

Alternaria

alternata群病原菌における宿主特異的毒素生成の制御 (2)

化学物質による毒素生成の阻害

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Metabolic Regulation of Host-specific Toxin Production in *Alternaria alternata* Pathogens (2). Suppression of Toxin Production from Germinating Spores by Chemical Treatments*

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Abstract

The inhibitory effects of chemical treatments on toxin production during spore germination and pathogenicity of the spores of *A. alternata* pathogens were examined. The spores of *A. alternata* Japanese pear pathotype treated with cerulenin (20 ppm) or methionine (100 ppm) almost completely lost their pathogenicity to Japanese pear leaves susceptible to the black spot disease as well as productivity of host-specific AK-toxin during spore germination, whereas the abilities of spore germination, elongation of germ tube, appressorium formation and invading hypha formation were not significantly inhibited. These chemicals caused a similar reduction in pathogenicity and toxin production of apple pathotype (AM-toxin producer) and strawberry pathotype (AF-toxin producer) of *A. alternata*. The treatments of the spores of *A. alternata* Japanese pear pathotype with NH_4Cl , KH_2PO_4 , yeast extracts and L-cysteine also exhibited similar effects at higher concentrations. The above data suggest that the chemical treatments of the spores of *A. alternata* pathogens cause the reduction of their pathogenicity through the suppression of host-specific toxin productivity.

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Key Words : *Alternaria alternata* pathogen, host-specific toxin, metabolic regulation, methionine, cerulenin.

Introduction

Host-specific toxins known to date are considered to be secondary metabolites of plant pathogens. Generally, secondary metabolites have no benefit in the economy of the producing organisms. Host-specific toxins also are not exceptional cases. For example, even if *Alternaria alternata* pathogens have lost the ability to produce their toxins, they can grow on any culture media or any dead organic matters in the field, because they have saprophytic nature essentially, *viz.*, they change themselves from parasitic fungi to saprophytic ones in the mode of nutrition along with the alternation from toxin producer to nonproducer. In the field of plant pathology, however, host-specific toxins are found

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to play an important role in pathogenesis of plant disease. Many researchers working on host-specific toxins have attempted to understand the molecular basis of fungal infection and to evaluate the role of the toxins in the site of host-pathogen interaction⁶⁻⁸⁾. By the contribution of their researches, host-specific toxins have been demonstrated to be involved in initial colonization of the fungi in host tissues. The studies with a group of *A. alternata* pathogens showed that "release of toxin" from the germinating spores seems to lead to successful infection of spores in plant tissues^{3,12)}. Thus, if the toxin production is inhibited during the spore germination, the virulence of the pathogen can conceptually be expected to decrease.

Previous report¹⁶⁾ presented the evidence for the suppression of toxin production. Heat shock (*e. g.*, at 50 C for 10 sec in water) to the dormant spores of *A. alternata* Japanese pear pathotype (AK-toxin producer) before inoculation suppressed virulence to pear leaves; spore germination was not strongly inhibited, but the secretion of toxin from the germinating spores was significantly reduced.

The purpose of this work is to search some chemicals which have the inhibitory effect on the toxin productivity of spores of *A. alternata* during germination and to observe their effect on the pathogenicity. The present results should be helpful in understanding the importance of host-specific toxin as an initiation factor of successful pathogenesis. Brief reports of this work have been published^{9,14,15)}.

Materials and Methods

Plants. Japanese pear (*Pyrus serotina* Rhed.) cv. Nijisseiki susceptible to both of Japanese pear pathotype (AK-toxin producer) and strawberry pathotype (AF-toxin producer)⁴⁾ of *A. alternata*, and apple (*Malus pumila* Millar var. *domestica* Schneider) cv. Starking Delicious susceptible to *A. alternata* apple pathotype (AM-toxin producer) were used in this study, respectively. Freshly harvested young leaves of these two plants were used in all experiments.

Fungi. Highly virulent stock cultures of Japanese pear pathotype (No. 15A), apple pathotype (M-71) and strawberry pathotype (O-187b) of *A. alternata* were used in this study. Their spores were produced and maintained as described previously¹⁶⁾.

