paper (Toyo No. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to remove the spores.

Isolation of ophiobolins. Culture filtrates and spore-germination fluids were subjected to the same procedure. At one time, 10 L of culture filtrates or spore-germination fluids of *B. oryzae* were collected and extracted three times with equal volumes of ethyl acetate. The extracts were evaporated to dryness at 40 C under reduced pressure. The residue was subjected to silica gel (Wako gel C-200, Wako Pure Chemical Industries, Ltd., Osaka, Japan) column chromatography, and the column was washed stepwise with chloroform, chloroform/acetone (9:1), chloroform/acetone (4:1), and acetone. Only the chloroform and chloroform/

acetone (9:1) eluates contained phytotoxic substances. These two fractions were further fractionated by preparative thin-layer chromatography (TLC) (silica gel 60/F-254, 0.5 mm in thickness, Merck) with a solvent system of benzene/ethyl acetate/acetic acid (50:50:1, v/v). Ophiobolins were detected on TLC plates by illumination at 254 nm and finally analyzed by high-performance liquid chromatography (HPLC) with a reverse phase column (ODS-7, 4.6 × 250 mm, Nomura Chemical Co., Ltd., Aichi, Japan). Samples were eluted with acetonitrile/water (80:20, v/v) at a flow rate of 0.5 ml/min and monitored with a multi channel detector (MULTI-320, Japan Spectroscopic Co., Ltd., Tokyo, Japan), which provided UV absorption spectra of each of the fractionated substances in the wavelength range between 200 and 350 nm. Substances with maximum absorption within 225 to 260 nm were pooled and further purified by a preparative HPLC system on a reverse phase column (ODS-10, 20 × 250 mm, Nomura Chemical Co., Ltd.) with a mobile phase of acetonitrile/water (80:20, v/v) at a flow rate of 5 ml/min. Chemical characterization of isolated substances was performed with a Ubest-30 UV-spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) and proton nuclear magnetic resonance (NMR) analysis with a JNM-FX 200 spectrometer (JEOL, Ltd., Tokyo, Japan). Information concerning chemical structures was obtained from proton-NMR spectra by comparison with those of ophiobolins previously reported (1,2,6,13-15,20,21).

Leaf puncture assay. Centers of detached plant leaves were nicked with needles, and the damaged leaf surfaces immediately were covered with 20 μl of sample solution. After a 48-hr incubation at 24 C in a moist chamber, development of chlorosis and/or necrosis around the nicked portion was observed.

Root assay. Seeds of rice and other plants were surface-sterilized with 1000 × Topsin water solution (Nipponsoda Co., Ltd., Tokyo, Japan) and germinated in distilled water. Seeds with signs of germination were selected and transferred to a filter paper (2 cm in diameter) in a petri dish (2.5 cm in diameter) containing 1 ml of sample solution to be tested. After a 72-hr incubation at 24 C under fluorescent light, root length of seedlings was measured.

Assay of susceptibility-inducing activity. Spores of a nonpathogenic isolate 0-94 of *A. alternata*, which is a stock culture in our laboratory that had been obtained from airborne fungi, were used as a living probe in this assay. The spores that were prepared as previously described (22) were suspended at a concentration of 10^6/ml in the test solution containing 0.05% Tween 80. One milliliter of the spore suspensions was then inoculated on three detached leaves by spraying with an atomizer. Spore suspensions without the sample and the sample solutions without spores were inoculated as controls. Inoculated leaves were incubated in a moist chamber at 24 C, and reactions of the plant leaves were scored at 48 hr.

RESULTS

Ophiobolin production. During isolation and characterization of compounds belonging to the ophiobolin series, we detected compounds with proton-NMR spectra identical to those of ophiobolin A, 6-epiophiobolin A, anhydroophiobolin A, 6-epianhydroophiobolin A, and ophiobolin I in culture filtrates; but only ophiobolin A and a compound identical to ophiobolin B were found in spore-germination fluids (Fig. 1). The retention times in HPLC analysis, R_f values, wavelength of maximum UV absorption, and concentrations of each compound detected in culture filtrates and spore-germination fluids are summarized in Table 1.

Phytotoxic activity of ophiobolins. When each ophiobolin was tested for activity to inhibit root elongation of rice seedlings, ophiobolin A and 6-epiophiobolin A were found to be more toxic as compared with anhydroophiobolin A, 6-epianhydroophiobolin A, and ophiobolin I (Fig. 2). The ID_{50} values of ophiobolin A and 6-epiophiobolin A were approximately 5 and 10 μg/ml, respectively. The ID_{50} values of anhydroophiobolin A and 6-epianhydroophiobolin A were approximately 100 μg/ml. Ophiobolin I showed very weak phytotoxicity with an IR_{50} value of more than 500 μg/ml. Ophiobolin A was further analysed for inhibition of root elongation of other plants, and their sensitivities were found to be similar to that of rice (Fig. 3).

