The Structure and Biological Properties of Secondary Metabolites Produced by *Peltaster fructicola,* a Fungus Associated with Apple Sooty Blotch Disease

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ABSTRACT

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Peltaster fructicola, one of the fungi that cause sooty blotch of apple (*Malus* × *domestica*) fruit, produced four metabolites in liquid culture that were isolated and structurally identified as trichothecolone, trichothecolone acetate, 6-methylsalicylic acid, and 2,5-dihydroxybenzoic acid. These toxins were not detected in a culture of *Leptodontidium elatius,* another fungus associated with apple sooty blotch. The four toxins were nonspecific; they caused necrosis when spotted on different cultivars or strains of apple and on eight weed species. In addition, trichothecolone acetate and 6-methylsalicylic acid showed strong antifungal properties against *Botryosphaeria dothidea, B. obtusa, Colletotrichum gloeosporioides,* and *C. acutatum* in vitro. Prior treatment of fruit with 6-methylsalicylic acid reduced infection of fruit inoculated with *C. acutatum.*

Sooty blotch is one of the most serious diseases of apples (*Malus* × *domestica* Borkh.) in the southeastern United States. For many years sooty blotch was considered to be caused by *Gloeodes pomigena* (Schwein.) Colby, and colonies with different morphologies were grouped into four mycelial types: ramose, fuliginous, punctate, and rimate (3). Recent studies have shown that sooty blotch is a disease complex caused by *Peltaster fructicola* Johnson, *Leptodontidium elatius* (G. Mangenot) De Hoog, *Geastrumia polystigmatis* Batista & M. L. Farr, and other fungi (7,8). The fungi that cause sooty blotch grow primarily on the surface of the cuticle of the fruit and result in a downgrading in the quality of the affected fruit. Control of sooty blotch in the southeastern U.S. is based on cultural practices such as pruning (1,9), which facilitate drying of fruit on the tree, and fungicides, which are applied on a 2-wk schedule throughout the summer.

During the course of fungicide trials it was observed that fruit rots (caused by *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not. and *Colletotrichum* spp.) often were equally severe or more severe on fungicide-sprayed fruit than on nonsprayed ones (16–19). Under conditions in North Carolina, 60–90% of the surface area of nonsprayed fruit usually is covered with sooty blotch and flyspeck (caused by *Zygophiala jamaicensis* E. Mason). This suggested to us that the fungi that cause sooty blotch or *Z. jamaicensis* might protect fruit from infection by *B. dothidea* or *Colletotrichum* spp. by producing one or more inhibitory chemicals. Thus, the objective of this study was to determine if two of the fungi that cause apple sooty blotch produce toxin (s) in vitro, and if the toxin(s) is inhibitory to different fungi that cause apple fruit rots. A second objective was to identify potential phytotoxins as part of the ongoing search for new fungal metabolites as herbicides or models for synthetic herbicides (21–26).

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MATERIALS AND METHODS

Isolates. Isolates of apple sooty blotch fungi were selected from a collection of 200 isolates obtained from infected apple fruit in North Carolina. Isolates in this collection were originally classified according to the four mycelial types described for *G. pomigena* by Groves (3). Stock cultures were maintained on potato-dextrose agar (PDA, Difco) slants. One fuliginous mycelial type (2C3F1), two punctate types (ES5P6 and C4P8), and one ramose type (B4Ra4b) were screened in this study. Isolate ES5P6 is *L. elatius*. Isolates B4Ra4b, C4P8, and 2C3F1 are *P. fructicola.* Isolate 2C3F1 was selected for large-scale fermentation based on biomass and quantity of crude toxins produced.

Plants. Eight different cultivars or strains of apple (Law Rome, Paula Red, Golden Delicious, Super Gold, McIntosh, and Silver Spur, Red Chief, and Oregon Spur strains of Delicious) and eight species of weeds (*Sida spinosa* L. [prickly sida], *Chenopodium album* L. [lambsquarters], *Ipomoea* sp. [morning-glory], *Datura stramonium* L. [jimsonweed], *Sorghum bicolor* (L.) Moench [volunteer sorghum], *S. halepense* (L.) Pers. [johnsongrass], *Nasturtium officinale* R. Br. [watercress], and *Cassia obtusifolia* L. [sicklepod]) were maintained in a greenhouse and used for the in vitro leaf bioassay (see below). Nonfungicide-sprayed fruit of the cultivar Golden Delicious were collected from an orchard at the Mountain Horticultural Crops Research Station, Fletcher, NC, and used to test the in vivo antifungal activity of 6-methylsalicylic acid.

