Mandarin Fruit Rot Caused by *Alternaria alternata* and Associated Mycotoxins

A. LOGRIECO and A. VISCONTI, Istituto Tossine e Micotossine da Parassiti Vegetali, Consiglio Nazionale delle Ricerche, and A. BOTTALICO, Dipartimento di Patologia Vegetale dell'Università degli Studi, 70126 Bari, Italy

ABSTRACT

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Mandarin (*Citrus reticulata*) fruits naturally infected by *Alternaria alternata* had two different symptoms: black and gray heart rot. Two samples of the black rot contained, respectively, tenuazonic acid at 21.0 and 87.2 mg/kg, alternariol monomethyl ether at 0.5 and 1.4 mg/kg, and alternariol at 1.0 and 5.2 mg/kg, whereas one sample of gray rot contained only tenuazonic acid at 173.9 mg/kg. The causal agents isolated from the two types of heart rot differed in toxin production, colony morphology, and pathogenicity to lemon and orange fruit.

The genus *Alternaria* is ubiquitous and includes both plant-pathogenic and saprophytic species. The most common species, *A. alternata* (Fr.:Fr.) Keissl., is widespread among plants, seasons, and geographic regions (1). This species is able to synthesize in culture secondary metabolites that have been demonstrated to be toxic to a variety of organisms (3,7). The five major mycotoxins belong to three structural classes: the benzopyrone derivatives, alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ANE); a perylene derivative, altertoxin I (ATX-I); and a tetramic acid derivative, tenuazonic acid (TA). These mycotoxins have been detected in only a few naturally infected plant products (5,6,9). However, Stinson et al (8) demonstrated the formation of some toxins in oranges and lemons that had been inoculated with *A. citri* Ellis & N. Pierce in N. Pierce.

Recently, mandarin (*Citrus reticulata* Blanco) fruits with symptoms of black rot were observed frequently in several localities in southern Italy. We undertook this investigation to determine the species of *Alternaria* responsible for the disease and to determine whether mycotoxins were present in the diseased fruits. In addition, we investigated some morphological and physiological characteristics of the different strains of *Alternaria* isolated from the diseased fruits.

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MATERIALS AND METHODS

Source of samples. During the winter of 1987, mandarin fruits in groves in Basilicata in southern Italy were observed to be affected with heart rot. At an advanced stage of infection, these fruits showed a discoloration of the rind, particularly near the peduncle, and longitudinal sections revealed internal decay of the tissues with two pigmentations: black and gray (Fig. 1A,B). For mycotoxin analysis, 100 naturally infected mandarin fruits were divided into three groups (two of black and one of gray heart rot) according to their internal pigmentation and degree of rot.

Isolation and identification of *Alternaria* species. Tissue fragments were excised from diseased fruits, plated on water agar supplemented with 10 mg of streptomycin sulfate per liter, and incubated for 5–7 days at 25 C. The fungal colonies were then transferred to plates of potato-sucrose agar (PSA) and incubated for 7 days at room temperature under fluorescent lamps (12-hr photoperiod). Single-spore cultures of *Alternaria* spp. were then obtained on PSA plates to test the color and morphology of the colonies. Conidial morphology was observed using potato-carrot agar (PCA) or a sterile carnation leaf on water agar (4). The latter medium, used in particular for the identification of *Fusarium* spp., also proved useful for *Alternaria,* because the isolates sporulated well on the carnation leaf.

Fungal characteristics. Five single-conidia cultures of strains isolated from each kind of rot were plated on PSA and inoculated in the dark at 5, 15, 20, 25, 28, and 30 C, and the diameters of the resulting colonies were measured 5 and 8 days later. Conidia (200 per strain) from colonies grown on PCA were examined under the microscope and compared. The species were then identified in accordance with the nomenclature of Ellis (1,2).

Postharvest pathology. Ripe fruits of mandarin, orange, and lemon were submerged in 2% sodium hypochlorite for 2 min, then rinsed with distilled water and cut into slices 2 cm thick. The slices (three per citrus fruit) were inoculated in the middle with a small piece of colonized PCA (about 1 mm^2) and incubated at 25 C in a sterile moist chamber for 10 days (corresponding to the time necessary for the pathogen to colonize all the tissue of mandarin fruit).

Toxin production. Four strains (two isolates from each kind of heart rot) were cultured on rice kernels as well as on mandarin fruits to evaluate toxin production. The rice (200 g) was brought up to 45% moisture in 500-ml Erlenmeyer flasks, autoclaved for 20 min at 120 C, and then inoculated with 1 ml of a conidial suspension (3×10^5 conidia/ml) containing 0.05% Triton X-100. The cultures were incubated in the dark for 3 wk at 25 C.

