

イチゴ黒斑病菌 (*Alternaria alternata* strawberry pathotype)の生成する宿主特異的毒素(AF - 毒素)に関する研究 (1)

宿主特異的毒素の生成とその生物活性

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Studies on Host-Specific AF-toxins Produced by *Alternaria alternata* Strawberry Pathotype Causing Alternaria Black Spot of Strawberry (1) Production of Host-Specific Toxins and Their Biological Activities*

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Abstract

The causal pathogen of Alternaria black spot of strawberry was identified as a distinct pathotype of *Alternaria alternata* (Fries) Keissler. Only one strawberry cultivar Morioka-16 was susceptible to the pathogen. Several cultivars of Japanese pear including Nijisseiki which were susceptible to *A. alternata* Japanese pear pathotype were also diseased by this pathogen. This fungus produced at least three species of host-specific toxins (named AF-toxin I, II and III, respectively). AF-toxin I and II were considered the major toxins. AF-toxin I induced necrosis and potassium leakage on the susceptible cvs. Morioka-16 and Nijisseiki. AF-toxin II induced only potassium leakage from cv. Morioka-16 but did not induce necrosis. AF-toxin II as well as AF-toxin I caused necrosis and potassium leakage from the cv. Nijisseiki.

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Key Words: Alternaria black spot of strawberry, host-specific toxin, AF-toxin I, II and III.

Introduction

In about 1975, a new disease of strawberry (*Fragaria glandiflora* Ehrh.) occurred in Tohoku district of Japan, shortly after the widespread cultivation of the newly bred strawberry cultivar Morioka-16; the disease was first described by Watanabe and Ume-kawa, who identified the causal pathogen as an *Alternaria* sp. and named the disease Al-

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ternaria black spot of strawberry¹⁴. Among the several strawberry cultivars tested, the causal isolates were found to be only pathogenic to the strawberry cv. Morioka-16. Nishimura *et al.*⁸ demonstrated that the pathogenic isolates were identical to *Alternaria alternata* (Fries) Keissler in morphological and cultural characters, but differed from the known pathotypes of *A. alternata* in their pathogenicity and host-specific toxin production. Interestingly, these isolates were highly pathogenic not only to the original host cv. Morioka-16 of strawberry, but also to certain cultivars of Japanese pear to which the Japanese pear pathotype of *A. alternata* is pathogenic^{9,10}. Culture filtrates of the pathogen were found to exhibit the same host-specific toxicity as the pathogen exhibited strict pathogenicity to certain cultivars of strawberry and Japanese pear⁹. These isolates were then characterized as a new distinct pathotype of *A. alternata*⁸.

Examination of a large volume of culture filtrates of the pathogen revealed the *in vitro* production of three detectable host-specific toxins (AF-toxin I, II and small portion of III)⁵. We report herein the methods employed for isolating these homogeneous toxins from the culture filtrates and also on some biological activities of these toxins on strawberry and pear hosts. Brief reports of portion of this study have been previously published^{5,8,9,15,16}.

Materials and Methods

The highly virulent isolate O-187 of *A. alternata* strawberry pathotype was used for the isolation of the host-specific toxins, unless otherwise noted. For toxin production *in vitro*, the isolate was still-cultured in 1,000 ml Roux bottles containing 200 ml each of potato dextrose broth for 14 days at 26 C.

Detection of the toxin was made by a leaf-necrosis assay using the susceptible strawberry cv. Morioka-16 and the susceptible Japanese pear cv. Nijisseiki. Leaves of resistant strawberry cv. Hokowase and resistant Japanese pear cv. Chojuro were used as the control. Young leaves detached from these susceptible and resistant cultivars were cut into small pieces (each 2 to 4 cm²). The center of each piece of leaf was then wounded slightly by pricking the underside with a needle. The wounded portion was immediately treated with a few drops of the prepared sample solution (50 μ l) to be tested, and then placed on a sponge mat and incubated at 26 C for 24 hr in a moist chamber. Characteristic veinal necrosis developed around the treated portion of the susceptible leaves but not on the resistant leaves when the sample solution contained the toxin. Data were taken 24 hr after the sample application. In the case of column chromatography, a piece of filter paper (2 $\times$ 2 mm) was immersed in each fraction to be tested. After removal of the solvent by air drying, the filter paper pieces were placed on the prepared leaves as described above, and wet with droplets of distilled water (50 μ l).