Ophiobolin A was also the most toxic one to leaves of all plants tested, but it exhibited no host selectivity in a leaf puncture assay (Table 2). Ophiobolin A induced leaf chlorosis on all plant leaves tested at a minimum concentration of 1–5 μg/ml. The other less toxic ophiobolins also showed nonselective leaf chlorosis-inducing activity and affected both host and nonhost leaves.

Susceptibility-inducing activity of ophiobolins. When ophiobolin A at concentrations higher than 3.1 μg/ml was mixed with a spore suspension of *A. alternata* and sprayed on detached leaves of rice plants, disease lesions appeared within 2 days (Fig. 4). Spraying of ophiobolin A alone also caused toxic lesions on rice leaves, but only at concentrations higher than 12.5 μg/ml. Application of the spores and ophiobolin A at concentrations higher than 12.5 μg/ml induced more numerous and larger-sized lesions than those of ophiobolin A alone at all concentrations tested.

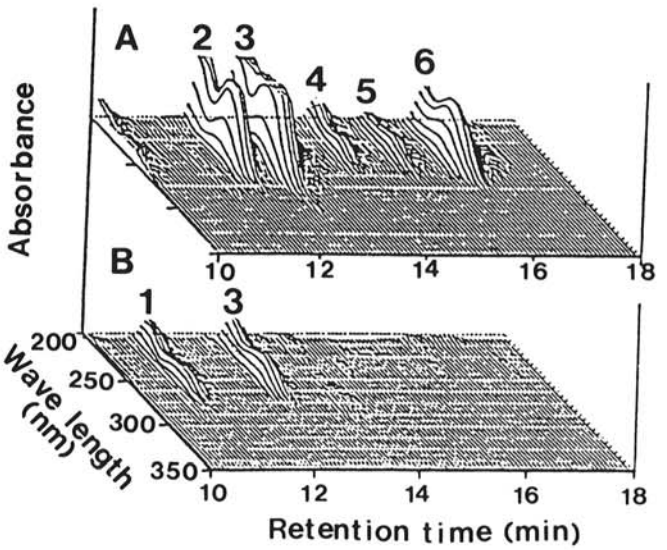

Fig. 1. High-performance liquid chromatography of the mixture of ophiobolins isolated from **A,** culture filtrates and **B,** spore-germination fluids of *Bipolaris oryzae*. The culture filtrates and spore-germination fluids were harvested after 2-wk culture and 24-hr incubation, respectively. 1, ophiobolin B; 2, 6-epiophiobolin A; 3, ophiobolin A; 4, ophiobolin I; 5, 6-epianhydroophiobolin A; and 6, anhydroophiobolin A.

TABLE 1. Characteristics of ophiobolins isolated from culture filtrates and spore-germination fluids (SGF) of *Bipolaris oryzae*

Compound	R_T[a] (min)	R_f[b]	λmax (nm)	Concentration (μg/ml)[c] CF[d]	SGF[e]
Ophiobolin A	14.1	0.60	236	43.0	2.7×10^{-3}
Anhydroophiobolin A	18.0	0.66	230	11.9	ND
6-Epiophiobolin A	13.2	0.55	235	15.5	ND
6-Epianhydroophiobolin A	16.8	0.62	229	4.3	ND
Ophiobolin B	12.5	0.37	237	ND	11.0×10^{-3}
Ophiobolin I	15.7	0.44	229	3.0	ND

[a] Retention time (R_T) was determined by high-performance liquid chromatography (HPLC) (ODS-7μm, 4.6 × 250 mm) with a mobile phase of 80% CH_3CN at a flow rate of 0.5 ml/min.
[b] R_f was determined by thin-layer chromatography with a solvent system of benzene/ethyl acetate/acetic acid, 50:50:1 (v/v).
[c] The crude ethyl acetate-extracts of culture filtrates and spore-germination fluids were fractionated by silica gel column, and the eluates containing ophiobolins were directly subjected to analytic HPLC for determination of ophiobolin contents.
[d] Filtrates of 2-wk old cultures (CF).
[e] SGF after incubation for 24 hr.
[f] Not detected.