Chemical treatments of spores and assay for their effects. The effects of different classes of chemicals on toxin production from germinating spores of *A. alternata* pathogens were examined by the following methods. The spores were suspended in water at 2×10^6 spores ml⁻¹. Equal volumes of the spore suspension and chemical solution to be tested were mixed, placed on paper towels and incubated at 25 C. After specified incubation periods, spore-germinated fluids were harvested from the towels by squeezing and centrifuged at 2,000rpm for 5 min. The supernatants were then bioassayed for toxin production. The test solution were prepared by successive 2-fold dilutions with distilled water, and one drop of each solution was placed on a cross scratch of susceptible plant leaves. The effects of test chemicals were determined by comparing the maximum dilution end point for toxin-induced leaf necrosis of the germination fluids of chemical-treated spores with that of untreated ones (control).

In order to observe the infection behaviors of spores, the mixtures of spore suspen-

sion and chemical solution or distilled water were placed on small cellophane sheets (3 × 1 cm in size), prepared from Cellophane Tubing-Seamless for dialysis (Wako Pure Chemical Industries, Ltd.), and incubated in a moist chamber at 25 C. After various incubation periods, spore germination, elongation of germ tube, appressorium formation and invading hypha formation were observed. To determine the pathogenicity, detached young leaves of susceptible host plant were cut into two parts along their midribs. One part was inoculated with the spores suspended in chemical solution at 5×10^5 spores ml^{-1} , and the other part was inoculated with the spores suspended in distilled water at the same spore concentration. After incubation in a moist chamber at 25 C for 20 hr, the numbers of black necrotic spots appeared on the leaves were counted.

Results

Detection of chemicals having inhibitory effect on AK-toxin production from germinating spores of A. alternata Japanese pear pathotype

Many diverse chemicals were tested for the inhibitory effect on AK-toxin production during spore germination (Table 1). Among 26 chemicals tested, cerulenin, ethionine and methionine markedly inhibited the toxin production without significant inhibition of spore germination. Cerulenin and methionine gave obviously no effect to the elongation of germ tube, appressorium formation and invading hypha formation, but ethionine considerably retarded them. There was no difference in the degree of inhibitory effects of L-, D- and DL-methionines on the AK-toxin production. KH_2PO_4 , NH_4Cl , yeast extracts and L-cysteine showed moderately inhibitory effects at rather high

Table 1. Effects of different classes of chemicals on AK-toxin production from germinating spores of *A. alternata* Japanese pear pathotype

Chemical	Concentration (ppm)	Inhibitory effect on ^{a)} AK-toxin production
KH_2PO_4	5000	+
NH_4Cl	5000	+
NH_4NO_3	5000	—
NaCl	5000	—
Peptone	5000	—
Yeast extracts	5000	+
L-Arginine	5000	—
L-Cysteine	500	+
L-, D- & DL-Methionines	250	++
DL-Phenylalanine	5000	—
DL-Tryptophan	5000	—
L-Valine	5000	—
DL-Ethionine	250	++
N-Acetyl-D- β -phenylalanine	1000	—
Sodium N-lauroyl-L-valinate	500	—
γ -Aminobutyric acid	5000	—

Carnitine	500	—
Cerulenin	20	++
Coenzyme A (Co A)	500	—
Palmitoyl Co A	500	—
4-Pentenoic acid	500	—
Chymostatin	10	—
Pepstatin A	10	—
Trypsin inhibitor	10	—

- a) Spores suspended in chemical solution or in distilled water were placed on paper towel and incubated at 25 C for 24 hr. Inhibitory effects were determined by comparing the maximum dilution end point for toxin-induced leaf necrosis of spore-germinated fluids of chemical-treated spores with that of untreated ones and were classified as highly active (++), moderately active (+) and inactive (—).

concentrations. Other chemicals such as non-sulfur-containing amino acids and known protease inhibitors showed no significant effect.

