Occurrence of the Strawberry Pathotype of *Alternaria alternata* in Italy

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ABSTRACT

Wada, H., Cavanni, P., Bugiani, R., Kodama, M., Otani, H., and Kohmoto, K. 1996. Occurrence of the strawberry pathotype of *Alternaria alternata* in Italy. Plant Dis. 80:372-374.

Recently in Italy, Alternaria black spot–like symptoms were observed on leaves and petioles of the strawberry cultivars Cesena, Dana, and Miss. The pathogen was identified as *Alternaria alternata* based on conidial morphology. Isolates of the fungus from Italy were pathogenic to susceptible cultivars of Japanese pear as well as strawberry among differential plants used to determine susceptibility to host-specific *Alternaria* toxins. Bioassay and high-performance liquid chromatography analysis showed that the fungus released host-specific AF-toxin I during spore germination. These data strongly suggest that the outbreak of Alternaria black spot of strawberry in Italy is caused by the strawberry pathotype of *A. alternata.*

Additional keywords: etiology, *Fragaria* × *ananassa*

Black spot of strawberry (*Fragaria* × *ananassa* Duchesne), caused by a distinct pathotype of *Alternaria alternata* (Fr.:Fr.) Keissl., was first recorded in Japan in 1977 (22,23) and then in Korea in 1980 (5). The pathogen had highly selective pathogenicity; only two strawberry cultivars, Morika-16 and Robinson, were susceptible to the pathogen among more than 60 cultivars examined (5,18). The host range of the pathogen appears to be determined by the action of a host-specific toxin, AF-toxin, which is released during spore germination (8,12,25). Nakatsuka et al. (14) elucidated the structure of AF-toxin, which contains 8-substituted-9,10-epoxy-9-methyl-decatrienoic acid, and is similar to AK-toxin (16) of the Japanese pear pathotype of *A. alternata* and ACT-toxin (10,11) of the tangerine pathotype of *A. alternata.*

A disease very similar to black spot of strawberry in Japan was reported in Holland in 1978 (6), in Germany in 1981 (2, 17), and in Belgium in 1984 (1). The cultivars affected were Sivetta, Tamella, Tenira, Elista, Bogota, and Tago. However, no investigations have been conducted to date to determine the exact cause of the disease.

In Italy, new strawberry cultivars, Dana (MD US3700 × Belruby) and Cesena (MD US3816 × Tago) were developed in 1973 and introduced in 1983 into the Emilia Romagna region. In 1992, a new leaf spot disease of strawberry was observed on the cultivars Dana and Cesena in Italian fields. The disease symptoms were similar to those of black spot, and Cavanni et al. (4) identified the pathogen as *A. alternata.* The disease became epidemic in several strawberry fields of these cultivars in the late summer when heavy rainfalls, high relative humidity, and daily mean temperatures ranging from 20 to 25°C favorable for disease development occurred. In subsequent years, most of the fields of Dana and Cesena in the Emilia Romagna region were severely affected by the disease.

Preliminary studies suggested that the causal pathogen produced toxigenic lesions on leaves of susceptible cultivars, but not on the resistant ones, when inoculated with a drop of conidial suspension of a monosporic isolate of the fungus from diseased strawberries (4). Similar trials were repeated using germ-free culture filtrates of the fungus and gave the same results (P. Cavanni et al., unpublished data).

The objective of this study was to confirm that the pathogen of strawberry in Italy is the strawberry pathotype of *A. alternata,* to investigate whether Italian isolates produce AF-toxin during spore germination, and to test their pathogenicity on leaves of other plants susceptible to the other pathotypes of *A. alternata* that produce host-specific toxins. An abstract of the work has been presented (3,21).

MATERIALS AND METHODS

Plants. Young leaves of the following plants were used: strawberry susceptible cv. Morioka-16 and cvs. Hoko-wase and Reiko, which are resistant to the strawberry pathotype of *A. alternata;* apple *(Malus* × *domestica* Borkh.) susceptible cv. Red Gold, moderately susceptible cv. Jonathan, and cv. Mahe 7, which is immune to the apple pathotype of *A. alternata*; tangerine *(Citrus reticulata* Blanco) cvs. Dancy tangerine and Emperor mandarin, both susceptible to the tangerine pathotype of *A. alternata*; rough lemon *(Citrus jambhiri* Lush.) susceptible to the rough lemon pathotype of *A. alternata*; and Japanese pear (*Pyrus pyrifolia* (N. L. Burm.) Nakai var. *culta* Rhed.) susceptible cv. Nijisseiki and cv. Chojuro, which is immune to the Japanese pear pathotype of *A. alternata.* Susceptibility of these differential cultivars had been determined previously (10–13,16).

