Studies on Apple Canker Disease. The Necrotic Toxins Produced by *Valsa ceratosperma*[†]

Hidehiko NATSUME, Haruo SETO and Noboru ÔTAKE

*Institute of Applied Microbiology, The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan*

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Several metabolites responsible for the toxic manifestations of *Valsa ceratosperma* (Toda et Fries) Maire, a phytopathogenic fungus of the Japanese apple canker, have been isolated from its culture filtrate after growth on apple branch extract. Chemical and spectrometric studies revealed the products to be degradation products of phlorizin which is a dominant component distributed in leaves, stems, fruits and roots of apple. The toxic substances were identified as 3-(*p*-hydroxyphenyl) propionic acid, phloroglucinol, *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid and protocatechuic acid. All of these compounds except *p*-hydroxyacetophenone were detected in the lesions of apple trees infected by *V. ceratosperma*. The fungus cultivated in a medium containing added phlorizin also produced the five toxic substances mentioned above. These results suggest that phlorizin is involved in the specific relationship between the host and the pathogen, indicating that the degradation products of phlorizin play important roles in the production of symptoms of infected apple trees.

Apple canker disease is now epidemic in many areas in Japan, and is a serious economic problem in apple production. The symptoms of several plant diseases are known to be caused by host-specific toxins produced by the pathogenic fungi. The characteristic symptom observed of apple trees infected by *V. ceratosperma* is necrosis of branches and trunks.

In spite of intensive investigations,¹⁾ the primary cause of necrosis due to the fungus remained to be clarified. Now we have succeeded in isolating and identifying the toxic substances produced by *V. ceratosperma*, and in revealing the mechanism of the necrosis caused by the pathogen.

In our experiments, toxic substances present in spreading lesions were investigated from the viewpoint of phytopathology, with the main interest being in the specific relationship between the host and the pathogen, *V. ceratosperma*.

The results indicated that the apple canker was a host-dependent disease whose cause has

been elucidated for the first time.

MATERIALS AND METHODS

Bioassay. At the beginning of the isolation process, we used a cut branch method as well as a lettuce germination test for bioassaying of toxic substances. After confirmation of the difference between the two methods for evaluating the toxicity, the latter was used for toxic materials, since the former was too timeconsuming. The cut branch method was utilized again in the final purification stage to confirm the activities of the isolated toxins.

Cut branch method. Absorbent cotton impregnated with 2.5 ml of an aqueous solution of a test sample was placed on the top cut end of a one or two year old apple branch which was about 1 cm in diameter and 15 cm in length. The cotton was covered with a piece of aluminum foil to prevent evaporation of water from the top end. After 3, 5, 7 and 14 days at 20°C, symptoms of necrosis were carefully examined.

Lettuce germination test. Seeds of lettuce (Great Lakes 366) were placed on filter paper impregnated with a sample solution in a Petri dish and 0.4 ml of water was added. The dish was incubated in the dark at 20°C. Inhibition of seed

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germination was examined after two days.

Culture media. Of the several media tested, the apple branch extract medium was the only satisfactory one for the toxin production by *V. ceratosperma*. The medium consists of apple branch extract, prepared by boiling 150 g of branch in 500 ml of water for 30 min, 0.2% yeast extract powder and 4% sucrose. The pH was adjusted to 5.8

TABLE I. YIELD OF TOXINS IN
VARIOUS KINDS OF MEDIA

Medium	Submerged culture	Stationary culture
Glucose-Czapek + yeast extract	—	—
Raulin-Thom + yeast extract	—	—
Richards + yeast extract	—	—
Yeast-sucrose	—	—
Potato-sucrose	—	—
Prune-sucrose	—	—
Apple branch extract	+++	+++

before sterilization. *V. ceratosperma* was submerged-cultured and the toxin titres obtained within a few days were almost equivalent to those produced under stationary cultural conditions for 3~4 weeks. Therefore, the submerged culture was adopted throughout this experiment. The yields of toxins in various kinds of media are summarized in Table I.

Isolation of toxins. *V. ceratosperma* was maintained on apple branch extract agar slants containing 4% agar. After incubation at 27°C for 21 days, sterilized water (2 ml) was added to each slant and the fungus was scraped out to prepare a spore suspension.

Two ml of this inoculum was added to 200 ml of the apple branch extract medium in 500 ml Erlenmeyer flasks, and cultivation was conducted at 27°C for 6 days on a reciprocal shaker.