Effects of cerulenin and methionine on pathogenicity of *A. alternata* pathogens

The treatment of spores of *A. alternata* Japanese pear pathotype with cerulenin or methionine led to a remarkable reduction of toxin productivity during spore germination. Then, cerulenin and L-methionine were employed for further observation of their effects on pathogenicity and infection behaviors (Table 2). By the treatment with 20 ppm cerulenin or 250 ppm L-methionine, the spores almost completely lost their pathogenicity without any visible damages on other infection behaviors such as germination, growth of germ tube, appressorium formation and invading hypha formation.

Time courses of spore germination and AK-toxin production of spores treated with

Table 2. Effects of treatments with cerulenin and L-methionine on infection behaviors and pathogenicity of virulent spores of *A. alternata* Japanese pear pathotype

Treatment	Concentration (ppm)	Infection behaviors				Pathogenicity ^{e)} (%)
		Spore germination ^{a)} (%)	Elongation of germ tube ^{b)} (%)	Appressorium formation ^{c)} (%)	Invading hypha formation ^{d)} (%)	
Control	—	100	100	100	100	100
Cerulenin	20	98.1±5.0	91.8±5.6	91.2±9.8	102.7±2.3	19.4±2.8
	40	91.8±7.2	85.9±6.3	92.0±14.6	89.3±13.0	11.5±3.5
L-Methionine	100	92.6±5.6	97.1±7.5	92.9±15.3	97.5±12.0	17.0±16.1
	250	91.4±3.4	94.7±8.4	96.5±9.2	99.0±23.3	10.1±9.5

- a) Spore germination represents the percentage of rate of spore germination of treated spores to that of untreated spores after 20 hr of incubation.
b) Elongation of germ tube represents the percentage of length of germ tubes of treated spores to that of untreated spores after 9 hr of incubation.
c) Appressorium formation represents the percentage of rate of appressorium formation of treated spores to that of untreated spores after 20 hr of incubation.
d) Invading hypha formation represents the percentage of rate of invading hypha formation of treated spores to that of untreated spores after 20 hr of incubation.
e) Pathogenicity represents the percentage of number of black necrotic spots on the half leaf inoculated with treated spores to that on the other half inoculated with untreated spores after 20 hr of incubation.

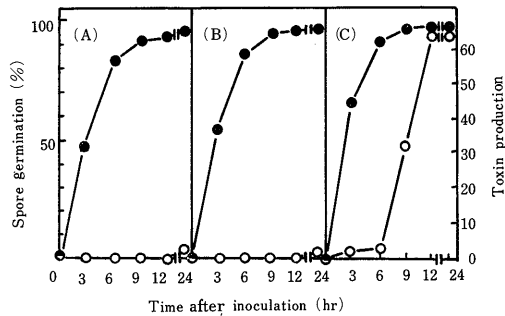


Fig. 1. Time course of spore germination (●) and toxin production from germinating spores (○) of *A. alternata* Japanese pear pathotype treated with 20ppm cerulenin (A), 250ppm L-methionine (B) and untreated control (C). Toxin production represents the dilution end point for toxicity to Japanese pear leaves.

20 ppm cerulenin or 250ppm L-methionine were studied. Untreated spores were served as a control (Fig. 1). Treated spores with cerulenin or methionine began to germinate by 3 hr after inoculation, and more than 90% of the spores completed germination by 9hr. Such time courses of spore germination were almost the same as those of untreated spores. However, release of AK-toxin from the treated spores became detectable at only 24 hr after inoculation, whereas the toxin from untreated spores was detected obviously in 3 hr incubation and increased markedly after 6 hr incubation.

As to the reason for the inhibitory effects of cerulenin and methionine on toxin production, an explanation, but less likely one, was that the AK-toxin molecules from germinating spores react with supplemented cerulenin or methionine, and less their toxicity. This possibility was rejected by the result of a following experiment. Cerulenin (at 50 ppm of final concentration) or L-methionine (at 1,000 ppm of final concentration) was added in a given AK-toxin solution. The mixed toxin solution was kept at 25 C for

Table 3. Effects of treatments with 20 ppm cerulenin and 500 ppm L-methionine on pathogenicity and toxin production from germinating spores of apple pathotype and strawberry pathotype of *A. alternata*

Pathogen	Treatment	Pathogenicity ^{a)} (%)	Toxin production ^{b)}
<i>A. alternata</i> apple pathotype	Control	100	32
	Cerulenin	28.9±13.5	2
	L-Methionine	12.8± 8.1	2
<i>A. alternata</i> strawberry pathotype	Control	100	4
	Cerulenin	34.7±18.1	1
	L-Methionine	13.9± 6.4	1

a) Pathogenicity represents the percentage of number of black necrotic spots on the half leaf inoculated with treated spores to that on the other half inoculated with untreated spores after 20 hr of incubation.

b) Toxin production represents the dilution end point for toxicity of spore-germinated fluid harvested after 24 hr of incubation.