Different chromatographic techniques were employed for the isolation of the toxins from culture filtrates. Silicic acid adsorption column chromatography (Wakogel C-200) was conducted by successive elution with benzene and acetone increasing stepwise the content of the latter. Sephadex LH-20 column chromatography was developed with me-

thanol. Droplet countercurrent chromatography (DCC-A, Tokyo Rikakikai Co. Ltd.) was used as a further purification step (solvents; benzene: chloroform: methanol: distilled water, 15 : 15 : 23 : 7, v/v/v/v) for the isolation of AF-toxin I, II and III. Thin layer chromatography (TLC) was performed on plates of Merck's silica gel 60 F-254 (solvents; benzene: acetone: *n*-hexane: acetic acid, 50 : 20 : 20 : 1, v/v/v/v, 3 times development), for final purification of the toxins. These chromatographic techniques will be discussed in more detail later in this paper.

Spores to be used for both pathogenicity tests and toxin production by germinating spores were prepared by the following method: 14-day-old mycelial mats of isolate O-187, which was cultured on potato dextrose broth described above, were washed fully in tap water to remove excess culture medium. After excess water of the mats was removed by pressing the mats between filter papers, these mats were placed in a moist chamber. After 24 hr incubation at 26 C, abundant sporulation was observed on the mats. Spores that were produced were then harvested by blushing the mycelial surface and flushing with distilled water. The resultant suspension was then filtered through two layers of gauze to collect only spores and eliminate mycelial fragments. The spore suspension was then centrifuged at $350 \times g$ for 5 min, and the sediment was resuspended in distilled water to give a final concentration of 5×10^5 spores/ml, unless otherwise noted. To determine the pathogenicity, the prepared spore suspensions were sprayed on detached leaves of susceptible and resistant strawberry and Japanese pear by an atomizer, and incidence of leaf spot was assessed following incubation of the treated leaves in a moist chamber for 24 hr at 26 C. Control leaves were sprayed with distilled water only. Pathogenicity was expressed in terms of the number of the necrotic spots per 1 cm² leaf surface area.

To assay the toxin production from germinating spores, a spore suspension (100 ml, 5×10^5 spores/ml) was sprinkled onto 16 sheets of tissue paper in a moist chamber and incubated for 24 hr at 26 C. The spore-treated papers were then pressed and the germination fluids were collected and filtered to remove the germinated spores. The resultant fluid was extracted with ethyl acetate at pH 3.0, and the extracts were evaporated under vacuum and subjected to the detection of each AF-toxin by TLC: gel was removed from zones on the TLC plates, placed on assay leaves and wet with drops of distilled water (50 μ l). The gel sample from R_f zone containing the toxin caused necrotic response on the susceptible but not on the resistant leaves. Toxic activity was presented as a value of maximum dilution for exhibiting leaf-necrosis to the susceptible leaves, which had been determined by using successive 2-fold dilution with distilled water.

To test the effect of each toxin on potassium leakage from leaf tissue, the following method was used: five discs (1 cm in diameter) were cut from the freshly detached leaves of both the susceptible and resistant cultivars with a paper punch and then infiltrated under vacuum with de-ionized distilled water. The discs were then rinsed in de-ionized distilled water and transferred into polyethylene tubes containing 10 ml of de-ionized distilled water. The tubes were pre-incubated for 1 hr on a reciprocal shaker (100 strokes/min) at 26 C. The ambient solution was exchanged by 5 ml of

1 ppm toxin solutions, which were 10-fold concentration of dilution end point for necrosis-inducing toxicity. The discs were transferred to another 5 ml of toxin solution at intervals of 1 hr up to 3 hr. Each ambient solution was examined for the content of potassium leakage from leaf tissue by the use of a Hitachi 170-30 atomic absorption spectrophotometer.