The isolation procedures for the toxins are summarized in Chart 1.

The broth filtrate (6 liters, pH 4) was adjusted to pH 3 with 1N HCl solution and extracted with ethyl acetate. The organic phase was concentrated *in vacuo* to remove the solvent. Then, the extract was purified by silica gel column chromatography using a solvent system of chloroform-methanol (5:1) to give three toxic fractions. Further purification of these fractions was achieved separately by common procedures, *i.e.* column chromatography on Sephadex LH-20 (solvent: methanol) and on silica gel (solvent system: chloroform-methanol = 50:1).

CHART 1. Isolation and Purification Procedures for VC Toxins 1, 2 and 3.

CHART 2. The Procedures for Detection of Toxic Substances.

The purified toxic fractions were evaporated *in vacuo* to give crude crystals. Recrystallization from ethyl acetate-chloroform or chloroform-hexane gave colorless needles of VC toxins 1, 2 and 3.

Since the toxins were produced only in the medium supplemented with apple extract, it was necessary to clarify whether they are inherent constituents of apple plants or metabolites produced by *V. ceratosperma*. To this end, healthy apple branches (120 g) and diseased ones (120 g) infected by the pathogen were extracted separately with methanol, and the extracts were subjected to GC-MS (1.5% OV-1, 3 mm × 200 cm, 140°C) analysis. In addition, metabolites of the fungus produced under submerged conditions were also investigated by GC-MS analysis of the ethyl acetate extract of the fermentation broth. The procedures for detection of toxic substances are summarized in Chart 2. Branches or trunks of infected apple trees were crushed into small pieces and extracted with methanol. The solution was concentrated *in vacuo* to a small volume, and the residue, after dilution with water, was extracted with ethyl acetate. The toxic compounds were then transferred to a 0.05 N NaOH solution, and reextracted with ethyl acetate at pH 3. The extract, which was methylated with diazomethane, was subjected to GC-MS analysis. The same procedures were applied to healthy apple plants. The analysis of broth filtrate was carried out as described previously.

RESULTS

The isolated metabolites, named VC toxins

1, 2 and 3, were identified as 3-(*p*-hydroxyphenyl) propionic acid, phloroglucinol and *p*-hydroxyacetophenone, respectively, on the basis of NMR (JEOL, JNM-FX-400 (400 MHz)), mass and IR spectral data and their physicochemical properties as summarized in Table II. Their structures were further supported by comparison with authentic samples. The following amounts of VC toxins 1, 2 and 3 were obtained from 6 liters of cultured broth; VC toxin 1 (946 mg), VC toxin 2 (805 mg) and VC toxin 3 (67 mg). In addition to these compounds, GC-MS analysis revealed the presence of two new toxic substances, *i.e.* *p*-hydroxybenzoic acid (VC toxin 4; 206 mg) and protocatechuic acid (VC toxin 5; 722 mg) in the broth filtrate. The structures of these two metabolites were confirmed by direct comparison with authentic specimens. In affected apple plants, four toxic substances, VC toxins 1 (400 mg), 2 and 4 (614 mg), and 5 (175 mg) had been detected before. On the other hand, VC toxin 1 (2 mg) was detected in healthy apple, but the amount was very small. The structures of these compounds indicated that VC toxins 1~5 were the degradation products of phlorizin^{2~8)} which is abundantly distrib-