Chromatographic materials and methods. E. Merck Silicagel 60 (230-400 mesh) (Merck & Co., Inc., West Point, PA) was used for low pressure flash column chromatography. Analytical thin layer chromatography (TLC) was carried out on E. Merck DC Alufolien, Kieselgel 60 F-254 (0.2-mm thickness). The chromatograms were examined under 254 and 365 nm UV light to detect fluorescent and quenching metabolites. Spots were visualized by dipping in phosphomolybdic acid reagent. Preparative TLC was carried out on E. Merck precoated plates, Silicagel 60 F-254 (20 × 20 cm, 0.5-mm thickness). Metabolites were detected by examining the plates under a UV lamp (254 nm). Dihydroxybenzoic acids standards (Sigma Chemical Co., St. Louis, MO) were distinguished by silica gel TLC in the following solvents: chloroform/methanol 7:3; chloroform/methanol/acetic acid 80:20:1; and toluene/ethyl ether 4:6.

For high-pressure liquid chromatography (HPLC) analysis an HP 1090 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) was used with a DR-5 solvent delivery system, variable volume autoinjector, autosampler, HP 1090 Series II diode array detector system, and MS-DOS software. Three detection wavelengths (220, 254, 340 nm) were used to scan the samples. A 100 × 2.1 mm column filled with 5 μm of Hypersil was eluted with water and a linear gradient of 10–90% acetonitrile containing 0.05% trifluoroacetic acid in 20 min at a flow rate of 0.4 ml/min. Samples of the neutral fraction and acid fraction of 500-ml culture filtrates of *P. fructicola* B4Ra4b and C4P8 and *L. elatius* ES5P6 were prepared in the same manner as used in purification of the toxins from *P. fructicola* 2C3F1.

Preparation, extraction, and purifica-

tion of toxins. An agar block from a 10-day-old culture of *P. fructicola* 2C3F1 (ATCC # 76560) grown on V8-PDA was transferred to each of 20 0.5-L portions of potato-dextrose broth in 2-L flasks containing baffles to increase turbulence during shaking. Flasks were incubated in a rotary shaker (New Bruswick) at 100 rpm at 26 C for 2 wk. The cell-free culture filtrate (10 L) was adjusted to pH 3 with 6 N HCl and extracted three times with 10 L of ethyl acetate. The water layer, which was inactive in the detached leaf toxicity assay, was discarded. The layer of ethyl acetate was evaporated to dryness in a rotary evaporator under reduced pressure at 40 C. All biological activity was in the 2.2 g residue left after evaporation of ethyl acetate. The partially crystalline residue was partioned between ethyl acetate and 0.4% aqueous sodium carbonate. The phenolic fraction was recovered from aqueous sodium carbonate by acidification and back extraction into ethyl acetate. After solvent evaporation, the neutral fraction weighed 245 mg and the phenolic fraction 1.15 g.

The neutral fraction was subjected to preparative TLC in the solvent system of toluene/acetone, 60:40. The phenolic fraction was subjected to low pressure flash silica gel column chromatography on a 5 × 55 cm column packed in methylene chloride (15). The column was eluted sequentially with 100 ml each of methylene chloride containing 1, 10, 25, 40, and 60% acetone.

Impure dihydroxybenzoic acid from chromatography of the phenolic fraction was purified by HPLC on a 4.6 × 25 mm Vydac C18 column eluted at 1.5 ml/min with acetonitrile containing 3% aqueous 5 mM phosphoric acid. The elution was monitored at 330 and 262 nm.

Leaf bioassay for toxicity. Phytotoxicity was determined on apple cultivars and strains and weed species by a detached leaf assay (23). All apple cultivars or strains were used in each test except for the cell-free culture filtrate toxicity in which only Super Gold, Golden Delicious, and Delicious were used. A drop (20 μl) of water containing 10–1,000 μg/ml of toxin was placed on the detached leaf. Only data from the 100 μg/ml concentration are presented. Compounds that produced a necrotic reaction were considered to be phytotoxins. The extent of necrosis was measured by tracing the necrotic area on transparent graph paper and counting the number of grids. Distilled water and solvent checks were maintained in each test. Four leaf pieces were used in each test and the tests were performed twice. The data were subjected to an analysis of variance and means were separated utilizing the Waller-Duncan k-ratio t test (12).