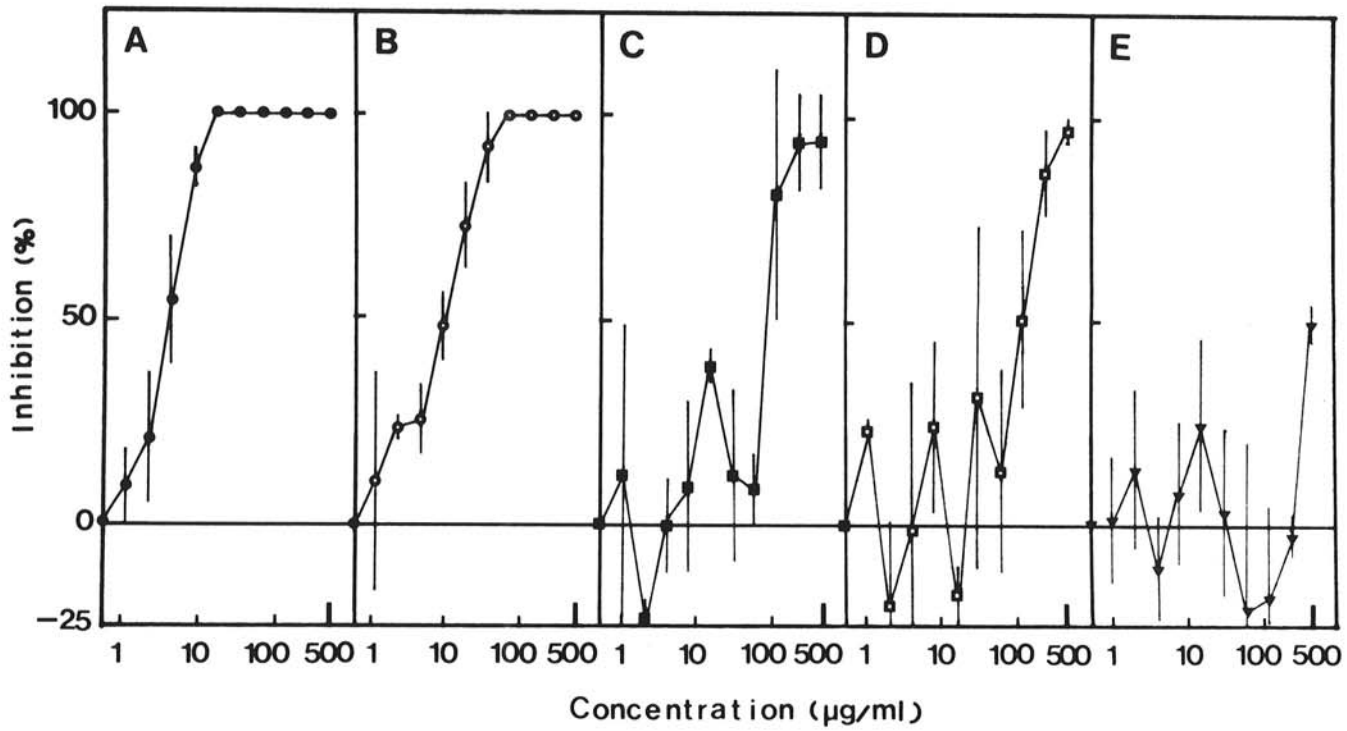

Fig. 2. Inhibitory effects of ophiobolins produced by *Bipolaris oryzae* on root elongation of rice seedlings. Seeds (cv. Sekiguchi-Asahi) were immersed in 1 ml of water containing each ophiobolin at designated concentrations for 72 hr, and then the root length of each seedling was measured. Percent inhibition is relative to a water control. Each value represents the mean of three determinations, and the vertical bar shows the standard deviation. **A,** ophiobolin A; **B,** 6-epiophiobolin A; **C,** anhydroophiobolin A; **D,** 6-epianhydroophiobolin A; and **E,** ophiobolin I.