Results

Isolation and purification of host-specific toxins from culture filtrate

Cell-free culture filtrates (30 liters) of the highly virulent isolate O-187 were adjusted to pH 3.0 with HCl, and extracted two times with ethyl acetate. The ethyl acetate phases were harvested, dried with anhydrous Na_2SO_4 and then evaporated under vacuum at 40 C. The toxic oily residues were chromatographed on a silicic acid column with benzene-acetone mixtures. The toxin was eluted from benzene only to 30 % acetone in benzene. These active fractions were combined and flash-evaporated to dryness. The residues were dissolved in an appropriate volume of methanol, and then insoluble materials were removed by filtration. The sample dissolved in methanol was chromatographed on the Sephadex LH-20 (column size: 38×4 cm). The column was eluted with methanol, and each of 10 ml fraction was collected. Only one elution zone was found to

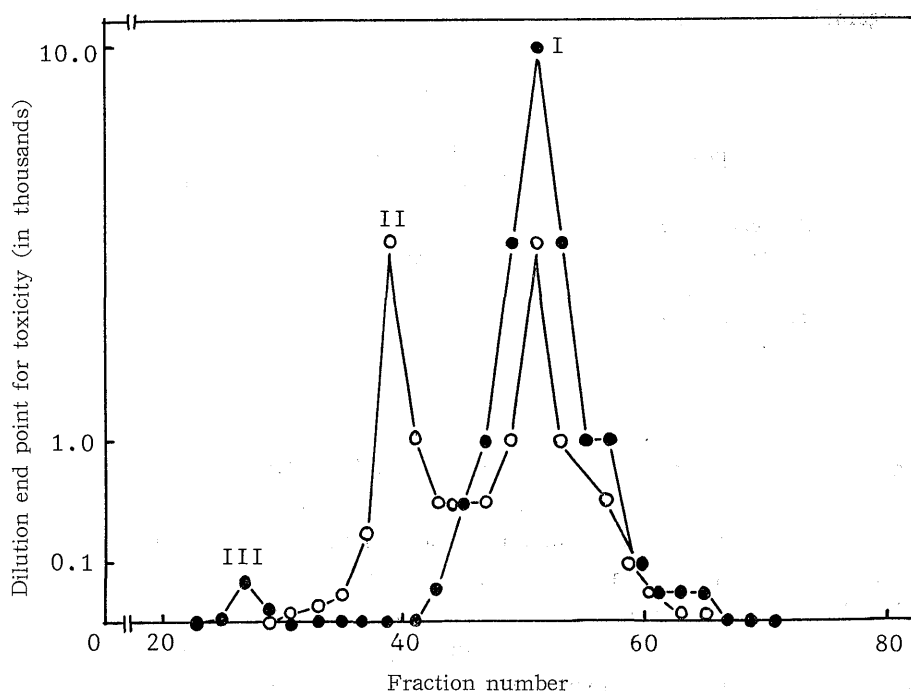


Fig. 1. Fractionation of Peak A by droplet countercurrent chromatography. Each fraction (10ml) was collected and bioassayed for host-specific toxicity. Toxic activities were determined by maximum dilution end point for causing necrosis on leaves of strawberry cv. Morioka-16 (—●—) and Japanese pear cv. Nijisseiki (—○—).

be toxic in the leaf-necrosis bioassay with Morioka-16 strawberry and Nijisseiki pear leaves. Active fractions (tube numbers 31-38) were combined and, following removal of methanol by flash evaporation, the resultant residues were re-applied to the Sephadex LH-20 column (132×2 cm) with methanol. Two peaks (peak A and B) had toxic activity although both peaks slightly overlapped each other. Peak A was toxic to both the susceptible strawberry and Japanese pear leaves, whereas peak B was only toxic to Japanese pear leaves. Peak A (tube numbers 32-36) was collected, flash evaporated to dryness and applied to droplet countercurrent chromatography. Eluate samples of 10 ml each were collected. The toxic activities were separated into three fractions, designated AF-toxin I, II and III, respectively (Fig. 1). AF-toxin I caused necrosis to both the susceptible strawberry and Japanese pear leaves and AF-toxin II only to Japanese pear leaves. On the other hand, AF-toxin III caused necrosis only to strawberry, but the biological activity was much less than those of AF-toxin I or II. Further purification of each AF-toxin was carried out on TLC plates. The R_f value of each AF-toxin was as follows: AF-toxin I; 0.35-0.45, AF-toxin II; 0.65-0.75, AF-toxin III: 0.70-0.80. On the basis of comparison with R_f value on the TLC plate, the toxic principle eluted in the peak B of the second LH-20 column chromatography was found to be identical to AF-toxin II, therefore, the two fractions which were toxic only to Japanese pear were combined as the AF-toxin II fraction. Since AF-toxin III seemed to be a minor toxin, AF-toxin I and II were examined for their necrosis-inducing and potassium leakage activities against strawberry and Japanese pear leaves (Table 1 and Fig. 2). AF-toxin I