Fungi. Two Italian isolates, N-14 and N-15, isolated in 1992 from diseased strawberry cvs. Cesena and Dana in different fields (in Ferrara and Forli, respectively) in the Emilia Romagna region of Italy, were used in the study. Two Japanese isolates, M-30 and T-32, of the strawberry pathotype of *A. alternata* and two reference isolates, CMI89343 and EGS35-193, of saprophytic *A. alternata* from stock cultures at the plant pathology laboratory in Tottori University, were also used.

Conidial morphology. For direct comparison, conidia were produced on V8 juice agar medium under near UV (blue black fluorescent light, FL-20SBLB, Matsushita Electric Industrial Co., Ltd., Osaka) at 22°C for 10 days (20). The length, width, and beak length of at least 100 conidia of each isolate were measured.

Pathogenicity tests. Two milliliters of conidial suspension (10^6 conidia per ml) in distilled water was sprayed with an atomizer on the lower surface of five detached young leaves of each test plant. Inoculated leaves were placed on moistened polyurethane foam mats in a moist chamber. The number of black necrotic lesions that formed on the leaves was counted after 30 h incubation at 26°C. The test was conducted three times.

Phytotoxicity of spore-germination fluid. One hundred milliliters of a conidial suspension in distilled water (10^6 conidia per ml) was uniformly sprinkled onto paper towels in water-tight chambers and incubated at 26°C for 24 h. The germination rates were over 90%. The spore-germination fluid was harvested by squeezing the towels and filtering the resulting suspension through filter paper (Whatman 50, Madstone, UK) and membrane filter (Minisart, pore size 0.2 μm, Sartorius AG, Göttingen, Germany) to remove the germinated conidia.

The center of the lower surface of five detached young leaves of each test plant was slightly scratched, making a cross sign

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Accepted for publication 6 December 1995.

Publication no. D-1996-0131-04R

(3 mm) with a needle. A drop (20 µl) of the spore-free germination fluid was placed on each wound site. Leaves were incubated on moistened polyurethane foam mats in a moist chamber at 26°C for 48 h. Leaves were observed after 48 h for necrosis around the wound sites. The test was conducted three times.

High-performance liquid chromatography (HPLC) analysis of toxin. One hundred milliliters of the spore-germination fluid was adjusted to pH 5.5 with 1 M KH_2PO_4 and extracted twice with ethyl acetate. After removing the ethyl acetate by vacuum evaporation, the residue was dissolved in 1 ml of methanol, and a portion of the solution was analyzed with HPLC. The HPLC system (Hitachi Co., Tokyo) was composed of an L-6200 intelligent pump, an L-3000 photodiode array detector, and a D-6100 three-dimension chromatosystem. The column was a Develosil ODS-5, 4.6 × 250 mm (Nomura Chemical Co. Ltd., Tokyo), that was eluted with a mixture of methanol, acetic acid, and water (60:1:39, vol/vol/vol) at a rate of 1 ml/min. Toxins were detected by monitoring absorbance at 290 nm (8,14). The UV absorption spectra of HPLC fractions were recorded with the D-6100 three-dimension chromatosystem.

RESULTS AND DISCUSSION

Conidial morphology. Conidial length, width, and beak length of the two Italian isolates were almost identical to those of the Japanese strawberry pathotype (M-30 and T-32) and saprophytic *A. alternata* isolates (CMI89343 and EGS35-193) (Table 1). Conidia of the Italian isolates were formed in chains similar to the reference isolates. These results support the hypothesis that the causal pathogen belongs to the species of *A. alternata* as previously suggested (4).

Pathogenicity and toxicity tests. Of the differential cultivars of apple, citrus, Japanese pear, and strawberry inoculated with the strawberry isolates N14 and N-15 (Italian) and M-30 (Japanese), only the susceptible strawberry cultivar Morioka-16 and the susceptible pear cultivar Nijisseiki developed lesions. Spore germination fluids of these isolates also produced necrosis on the leaves of the differential cultivars that are susceptible to the pathotypes of strawberry and Japanese pear. No necrosis was observed on leaves of water control. The selective pathogenicity correlated with the specific phytotoxicity of their germ-free, spore-germination fluids. These selective responses to the differential cultivars were compatible with those described in a previous report (12).

HPLC analysis of toxins released by germinating conidia. HPLC analysis revealed that the Italian isolates release AF-toxin I during spore germination (Fig. 1). The single peak at Rt 12.6 min, which has the maximum UV absorption at 292 nm, coincides in co-chromatography with the retention time and the UV spectrum of the authentic AF-toxin I (8,14).