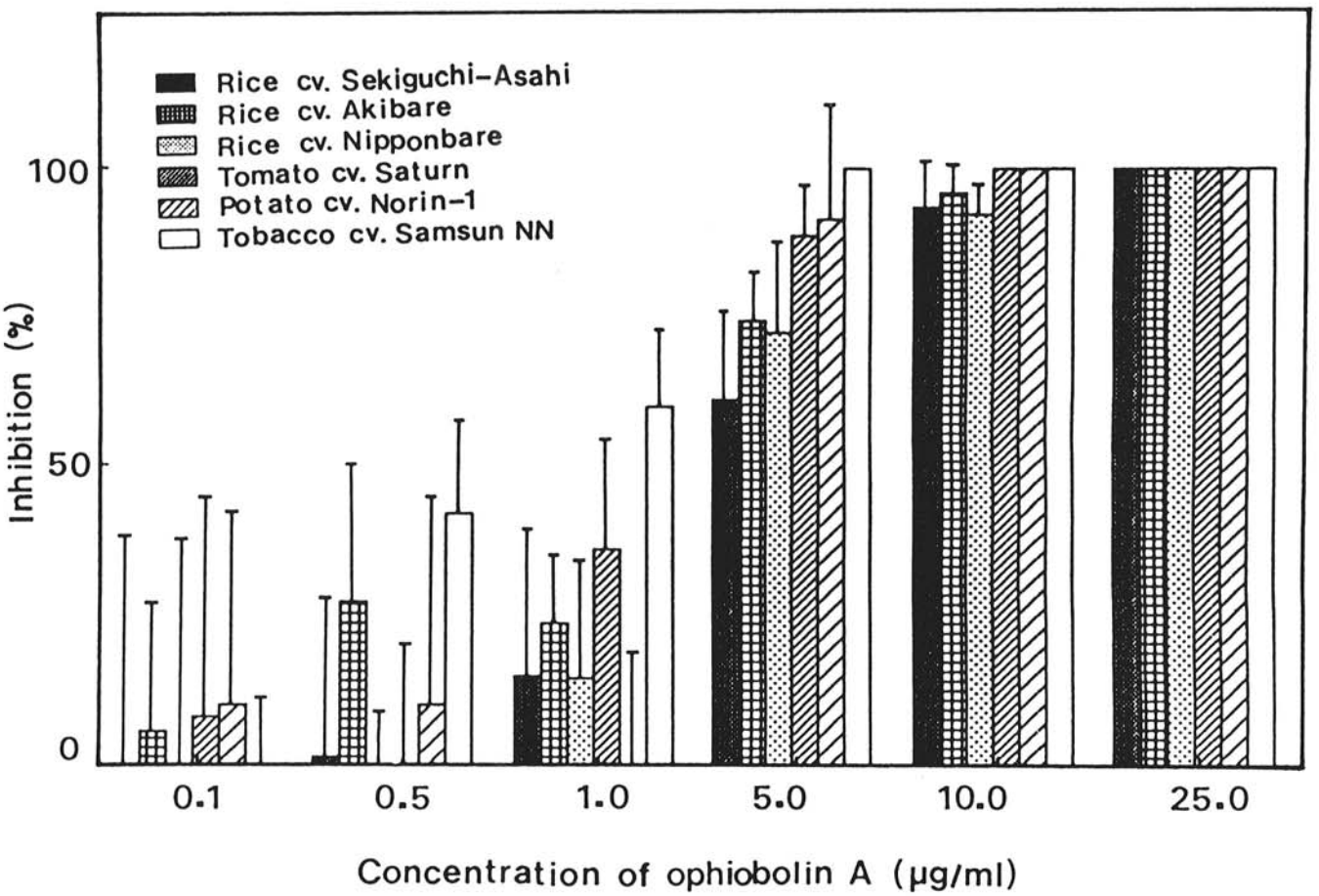

Fig. 3. Effect of ophiobolin A on root elongation of rice, tomato, potato, and tobacco seeds. Assay conditions are given in Fig. 2. Each value represents the mean of three determinants, and the vertical bar shows the standard deviation.

TABLE 2. Phytotoxicity of ophiobolins from *Bipolaris oryzae*

Plant[a]	Phytotoxicity[b] Concentration (μg/ml) 500	100	50	10	5	1	0.5
Ophiobolin A							
Cabbage	++	++	++	+	+	+	−
Corn	+++	+++	++	++	++	+	−
Rice	+	+	+	+	+	−	−
Tobacco	+	+	+	+	+	−	−
Anhydroophiobolin A							
Cabbage	+	+	+	−	−	−	−
Corn	+++	++	+	−	−	−	−
Rice	++	+	+	−	−	−	−
Tobacco	+	+	−	−	−	−	−
6-Epiophiobolin A							
Cabbage	+++	++	+	+	−	−	−
Corn	++	+	−	−	−	−	−
Rice	++	+	+	−	−	−	−
Tobacco	+	+	+	−	−	−	−
6-Epianhydroophiobolin A							
Cabbage	+	+	+	−	−	−	−
Corn	+	+	−	−	−	−	−
Rice	+	−	−	−	−	−	−
Tobacco	−	−	−	−	−	−	−
Ophiobolin B							
Cabbage	+	+	+	+	−	−	−
Corn	+	+	+	−	−	−	−
Rice	++	+	+	+	−	−	−
Tobacco	+	+	−	−	−	−	−
Ophiobolin I							
Cabbage	+	+	−	−	−	−	−
Corn	−	−	−	−	−	−	−
Rice	+	−	−	−	−	−	−
Tobacco	+	−	−	−	−	−	−

[a] Cabbage cv., Komochikanran; corn cv., BssS; rice cv., Sekiguchi-Asahi; and tobacco cv., Samsun NN.