Toxin extractions and analyses. Internal tissues of infected mandarin fruits of each group were collected up to a weight of 100 g (usually six to eight fruits). Two milliliters of 2 N hydrochloric acid and 200 ml of chloroform-ethanol (4:1) were added to each sample. Samples were homogenized in a blender for 2 min. The homogenate was then centrifuged at 5,000 rpm for 10 min. The organic phase was removed, dried with anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in 2 ml of methanol. The extracts were analyzed by thin-layer chromatography and high-pressure liquid chromatography for TA, AME, AOH, ATX-I, and ANE as reported elsewhere (9).

The rice cultures were extracted and analyzed according to the procedures previously described (9).

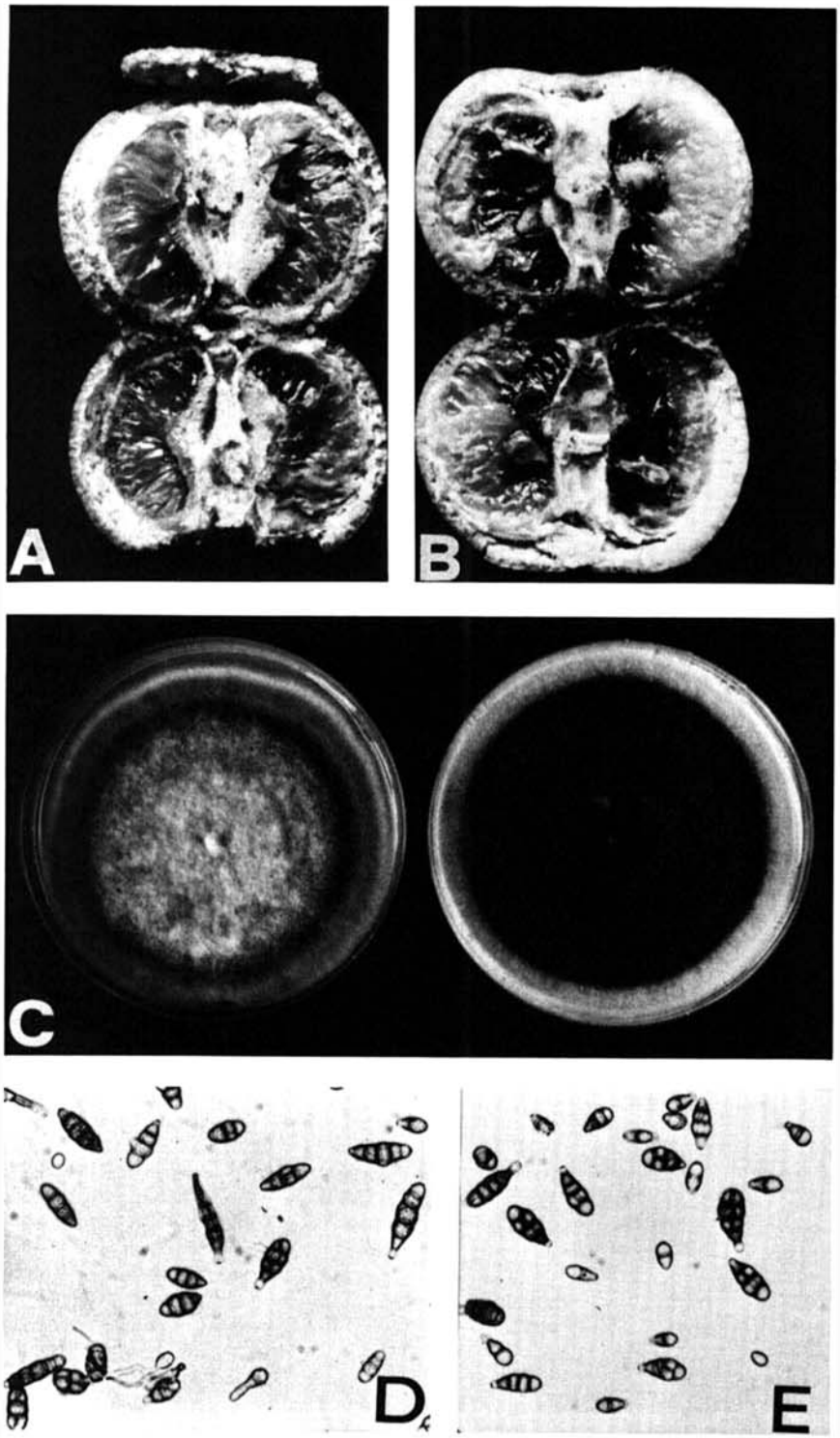

Fig. 1. Gray **(A)** and black **(B)** heart rot of mandarin caused by *Alternaria alternata;* gray (left) and black (right) strains of *A. alternata* on potato-sucrose agar **(C);** and conidia of the two strains from colonies on potato-carrot agar **(D and E)** (×200).