24 hr and then its dilution end point of toxicity was determined by a leaf-necrosis assay. The same AK-toxin solution lacking the chemicals was served as a control. The dilution end points of both solutions were the same to susceptible pear leaves.

The spores of apple pathotype and strawberry pathotype of *A. alternata* were subjected to the treatments with cerulenin and L-methionine, and their pathogenicity was examined (Table 3). These chemicals considerably suppressed pathogenicity expression of spores of both pathotypes of *A. alternata*. Toxin productivity of the spores was also reduced by the treatments.

Effects of KH_2PO_4 , NH_4Cl , yeast extracts and cysteine on pathogenicity of *A. alternata* Japanese pear pathotype

KH_2PO_4 , NH_4Cl , yeast extracts and cysteine had moderately inhibitory effect on AK-toxin productivity during spore germination. Thus, the effects of these chemicals on pathogenicity expression of the spores of *A. alternata* Japanese pear pathotype were observed (Table 4). Necrotic spot formation on Nijisseiki pear leaves by the spores treated with these chemicals was remarkably reduced in number as compared with that by untreated spores, while no significant decrease was observed in the rates of spore germination on both leaves.

Table 4. Effects of treatments with KH_2PO_4 , NH_4Cl , yeast extracts and L-cysteine on spore germination, pathogenicity and toxin production from germinating spores of *A. alternata* Japanese pear pathotype

Treatment	Concentration (ppm)	Spore germination ^{a)} (%)	Pathogenicity ^{b)} (%)	Toxin production ^{c)}
Control	—	100	100	64
KH_2PO_4	1000	97.9 ± 2.0	87.3 ± 11.1	64
	5000	98.5 ± 4.1	41.0 ± 8.5	16
NH_4Cl	1000	99.5 ± 1.2	89.9 ± 7.2	64
	5000	101.0 ± 4.3	22.4 ± 23.6	4
Yeast extracts	1000	104.0 ± 3.7	99.8 ± 19.8	64
	5000	104.1 ± 2.5	32.8 ± 27.0	4
L-Cysteine	100	98.3 ± 2.8	87.8 ± 20.4	64
	1000	96.8 ± 7.1	32.6 ± 19.5	8

a) Spore germination represents the percentage of rate of spore germination of treated spores to that of untreated spores after 20 hr of incubation.

b) Pathogenicity represents the percentage of number of black necrotic spots on the half leaf inoculated with treated spores to that on the other half inoculated with untreated spores after 20 hr of incubation.

c) Toxin production represents the dilution end point for toxicity of spore-germinated fluid harvested after 24 hr of incubation.

Discussion

In the present work, 26 chemicals were applied to the spores of a virulent isolate of *A. alternata*, which pathogenicity is depending on host-specific toxin, to know whether the chemicals can suppress toxin productivity without any significant effects on the other infection behaviors or not. Results showed that the treatments of spores with

cerulenin, methionine, KH_2PO_4 , NH_4Cl , yeast extracts and cysteine suppressed the toxin production during spore germination. Specific virulence of these *A. alternata* pathogens could not be exhibited if the toxin production was inhibited.