Antifungal activity in vitro. Chromatographically pure, recrystallized trichothecolone acetate, and recrystallized 6-methylsalicylic acid isolated from *P. fructicola* culture filtrate were used to prepare ethyl acetate solutions containing known concentrations of the solute. Antibiotic assay disks (8 mm in diameter) were impregnated with 20 μl of an ethyl acetate solution containing different concentrations of appropriate toxin. The ethyl acetate was allowed to evaporate. Treated disks were placed on PDA in 100-mm petri dishes containing 20 ml of medium that had been seeded with a lawn of conidia of each test fungus: *Colletotrichum acutatum* J. H. Simmonds (Lab # NC 1708) (cause of bitter rot), *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (NC 1700) (cause of bitter rot), *Botryosphaeria obtusa* (Schwein.) Shoemaker (NC 087) (cause of black rot), *B. dothidea* (NC 1502) (cause of white rot), and *P. fructicola.* Disks used as controls were treated with 20 μl of ethyl acetate, and the ethyl acetate was allowed to evaporate before placing the disks on the agar dishes. The culture dishes were incubated at 26 C, observed at suitable intervals, and the diameters of the inhibitory zones were recorded after 48 hr. Three dishes were used for each toxin concentration and fungus combination. The experiment was repeated once with trichothecolone acetate and three times with 6-methylsalicylic acid. Data from experiments with trichothecolone acetate were subjected to an analysis of variance, and means were separated with the Waller-Duncan k-ratio t test. The response of each fungus to increasing concentrations of 6-methylsalicylic acid was examined with regression analysis (12).

Antifungal activity in vivo. An in vivo test was performed to determine if 6-methylsalicylic acid would reduce fruit infection by *C. acutatum.* A 2 × 2 cm area was marked off with a wax pencil on the surface of mature, nonfungicide-sprayed fruit of Golden Delicious. Methylsalicylic acid was solubilized in either methanol or water and each 2 × 2 cm area was wet uniformly with a 0 (methanol or water control), 250, 500, 1,000 or 5,000 μg/ml solution. Fruit were allowed to dry for 4 hr and then were inoculated with a 1×10^5 conidia per milliliter suspension of *C. acutatum.* The inoculation procedure consisted of dipping a piece of sterile 3 × 3 cm laboratory towel in the suspension, applying it to the treated surface of the fruit, and covering the piece of laboratory towel with aluminium foil to retard drying. Fruit were placed in plastic moist chambers for 48 hr, after which they were removed and the surface was wiped with 70% ethanol to remove any surface inoculum. The inoculated area was sectioned into 10 pieces of equal size (ca 40 mm^2 with 5 mm underlying tissue), which were incubated in a petri dish moist chamber for 1 mo and observed for sporulation of *C. acutatum.* Five fruit were used for each treatment. We were not able to satisfactorily wet the marked area on the fruit with the water suspension of 6-methylsalicylic acid; results were variable and consequently are not presented. Data from the experiments using methanol as the solvent were examined with a regression analysis (11).

RESULTS

Identification of toxic metabolites. The preparative TLC plates of the neutral fraction from *P. fructicola* 2C3F1 showed two distinct fluorescence-quenching zones when observed under UV light. These metabolites were eluted from the TLC zones with ethyl acetate and recrystallized from hexane-ethyl ether. The higher Rf metabolite yielded 20 mg of fine, white crystals (mp 102 C), and the lower Rf substance gave 10 mg colorless needles. The near identity of these two metabolites is evident in their ^{1}H nmr spectra, which differed by the presence of an acetate methyl (2.06 ppm) in the spectrum of the less polar metabolite. Both possess unsaturated ketone absorption in the infrared (1,680 cm^{-1}) and ultraviolet spectra (λ_{max} 225, a_M 20,000). In addition, the ir spectrum of the less polar, high Rf metabolite showed the presence of the acetate ester (1,735 cm^{-1}). The identity of the ester as an acetate was confirmed by the 42 a.m.u. difference between the molecular weights of the two metabolites (chemical ionization protonated molecular ions at m/z 307 and 265). The ir, uv, ms, and ^{1}H nmr data for the more polar, low Rf metabolite were identical to those reported for trichothecolone (4,5) . Thus, the less polar metabolite was identified as trichothecolone acetate. Its ^{1}H, and ^{13}C nmr spectra were consistent with the published spectra of trichothecolone acetate (13).

The phenolic fraction was resolved on a silica gel column by elution with methylene chloride and acetone. Fifty 10-ml fractions were collected. Evaporation of pooled fractions 11–30 gave a crystalline residue that was recrystallized in hexane-ethyl ether to yield 325 mg, m.p. 170 C. This metabolite was identified as 6-methylsalicylic acid by comparing its Rf, uv, and ^{1}H nmr spectra with those of a standard of 6-methylsalicylic acid (24). Pooled fractions 37–50 contained a highly polar material that was purified by preparative HPLC to obtain 30 mg of colorless crystals, m.p. 205 C. The mass spectral molecular weight (m/z 154), and ^{1}H nmr spectrum (7.28 ppm, J = 2 Hz,1H; 6.83 ppm, dd, J = 9, 2 Hz,1H; 6.63 ppm, d, J = 9 Hz, 1H) indicated the presence of a dihydroxybenzoic acid. The metabolite was identified as 2,5-dihydroxybenzoic acid by its melting point and by comparison with authentic standards of the isomeric dihydroxybenzoic acids on TLC in three solvent systems. The remaining phenolic

fractions were biologically inactive.