Table 1. Biological activity of AF-toxins

Plants	Minimum concentration to induce necrosis ($\mu\text{g/ml}$)	
	AF-toxin I	AF-toxin II
Strawberry cvs.		
Morioka-16	0.08	>1000
Hokowase	>1000	>1000
Japanese pear cvs.		
Nijisseiki	0.08	0.08
Chojuro	>1000	>1000

induced necrosis on susceptible cv. Morioka-16 and cv. Nijisseiki at concentration of 0.08 $\mu\text{g/ml}$, but not on the resistant leaves even at a concentration of 1,000 $\mu\text{g/ml}$. Potassium leakage from leaf tissue was also induced by AF-toxin I and only from the susceptible strawberry and pear leaves. In contrast, at the concentration of 1,000 $\mu\text{g/ml}$, AF-toxin II caused no necrosis on leaves of not only the resistant strawberry and Japanese pear but also the susceptible strawberry, which was the original host of this pathogen. The susceptible Japanese pear leaves, however, were induced necrosis at the same concentration as the AF-toxin I. The AF-toxin II caused potassium leakage from both the susceptible strawberry and pear leaves (Fig. 2), while the susceptible strawberry seemed to be insensitive to the AF-toxin II as far as the observation of visible necrosis.

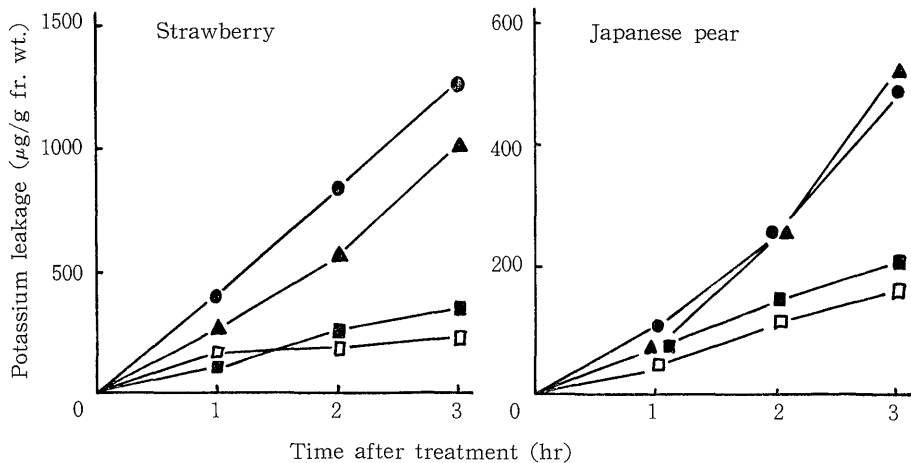


Fig. 2. Toxin-induced potassium leakage from leaves. Leaf discs were infiltrated with de-ionized water under vacuum. The discs were incubated in AF-toxin I solution (—●—), AF-toxin II solution (—▲—) or 1% methanol solution as a control (—■—). The potassium leakage from toxin treated and 1% methanol solution treated resistant leaves (cvs. Hokowase and Chojuro) are presented as the same symbol (—□—) since there were no significant differences between the treated and control leaves. Solid symbols (—●—, —▲—, —■—) indicate the leakage from cvs. Morioka-16 or Nijisseiki.

Correlation between host range and toxin-sensitive cultivars

To determine whether or not the toxin-sensitive cultivars were identical to the host range of this pathogen, leaves from commercially available cultivars of strawberry and Japanese pear were inoculated with the pathogen and treated with AF-toxins (Table 2). After 24 hr incubation, AF-toxin I caused veinal necrosis on the leaves of only cv. Morioka-16 in the strawberry plants tested. On the other hand, of the Japanese pear cultivars tested, nine cultivars including Nijisseiki responded to the toxin with necrosis on their leaves. AF-toxin II caused no necrosis on leaves of all the tested cultivars of strawberry including cv. Morioka-16, but induced necrosis on leaves of Japanese pear which were sensitive to the AF-toxin I. Among the Japanese pear cultivars, the host range of this pathogen and the range of AF-toxin sensitive plants were coincided with those of Japanese pear pathotype of *A. alternata* causing black spot of Japanese pear.