From the chemical structures of AK-toxin I and II [8- α -acetylamino- β -methyl- β -phenyl-propionyloxy)-9, 10-epoxy-9-methyl-2E, 4Z, 6E-decatrienoic acid and its β -demethyl derivative, respectively]⁹⁾, it is strongly suggested that metabolic systems of phenylalanine and fatty acids are concerning with synthesis of the toxin moieties. However, phenylalanine and its N-acetyl derivative, antagonists of fatty acid metabolism such as carnitine and palmytoyl Co A²⁾ and an inhibitor of fatty acid oxidation such as 4-pentenoic acid¹³⁾ were found to have no inhibitory effects on the toxin production even at relatively high concentrations. Only one potent inhibitor of toxin production was cerulenin (2R, 3S-epoxy-4-exo-7, 10-*trans*, *trans*-dodecadienoyl amide), which is originally an antibiotic from *Cephalosporium caerulens* and is known to be a specific inhibitor of the condensing enzyme in fatty acid biosynthesis¹¹⁾. Inhibitory effect of cerulenin on toxin production implies that cerulenin inhibits the synthesis of this toxin *in vivo* through fatty acid or the related intermediates. A similar effect of cerulenin on AM-toxin and AF-toxin production was observed since AM-toxin and AF-toxin have fatty acid-related moieties in their structures. Cerulenin might have inhibited the biosynthesis of fatty acid moieties of these three toxins almost to the same extent.

KH_2PO_4 , NH_4Cl and yeast extracts exhibited the inhibitory activity at 5,000ppm. In the field of fermentation technology (*e. g.*, antibiotics biosynthesis), it has been observed that when phosphate or nitrogen sources was added in culture media at relatively high concentrations, the production of secondary metabolites was suppressed without reduction the producer's growth^{1,10)}. The mechanism of such effect of phosphate has been concluded as the disturbance of energy metabolism, but that of nitrogen sources had not been clarified yet. The phosphate-induced reduction of toxin production during spore germination may be explained as a similar metabolic regulation. The results of yeast extracts treatment suggest that the excess amount of nutrients may be unfavorable for toxin production, because germ tubes from the treated spores were abnormally elongated.

In the case of cysteine and methionine, which contain sulfur in their chemical structures, the mechanism of inhibitory effect might be different from that of nitrogen sources because they could exhibit the effects at relatively lower concentrations. Methionine did not affect the toxicity of toxin directly. From the results described above, it was suggested that the inhibitory activities of these chemicals to pathogenicity of spores were mainly dependent on suppression of toxin productivity. However, the mechanism whereby these occur is unclear.

In addition to physical treatments of pathogenic spores¹⁶⁾, application of chemical method may be helpful as tools for studying the exact role of host-specific toxin in pathogenesis. Such research may also lead to a strategy for development of new type of disease control agents.

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和 文 摘 要

柘植尚志・西村正暘・大村 智・甲元啓介・尾谷 浩 : *Alternaria alternata* 群病原菌における宿主特異的毒素生成の制御 (2)化学物質による毒素生成の阻害

宿主特異的毒素を生成する *Alternaria alternata* 群植物病原菌の病原性発現および分生孢子発芽時の毒素生成に及ぼす化学物質の効果について検討した。ナシ黒斑病菌分生孢子懸濁液に、抗生物質セルレニンまたはメチオニンをそれぞれ 20 ppm, 100 ppm 以上の濃度で添加すると、孢子の発芽、付着器形成などは殆んど影響されなかったが、孢子発芽時の宿主特異的毒素(AK-毒素)の生成・放出は著しく抑制された。また二十世紀ナシ葉に対する病原性の低下が観察された。さらに、これらの化学物質は、リンゴ斑点落葉病菌の AM-毒素生成およびイチゴ黒斑病菌の AF-毒素生成も阻害し、両菌の病原性低下を引き起こした。また、 KH_2PO_4 , NH_4Cl , 酵母エキスおよびシステインも、ナシ黒斑病菌の孢子発芽にはほとんど影響することなく孢子発芽時の AK-毒素生成能力および病原性を阻害した。しかし、これらの化学物質の効果は、セルレニンやメチオニンほど顕著ではなく、比較的高濃度処理によって、阻害効果が認められた。以上の結果から、*Alternaria alternata* 群病原菌の分生孢子懸濁液に、ある種の化学物質を添加することにより、孢子発芽時の宿主特異的毒素生成が阻害され、その結果、病原性の低下が引き起こされるものと考えられ、本群菌の病原性発現における孢子発芽時の宿主特異的毒素の重要性が示唆された。