[b] Evaluated by chlorosis-inducing activity to plant leaves: −, none; +, chlorosis within wound; ++, chlorosis up to 2 cm from wound; +++, chlorosis more than 2 cm from wound.

Spraying *A. alternata* spores without ophiobolin A caused no visible symptoms on the rice leaves. Ophiobolin B also showed similar activity, but higher concentrations (>50–100 μg/ml) were needed.

Susceptibility-inducing activity of spore-germination fluids. Spore-germination fluids of *B. oryzae*, which were harvested after 24 hr incubation, were concentrated 50-fold at 45 C under reduced pressure. When spores of *A. alternata* were suspended in the concentrated fluids and sprayed on leaves of rice plants, the fungus induced abundant lesions on the rice leaves. To characterize the susceptibility-inducing activity of spore-germination fluids of *B. oryzae*, time course studies on the activity and ophiobolin production of the germinating spores were carried out (Fig. 5). Most spores completed their germination within 3 hr after incubation, and germ tubes elongated during further incubation. The spore-germination fluids were harvested at various intervals, concentrated 50-fold, and assayed for susceptibility-inducing activity. Significant activity was first detected in samples taken after 6-hr incubation. It reached a maximum value at 9-hr incubation, and then decreased with further incubation time.

By contrast, ophiobolin A and ophiobolin B were detectable in only trace amounts until 12-hr incubation and then gradually increased with further incubation time. After 9-hr incubation, when susceptibility-inducing activity of germination fluids showed its maximum, ophiobolin A and ophiobolin B were present in spore-germination fluids at concentrations of approximately 0.001 and 0.002 μg/ml, respectively, as determined by HPLC analysis. These concentrations represented only approximately 0.1% of the effective concentration required for a susceptibility-inducing activity similar to the one exhibited by the spore-germinated fluids.

To determine if there was a host-selective activity for inducing susceptibility and/or chlorosis, spore-germination fluids (50-fold concentrated) obtained at 9-hr incubation were sprayed together with spores of *A. alternata* on rice, oats, wheat, cabbage, eggplant, and strawberry leaves. At 2 days after inoculation, numerous lesions appeared only on rice leaves, but not on the other plants tested (Table 3). In a leaf puncture assay, the spore-germination