Table 1. Production of mycotoxins by strains of *Alternaria alternata* on mandarin fruits and rice kernels

Substrate / Strain type	Toxins (mg/kg)[a]				
	TA	AME	AOH	ANE	ATX-I
Mandarin fruits					
ITEM-558 (gray)	52.8	0.09	0.27	ND[b]	0.2
ITEM-559 (gray)	84.8	0.17	0.92	ND	0.2
ITEM-560 (black)	49.2	5.9	7.0	ND	ND
ITEM-561 (black)	93.1	6.3	8.7	ND	ND
Rice kernels					
ITEM-558 (gray)	6,850.0	7.5	12.5	5.0	83.3
ITEM-559 (gray)	4,200.0	5.0	12.5	25.0	83.3
ITEM-560 (black)	175.0	10.0	15.0	12.5	5.0
ITEM-561 (black)	177.0	20.0	20.0	50.0	7.5

[a]TA = tenuazonic acid; AME = alternariol methyl ether; AOH = alternariol; ANE = altenuene; ATX-I = altertoxin I.
[b]ND = not detected.

RESULTS AND DISCUSSION

Mandarin fruits with *Alternaria* black rot were observed in several localities in southern Italy at harvest time. In the first stage of the disease, the fruit did not show any symptoms on the outside, but later the surface turned dark starting from the peduncle, and in the advanced stage of the disease, the fruit generally fell to the ground. Two kinds of *Alternaria* heart rot were distinguished, based on the color of the diseased tissues (gray and black).

The causal agents produced gray and black colonies on all culture substrates used. However, the coloring was most evident when the strains were grown on PSA under light (Fig. 1C). The darker color was associated with sporulation, whereas the gray color was associated with felty gray mycelium with conidiophores growing on the aerial hyphae. In accordance with the Ellis nomenclature (1,2), both types were identified as belonging to *A. alternata*. The identity of the two representative strains was confirmed by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

The strains did not differ significantly in conidial characteristics (Fig. 1D,E) or growth rate at the different temperatures. Both strains grew best at 28 C.

The two strains colonized mandarin, orange, and lemon fruits to different degrees. Both strains colonized the same percentage of mandarin tissue, but the black strain colonized larger amounts of orange and lemon fruit tissues than the gray strains (on orange fruit, 89 and 34%, respectively; on lemon fruit, 63 and 6%, respectively; the differences were significant [$P = 0.01$] according to Duncan's test). Results were similar when whole citrus fruits were inoculated with conidial suspensions and incubated at 25 C for 10 days.

The two kinds of heart rot differed substantially, both qualitatively and quantitatively, in the production of the *Alternaria* toxins. Both black rot samples contained TA, AME, and AOH (the first, more rotted sample contained 87.2, 1.4, and 5.2 mg/kg, and the second contained 21.0, 0.5, and 1.0 mg/kg, respectively), whereas TA was the only detectable toxin in the gray rot sample (173.9 mg/kg). None of the naturally occurring gray rot samples contained detectable ATX-I or ANE.

The toxin production of all strains was better on the rice substrate than on mandarin fruit (Table 1). No ANE was detected in the mandarin substrate. Thus, as reported previously (9), rice is suitable for testing the ability of *Alternaria* strains to synthesize the major mycotoxins. Overall, based on the results reported in Table 1, the gray strains were able to synthesize more ATX-I than the black strains, which produced more benzopyrone derivatives (AME, AOH, and ANE). On rice, gray strains produced more TA than black strains, whereas on mandarin, no significant difference in these amounts was observed.

The differences in morphology, postharvest pathology, and toxigenicity of the black and gray strains demonstrate the occurrence of substantial variation within the *A. alternata* population. However, further investigations are needed because this species is widely distributed and includes a large population of very different isolates. The fact that this species has rarely been reported associated with citrus fruit rot may be partly because of the difficulties often encountered in identifying it, as it is very similar to *A. citri*.

The occurrence of large amounts of toxins in the naturally infected samples and the toxigenic potential of the strains may represent a potential health hazard, considering that the toxins may also be transferred into processed products. Further studies are necessary to investigate this possibility and to evaluate ways to prevent the disease.

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