Relationship between pathogenicity and AF-toxin production

In order to determine whether or not pathogenicity correlates with AF-toxin production by the germinating spores of the pathogen, eight isolates of *A. alternata* strawberry pathotype, *A. alternata* Japanese pear pathotype (H-218) and saprophytic *A. alternata* (O-94) were selected. Pathogenicity was determined as the number of black spots that appeared per cm² of leaf surface area. AF-toxin production by germinating spores was determined as a maximum dilution end point for necrosis. All of the isolates which showed pathogenicity to both the susceptible strawberry and Japanese pear cultivars also produced AF-toxin I, II and III (Table 3). The highly pathogenic isolates did not necessarily produce a high level of the toxins in germination fluid, but it was

Table 2. Comparison of different strawberry and Japanese pear cultivars in their response to the pathogen and AF-toxins

Cultivar	Treatment		
	Pathogen ^{a)}	AF-toxin I ^{b)}	AF-toxin II ^{b)}
Strawberry cv.			
America	—	—	—
Donner	—	—	—
Harunoka	—	—	—
Hogyoku	—	—	—
Hokowase	—	—	—
Hotta-wonder	—	—	—
Kogyoku	—	—	—
Morioka-16	+	+	—
Reiko	—	—	—
Takane	—	—	—
Japanese pear cv.			
Chojuro	—	—	—
Doitsu	+	+	+
Gion	—	—	—
Hakata-ao	+	+	+
Hakko	—	—	—
Hakuro	+	+	+
Hokkai-wase	—	—	—
Hosui	—	—	—
Ichihara-wase	—	—	—
Imamura-aki	—	—	—
Ishii-wase	—	—	—
Kikusui	—	—	—
Kimizuka-wase	+	+	+
Kogetsu	+	+	+
Kosui	—	—	—
Kumoi	—	—	—
Meigetsu	+	+	+
Niitaka	—	—	—
Nijisseiki	+	+	+
Okusankichi	—	—	—
Seigyoku	—	—	—
Seiryu	—	—	—
Shinchu	—	—	—
Shinko	—	—	—
Shinsui	+	+	+
Shirayuki	—	—	—
Suisei	—	—	—
Suiryu	—	—	—
Taihaku	—	—	—
Taiheiyo	—	—	—
Yakumo	—	—	—
Wase-aka	—	—	—
Wase-nijisseiki	+	+	+

a) Spore suspension was sprayed on the leaves as described in text.

b) AF-toxins were applied at the concentration of 1 ppm.

+ : Successful pathogenicity / positive reaction (necrosis).

— : No successful pathogenicity / negative reaction (no necrosis).

Table 3. Relationship between pathogenicity and AF-toxin productivity by germinating spores of several isolates of *A. alternata*

<i>A. alternata</i> isolate	Pathogenicity (spots/cm ²)		AF-toxin productivity ^{a)}		
	Morioka-16	Nijisseiki	I	II	III
M-9	19	40	16	32	2
M-24	5	15	4	8	1
M-25	11	21	4	4	1
M-30	11	16	8	4	1
O-165	16	21	1	4	1
O-187	10	20	128	128	2
T-7	23	43	16	64	2
X-20	10	9	16	4	2
H-218	0	27	0	0	0
O-94	0	0	0	0	0

a) Defined as a maximum dilution end point for necrosis.

confirmed to produce all the toxins (AF-toxin I, II and III) following germination when the isolate was pathogenic. However, AF-toxins detected herein were not detected in spore germination fluids of *A. alternata* Japanese pear pathotype (H-218) and saprophytic *A. alternata* (O-94) which were not pathogenic to the strawberry plant.

Discussion

The purpose of this paper was to report a fifth host-specific toxin produced by *A. alternata*, following the previously reported AK-, AM-, AC- and AL-toxins⁷⁾. AF-toxins should be in the category of these host-specific toxins because of the results reported herein, *i.e.* highly host-specific activities at extremely low concentrations, being produced by only the pathogenic isolates and in their spore germination fluids. Morphological study of conidia showed that conidium body was $17-50 \times 8-20 \mu\text{m}$, average $33.4 \times 13.3 \mu\text{m}$, with 2-7 transverse and longitudinal septa; beak length was up to $24 \mu\text{m}$. On the basis of these morphological characteristics, we propose that the causal *Alternaria* having the pathogenicity to both the strawberry cv. Morioka-16 and Japanese pear cv. Nijisseiki should be named a distinct pathotype of *A. alternata* (Fries) Keissler (*A. alternata* strawberry pathotype).