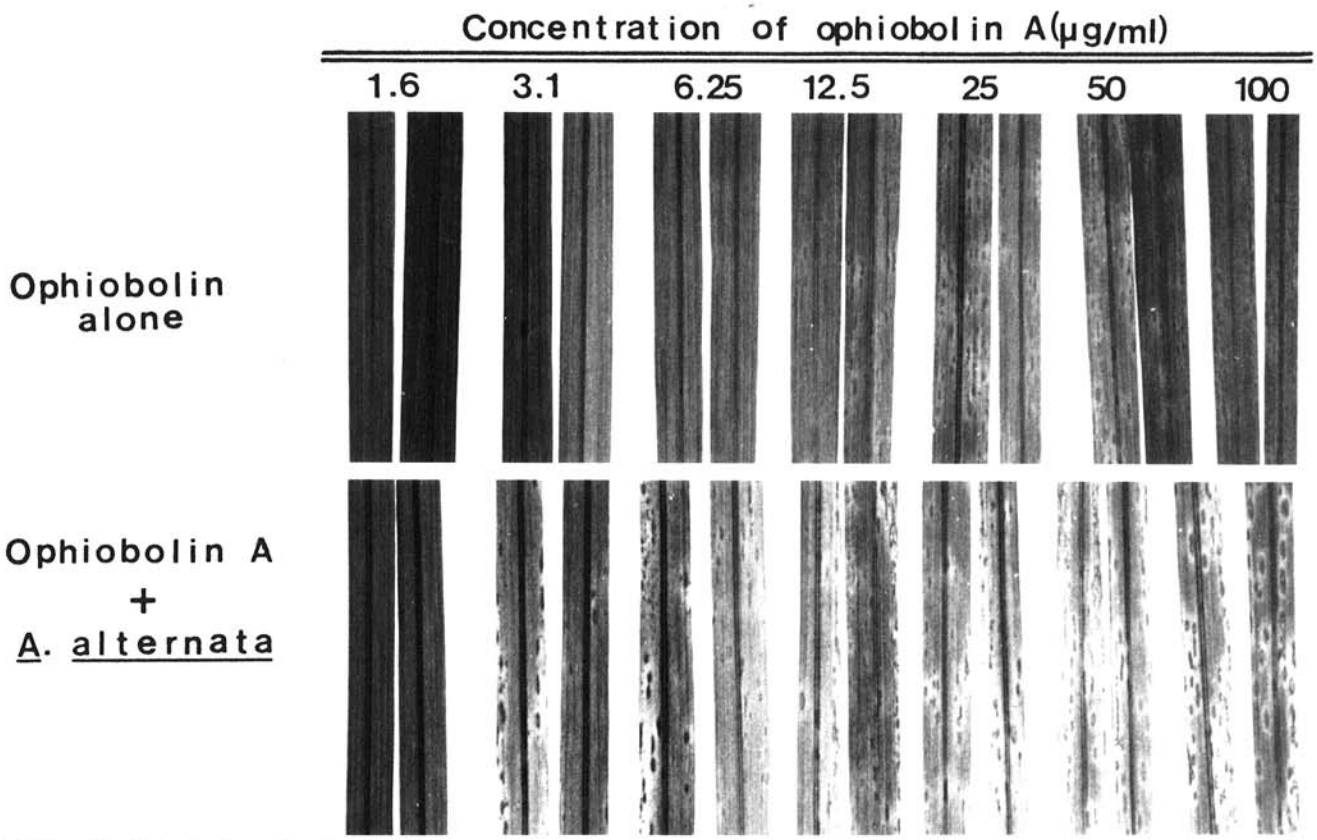

Fig. 4. Effect of ophiobolin A on the infection of rice leaves by saprophytic isolate 0-94 of *Alternaria alternata*. Rice leaves (cv. Sekiguchi-Asahi) were inoculated with spores (10^6 spores/ml) together with ophiobolin A at designated concentrations, or treated with ophiobolin solutions alone. Photograph was taken 48 hr after treatment.

fluids alone also selectively induced leaf chlorosis only on rice leaves (Table 3).

Effect of ophiobolin A and spore-germination fluids on colonization of rice leaf-sheaths by a nonpathogen. Spores of *A. alternata* were either suspended in ophiobolin A solution (50 μg/ml) or spore-germination fluids (50-fold concentrated) of *B. oryzae*, and then 0.5 ml of the suspension (1×10^4 spores/ml) was inoculated on the inner surface of detached rice leaf-sheaths and incubated as described for the assay of susceptibility-inducing activity. After 48 hr, spore germination, penetration, and hyphal growth inside the sheath tissues were examined microscopically. The spores with or without ophiobolin A and/or spore-germination fluids germinated well and formed appressoria and infection pegs on the sheath surface. Extensive inter- and intracellular hyphal growth was observed only in the sheath tissues that had been inoculated in the presence of ophiobolin A and/or spore-germintion fluids (Fig. 6). The spores without ophiobolin A or spore-germination fluids could not grow beyond the infection peg and did not grow inside the cell.

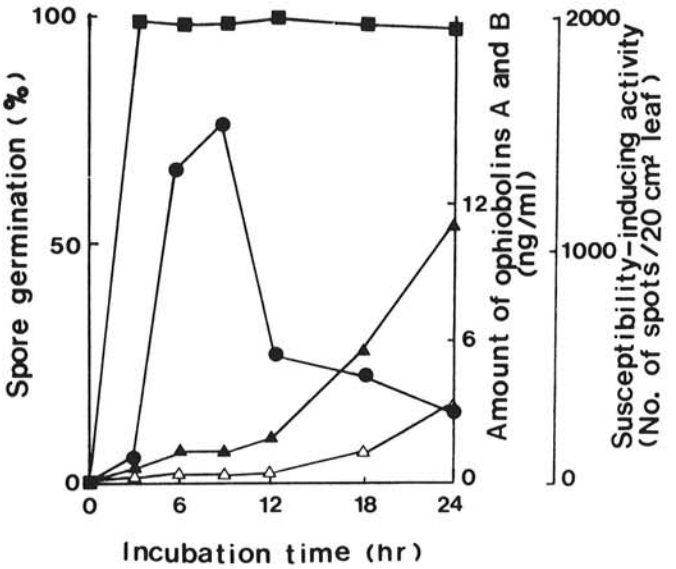

Fig. 5. Time course study of the concentrations of ophiobolin A (△) and ophiobolin B (▲), and of susceptibility-inducing activities (●) in spore-germination fluids of *Bipolaris oryzae*. Spore germination (■) was observed under the microscope. The susceptibility-inducing activity was assayed as described in Materials and Methods with rice cultivar Sekiguchi-Asahi. This experiment was repeated three times, and the results shown are from a single representive experiment.