TABLE II. PHYSICOCHEMICAL PROPERTIES OF TOXINS 1, 2 AND 3

	Toxin 1		Toxin 2		Toxin 3	
Appearance	Colorless needle		Colorless needle		Colorless needle	
Melting point	129 ~ 131°C		218 ~ 221°C		106 ~ 108°C	
Solubility	Sol. in MeOH, EtOAc, Acetone Insol. in C ₆ H ₆ , CCl ₄		Sol. in MeOH, EtOAc, Acetone Insol. in C ₆ H ₆ , CCl ₄		Sol. in MeOH EtOAc, CHCl ₃ Insol. in Hexane	
Elemental analysis	Found	Calcd.	Found	Calcd.		
C;	65.46	65.06	57.06	57.14		
H;	6.05	6.02	4.83	4.76		
O;	28.49	28.92	37.75	38.10		
Mass (EI) <i>m/z</i>	166		126		136	
$\nu_{\text{max}}^{\text{KBr}}$	3400, 1710 cm ⁻¹		3200 ~ 3470, 1620 cm ⁻¹		3140, 3280, 1660 cm ⁻¹	
¹³ C-NMR (δ) (100 MHz)	(CD ₃ OD) Functional group		(CD ₃ OD)		(CDCl ₃)	
	31.2	—CH ₂ —	95.5	=CH— × 3	26.3	—CH—CH ₃ O
	37.1	—CH ₂ —	160.1	=C—O— × 3 		
	116.2	=CH— × 2	[Toxin 2 acetate]		115.5	=CH— × 2
	130.2	=CH— × 2	(CDCl ₃)	O —C—CH ₃ × 3	129.7	=C—
	132.9	=C—	21.1	—C—CH ₃ × 3	131.2	=CH— × 2
	156.6	=C—O—	112.7	=CH— × 3	161.2	=C—O—
	176.9	—C—O— O	151.3	=C—O— × 3	198.3	—C— O
¹ H-NMR (δ) (400 MHz)	(CD ₃ OD) <i>J</i> (Hz)		(CD ₃ OD)		(CDCl ₃) <i>J</i> (Hz)	
	2.55 (t)	8 2H	5.79 (s)	3H	2.60 (s)	3H
	2.80 (t)	8 2H	[Toxin 2 acetate]		6.96 (d)	8 2H
	6.70 (d)	8 2H	(CDCl ₃)		7.40 (s) broad	1H
	7.05 (d)	8 2H	2.28 (s)	9H	7.96 (d)	8 2H
			6.84 (s)	3H		

uted in leaves, stems, fruits and roots of apple trees.

In order to elucidate the role of the parasite in relation to the metabolism of phlorizin, *V. ceratosperma* was cultivated in modified glucose-Czepek medium (500 ml) containing 0.2% yeast extract powder supplemented with phlorizin (0.5 mg/ml medium) for 4 days at 27°C on a reciprocal shaker. The broth filtrate was subjected to GC-MS analysis. VC-toxin 1 (3.3 mg), 2 and 4 (50 mg), 3 (16.7 mg) and 5 (30 mg) were detected in the broth filtrate to

TABLE III. MINIMUM BROWNING CONCENTRATION

1.	3-(<i>p</i> -Hydroxyphenyl)propionic acid	0.95 mg/ml
2.	Phloroglucinol	3.00 mg/ml
3.	<i>p</i> -Hydroxyacetophenone	0.95 mg/ml
4.	<i>p</i> -Hydroxybenzoic acid	2.50 mg/ml
5.	Protocatechuic acid	1.25 mg/ml

which phlorizin had been added. It is quite obvious, therefore, that these detected substances are fungal metabolites of phlorizin. The minimum browning concentrations of VC toxins 1~5 are summarized in Table III.

DISCUSSION

It is well known that phlorizin is widely distributed in each tissue of the apple tree. Our experiments indicated that *V. ceratosperma* can metabolize phlorizin, a typical constituent of the genus *Malus*, into five toxic substances, 3-(*p*-hydroxyphenyl) propionic

acid, phloroglucinol, *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid and protocatechuic acid.

Williams *et al.*^{9~11)} proposed a hypothetical metabolic scheme for degradation of phlorizin by *Venturia inaequalis* in 1961. The enzymatic degradation of phlorizin was assumed to be as in the works of Börner,²⁾ Sleeper and Stanier¹²⁾

CHART 3.

and Stanier *et al.*¹³⁾ On the basis of our experimental data and the scheme of Williams *et al.*, we propose the scheme summarized in Chart 3 for degradation of phlorizin by *V. ceratosperma* in the apple plant.

It is clear that phlorizin and its degradation products play an important role in the host-parasite interactions in the Japanese apple canker for the following reasons; (1) phlorizin exists in a high concentration in the host plant, (2) its distribution is characteristic of the genus *Malus*, (3) the parasite can metabolize phlorizin, and (4) the degradation products of phlorizin produce a symptom similar to that of bark infected with the pathogen. Therefore, it may be concluded that the symptom of apple canker, *i.e.* necrosis, is caused by the action of *V. ceratosperma* on the constituents of the apple plant. In this context, apple canker can be regarded as a kind of host-dependent disease.

During this work, we noticed that the sporulation and growth of the fungus were much better in apple branch extract medium than in other natural or artificial media. Similar results were not obtained in other culture media supplemented with phlorizin only. Therefore, constituents of apple plants other than phlorizin may also play some roles in the apple canker disease. Studies to clarify this problem are now under way.

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