This pathogen was found to produce three different host-specific toxins in culture and following in spore-germination fluid, which have different activities on the cvs. Morioka-16 and Nijisseiki, respectively; AF-toxin I caused necrosis on both the cultivars, AF-toxin II caused only to cv. Nijisseiki and AF-toxin III, in contrast to AF-toxin II, was likely to cause necrosis only to cv. Morioka-16; however, the small quantity of available AF-toxin III prevented any experiment to be conducted by using a relatively high concentration. These differences seemed to be unusual since among the host-specific toxin producing *Alternaria* so far reported, each fungus has been found to produce more than one toxins which have the same biological effects^{2-4, 6, 11, 13)}. Since AF-toxin I induced necrosis and potassium leakage on both the cvs. Morioka-16 and

Nijisseiki, toxin-sensitive plants is coincided with the host range of this pathogen. This toxin may be a major toxin of this pathogen. On the other hand, AF-toxin II induced necrosis only on the susceptible Japanese pear. It has not yet been determined whether AF-toxin II has any importance in the pathogenesis of this pathogen on the susceptible strawberry cv. Morioka-16 but does have on the susceptible Japanese pear cv. Nijisseiki, because AF-toxin II could induce only potassium leakage but not necrosis on the toxin-treated leaves of cv. Morioka-16 and could induce both on leaves of cv. Nijisseiki. The increased level of potassium leakage from the leaves of cv. Morioka-16 could indicate a induced physiological change by AF-toxin II in the leaf tissue. An ultrastructural observation has provided another evidence¹²⁾ that AF-toxin II could affect the cells of the vascular system in leaves of cv. Morioka-16. The biological activities of AF-toxin II on the strawberry host are rather complicated and, therefore, a more extensive investigation is needed to confer an interpretation for its decisive role in pathogenesis.

The increased level of potassium leakage from leaves, which was significant at least by 1 hr after the treatment with AF-toxins, would further suggest that modes of action of AF-toxin I and II are most likely to disturb the membrane integrity of the susceptible cells as it has been suggested for the modes of action of AK- and AM-toxins^{3,10)}. A similar interpretation has been made from the comparatively microstructural study of these AF-, AK- and AM-toxins¹²⁾.

The current experiments, therefore, aim at evaluating the role of these two host-specific toxins (AF-toxin I and II) involved in the infection stages, and elucidating the chemical characterization of these toxins.

Recently, the occurrence of *Alternaria* black spot of strawberry caused by the same *Alternaria* pathogen was reported in Korea¹⁾.

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和 文 摘 要

イチゴ黒斑病菌 (*Alternaria alternata* strawberry pathotype) の生成
する宿主特異的毒素 (AF-毒素) に関する研究 (1)
宿主特異的毒素の生成とその生物活性

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イチゴ黒斑病 (*Alternaria* black spot of strawberry) の病原 *Alternaria* は形態学的には集合種 *A. alternata* (Fries) Keissler の範疇に入るが、その病原性はイチゴでは盛岡16号品種のみに認められた。したがって、病原菌は *A. alternata* のイチゴ pathotype として位置付けるのが妥当であると考えた。なお、本菌はイチゴ以外に、日本ナシのうち、二十世紀など特定のナシ品種に対し病原性を示した。本菌の病原性保有菌株は培養液中に少なくとも3種類の宿主特異的毒素 (AF-毒素 I, II および III と命名) を生成することが見出された。そのうち毒素 I と II が主成分であった。AF-毒素 I は感受性イチゴと二十世紀ナシ葉に対して、黒色葉脈壊死の形成と K^+ イオンの異常漏出を誘起した。一方、AF-毒素 II は感受性イチゴ葉から K^+ イオンの異常漏出は誘起したが、壊死斑は形成しなかった。なお、AF-毒素 II は二十世紀ナシ葉に対して壊死斑形成と K^+ イオンの異常漏出の両方を誘起した。