TABLE 3. Susceptibility-inducing activity and phytotoxicity of spore-germination fluids (SGF) of *Bipolaris oryzae* to plant leaves

Plant[a]	Lesions per 20 cm[b]		Phytotoxicity[c]
	A. alternata + SGF	*A. alternata*	
Rice cv.			
Sekiguchi-Asahi	804[d]	0	+
Akibare	526[e]	0	+
Nipponbare	198[e]	0	ND[f]
Oats	4	0	−
Wheat	0	0	−
Cabbage	0	0	−
Eggplant	0	0	−
Strawberry	5	2	−
Tobacco	0	2	−
Tomato	2	0	−

[a] Oats cv., Sprinter; wheat cv., C4481; cabbage cv., Komochikanran; eggplant cv., Senryo-2; strawberry cv., Morioka-16; tobacco cv., Samsun NN; and tomato cv., Saturn.
[b] Number of lesions that appeared 48 hrs after inoculation with spores of *Alternaria alternata* isolate (0–94) in water or in 50-fold concentrated SGF.
[c] Evaluated by leaf chlorosis induced by 50-fold concentrated SGF on plant leaves in leaf puncture assay: −, none; +, severe.
[d] Large lesions with size of approximately 2 × 0.3 mm.
[e] Small lesions with size of approximately 0.2 × 0.1 mm.
[f] Not determined.

DISCUSSION

It is well documented that *B. oryzae* produces ophiobolin A and ophiobolin B in its mycelium or in culture medium (2,7,15,17). In the present study, six phytotoxic substances belonging to the ophiobolin series were detected as metabolites of *B. oryzae* (Table 1 and Fig. 1). 6-Epiophiobolin A, anhydroophiobolin A, 6-epianhydroophiobolin A, and ophiobolin I were confirmed as products of the fungus in addition to the two previously known compounds. *B. maydis, B. sorghicola, Helminthosporium zizaniae* Nisikado, *B. leersiae* (Akinson) Shoemaker, *Exserohilum turcicum* (Passerini) Leonard & Suggs, and *H. panici-miliacei* Nisikado also were reported to produce ophiobolins (3,4,12,19).

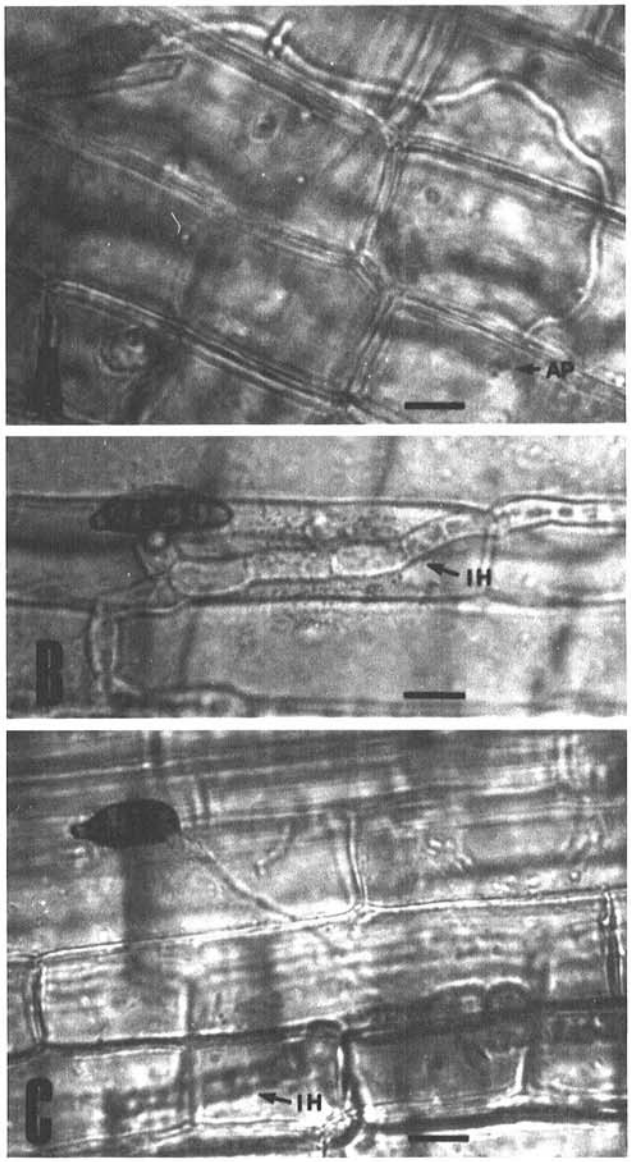

Fig. 6. Light micrographs of infection behavior of nonpathogenic *Alternaria alternata* on leaf-sheath of rice cultivar Sekiguchi-Asahi 48 hr after inoculation. **A,** Inoculated with *A. alternata* alone; **B,** inoculated with *A. alternata* in the presence of ophiobolin A (50 μg/ml); **C,** inoculated with *A. alternata* in the presence of spore-germination fluids of *Bipolaris oryzae* (50-fold concentrated). Abbreviations: AP = appressorium; IH = infection hypha. Each bar indicates 25 μm.