Table 1. Size of conidia of Italian and reference isolates of *Alternaria alternata*

	Conidial body					
	Length (µm)		Width (µm)		Beak length (µm)	
Isolate[a]	Range	Mean ± SD[b]	Range	Mean ± SD[b]	Range	Mean ± SD[b]
N-14	15 to 50	28.5 ± 4.6	5 to 24	12.2 ± 3.0	0 to 20	5.6 ± 3.2
N-15	16 to 52	29.8 ± 7.1	6 to 20	13.5 ± 2.8	0 to 20	6.0 ± 4.4
M-30	15 to 49	26.9 ± 3.8	8 to 22	13.2 ± 2.2	0 to 18	5.8 ± 4.1
T-32	15 to 54	28.3 ± 5.6	7 to 20	11.6 ± 2.5	0 to 16	5.5 ± 3.8
CMI89343	14 to 44	26.2 ± 5.3	7 to 26	11.7 ± 3.2	0 to 17	5.4 ± 3.7
EGS35-193	16 to 48	29.4 ± 6.0	6 to 18	12.8 ± 2.6	0 to 24	6.5 ± 4.5

[a] N-14 and N-15 are Italian isolates from black leaf spot of strawberry; M-30 and T-32 are the strawberry pathotypes of *Alternaria alternata* in Japan; and CMI89343 and EGS35-193 are saprophytic strains of *A. alternata*.
[b] Standard deviation.

These data indicated that a recent leaf spot disease observed on strawberries in the Emilia Romagna region in Italy was caused by the strawberry pathotype of *A. alternata*. The disease is very specific and catastrophic to a limited group of strawberry cultivars, such as Dana and Cesena, and to Morioka-16 in fields in Japan and Korea (5,12,18). Recently in Italy the disease has also been observed on the newly introduced cultivar Miss (Dana × 80.39.1).

Strawberry cultivars used for berry production have been among the crops most intensively bred (9). Recent genetic analysis shows that susceptibility to the disease and AF-toxin is determined by the presence of a single gene pair with semi-dominance (19,24). The susceptible cultivars Morioka-16, Dana, and Miss have a common cultivar, Midland, in their ancestry. Studies on the pedigree and molecular biology of the gene remain to be done.

A similar leaf spot of strawberry caused by *A. alternata* also was reported in New Zealand (7). There are no data available to determine whether *A. alternata* f. sp. *fragariae* coined by Dingley (7) in New Zealand is identical to the strawberry pathotype, AF-toxin producer, of *A. alternata*. However, Dingley (7) reported that the cultivar Redgauntlet was most susceptible, but Takahashi et al. (18) reported that the same cultivar was resistant to the AF-toxin producer in laboratory and field tests. This suggests that Dingley's strains may not include the strawberry pathotype of *A. alternata* that produces AF-toxin.

Opportunistic infection by saprophytic or weakly pathogenic *A. alternata* might occur under favorable environmental conditions. However, if a severe epidemic occurs every year on a few limited cultivars of a crop, it would be more likely that such *A. alternata* is a new pathotype arising from spontaneous mutation. Often such spontaneous mutants are also biochemical variants producing a host-specific toxin and affecting a distinct host range of the crop. Nishimura (15) reported that AK-toxin-producing mutants were detected in a large number of auxotrophic spores of saprophytic *A. alternata* in laboratory tests. Likewise, the sudden occurrence of the strawberry pathotype (AF-toxin producer) in the Emilia Romagna region in Italy might be a result of a genetic mutation rather than accidental introduction of the fungus on host. Although the latter possibility cannot be ruled out, there is no record suggesting the introduction of Japanese strawberry cultivars to Italy.

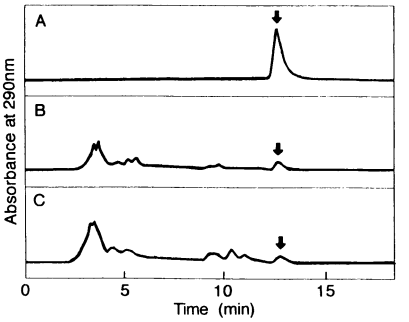

Fig. 1. Comparative profiles of high-performance liquid chromatography (HPLC) of AF-toxin I. **(A)** HPLC of authentic AF-toxin I, developed with a mixture of methanol, acetic acid, and water (60:39:1, vol/vol/vol). **(B)** HPLC of spore-germination fluid of Italian isolate N-14 of *Alternaria alternata,* developed with same mixture used in **A**. **(C)** HPLC of spore-germi nation fluid of Italian isolate N-15 of *A. alternata,* developed with same mixture used in **A.** Arrow indicates position of AF-toxin I.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support in part by research grants from the Ministry of Education, Science and Culture of Japan (No. 05044133 and No. 05404010). We also thank T. B. Sutton of North Carolina State University for critical reading of the manuscript and correcting the English.

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