B. maydis is known to produce ophiobolin A, 6-epiophiobolin A, 6-epianhydroophiobolin A, ophiobolin C, ophiobolin I, and 25-hydroxyophiobolin I, while *B. sorghicola* is known to produce ophiobolin A, 6-epiophiobolin A, ophiobolin I, and 25-hydroophiobolin I during culture (20). Kim et al reported the production of ophiobolin A and its analogs including 6-epiophiobolin A, anhydroophiobolin A, and 6-epianhydroophiobolin A by an unidentified isolate of *Helminthosporium* (6).

Until now, much effort was done to elucidate the chemistry structures of the ophiobolins, but not much emphasis was paid to illustrate the structure-biological activity. Our results indicated that ophiobolin A and its 6-epimer exhibited almost the same phytotoxicity and were the most phytotoxic among the ophiobolins. Anhydrogation of ophiobolin A at the 3, 4-position severely decreased the phytotoxicity, and hydrogenation of ophiobolin A at the 21-position almost abolished the toxicity. Consequently, the aldehyde group at 21-position and the hydroxy group at 3, 4-position appear to be essential to its phytotoxicity of the ophiobolins.

Among the six ophiobolins, only ophiobolin A and ophiobolin B were detectable in the spore-germination fluids of *B. oryzae*. It can been assumed that the fungus probably produces these two ophiobolins also during spore germination on infected leaves. Ophiobolin A was the most biologically active one of the isolated ophiobolins; it inhibited seedling root elongation (Fig. 2), caused leaf chlorosis (Table 2), and induced susceptibility on rice leaves to a nonpathogen (Fig. 4) at concentrations of approximately 5 μg/ml. Ophiobolin A had also been shown to occur in infected leaves at concentrations of more than 100 μg/g fresh weight (16). These results suggest that ophiobolin A, like other host-selective toxins, may be required for successful penetration by *B. oryzae* (9–11,19,25,26). However, the detection at only nanogram levels in the spore-germination fluids even 24 hr after the start of germination (Fig. 5) make it questionable, because it may be too low of a concentration of ophiobolin A to actually be effective in the early steps of infection by *B. oryzae*. Thus, the role of ophiobolins in the pathogenesis of *B. oryzae* may determine the disease severity as virulence factors (26) rather than be directly involved in the initial step of infection.

To find host-selective factor(s) in spore-germination fluids of *B. oryzae*, we initiated studies of factors other than ophiobolins. Saprophytic *A. alternata* spores inoculated together with spore-germination fluids of *B. oryzae* produced abundant visible lesions on rice leaves, but not on nonhost plant leaves (Table 3). Further, this susceptibility-inducing activity was detectable at a high level in the early period of spore germination and could not be explained by the trace amounts of ophiobolins present in the fluids (Fig. 5). Therefore, these results indicate the possibility that germinating spores may produce unknown compound(s) which play a role as host-specific determinants.

The method we employed here to detect susceptibility-inducing activity had been used in several plant disease systems where host-selective toxins are involved in pathogenesis (9–11,19,25,26). In our study, the nonpathogenic isolate of *A. alternata* employed as a living probe failed to infect plant tissues by itself, producing no visible symptom on rice leaves. When the ophiobolins and/or spore-germination fluids of *B. oryzae* were present, the nonpathogen invaded and colonized the leaf-sheath tissues and produced symptoms on the leaves in a similar manner as the pathogen itself. These results indicate that the substances added actually induced a susceptibility of rice tissues which leads to the successful invasion and colonization by the nonpathogen and symptom expression.

We are now trying to isolate the compound(s) showing susceptibility-inducing activity from the spore-germination fluids. However, further effort is necessary to critically evaluate the activity as well as to isolate the compound(s) in pure state. Vidhyasekaran et al (23) reported that a host-selective toxin was produced by *B. oryzae*. Their toxin showed phytotoxicity to susceptible plants at the concentration of 0.5 μg/ml and to resistant plants at 100 μg/ml. Our duplication of their experiments has failed to identify such a toxin in culture filtrates of our isolate of *B. oryzae* (*unpublished data*). The identity of the putative host-specific disease determinants of *B. oryzae* is a subject for further investigation.

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