Culture filtrates of *L. elatius* and two additional isolates of *P. fructicola* were subjected to acid-base extraction and examined for the presence of the toxins by HPLC with a photodiode array detector. The acidic fractions of *P. fructicola* C4P8 and B4Ra4b contained 6-methylsalicylic acid with retention time (4.95 min) identical to that of the purified standard. The identification was confirmed by overlay of the full UV spectrum with that of the standard. Trichothecolone (retention time 5.79 min) was readily detected in the neutral fraction from *P. fructicola* B4Ra4b and was also present in the neutral fraction from *P. fructicola* C4P8. Identification of trichothecolone was confirmed by comparison with retention time of pure trichothecolone and by UV spectral overlay. Neither trichothecolone nor 6-methylsalicylic acid could be detected in the neutral or acidic fractions of culture filtrate of *L. elatius* ES5P6.

Leaf bioassay for toxicity. The cell-free culture filtrate caused brown necrosis on apple cultivars and strains and weed species within 48 hr after application. There was variation in the size of necrotic spots among the three apple cultivars or strains screened. There was no significant difference in the size of the necrosis on Super Gold (5.7 mm^2) and Delicious (5.0 mm^2); however, the area of necrosis on both was significantly larger than the necrotic area on Golden Delicious (2.7 mm^2). Among weed species leaves of monocots (johnsongrass and sorghum) showed larger necrosis than dicots (Table 1). Sicklepod and lambsquarters were least sensitive to the culture filtrate.

6-methylsalicylic acid caused larger necrosis than trichothecolone acetate on all apple cultivars (Table 2). Super Gold, Silver Spur, and Golden Delicious were the most sensitive varieties or strains tested to 6-methylsalicylic acid; Super Gold, Oregon Spur, and Silver Spur were the most sensitive to trichothecolone acetate.

Trichothecolone acetate was very phytotoxic to all weed species. Sicklepod and morning-glory were most sensitive (Table 1). Water-soaked lesions were visible 12 hr after treatment on most of the weed leaves treated with trichothecolone acetate; the lesions turned light brown after 48 hr. Trichothecolone generally was less toxic than its acetate on weed leaves. However, it caused necrosis at 100 μg on all weed species. 6-methylsalicylic acid also caused necrosis on all weeds; jimsonweed leaves showed larger necrosis compared with other weed species. Dihydroxybenzoic acid was the least active phytotoxin, but caused necrosis on all weeds except prickly sida.

Antifungal activity in vitro. Trichothecolone acetate and 6-methylsalicylic acid

Table 1. Phytotoxicity of cell-free culture filtrate and purified toxins of *Peltaster fructicola* on different weed species

	Toxic metabolite[x]				
Weed species	**cf**	**1**	**2**	**3**	**4**
Sicklepod	2.3 f[y,z]	21.0 e	125.3 a	38.5 d	3.0 b
Morningglory	3.3 ef	31.3 bcd	136.0 a	128.5 a	2.0 b
Lambsquarters	3.3 ef	38.0 b	61.0 d	25.5 e	3.0 b
Jimsonweed	4.0 def	48.0 a	81.3 c	38.3 d	1.7 b
Watercress	5.3 cd	25.3 de	103.7 b	107.5 b	5.0 a
Prickly sida	6.7 bc	33.0 bc	74.3 cd	54.5 c	0.0 c
Sorghum	7.7 b	31.3 bcd	104.7 b	40.0 d	4.7 a
Johnsongrass	9.7 a	31.0 cd	82.7 c	60.0 c	1.7 b

[x]cf = concentrated five fold; 1 = 6-methylsalicyclic acid; 2 = trichothecolone acetate; 3 = trichothecolone; 4 = 2,5-dihydroxybenzoic acid. All pure toxins were tested at 100 μg/ml.
[y]Values presented are mean necrotic area (mm^2).
[z]Means within same column followed by same letter are not significantly different at $P = 0.5$ as determined by the Waller-Duncan k-ratio t test.

Table 2. Phytotoxicity of purified toxins of *Peltaster fructicola* (2C3F1) on different apple cultivars or strains

Apple cultivars or strains	Toxin[x]	
	6-methylsalicylic acid	**Trichothecolone acetate**
Oregon Spur[z]	78.7 ab[y]	18.0 b
McIntosh	38.3 d	11.3 c
Law Rome	74.7 abc	4.0 d
Red Chief[z]	63.3 c	3.3 d
Silver Spur[z]	82.0 ab	20.7 ab
Super Gold	84.0 a	25.3 a
Paula Red	70.0 bc	3.7 d
Golden Delicious	83.0 a	2.3 d

[x]Concentration of both toxins was at 100 μg/ml.
[y]Values presented are mean necrotic area (mm^2). Means within same column followed by same letter are not significantly different at $P = 0.5$ as determined by the Waller-Duncan k-ratio t test.
[z]Strains of the cultivar Delicious.

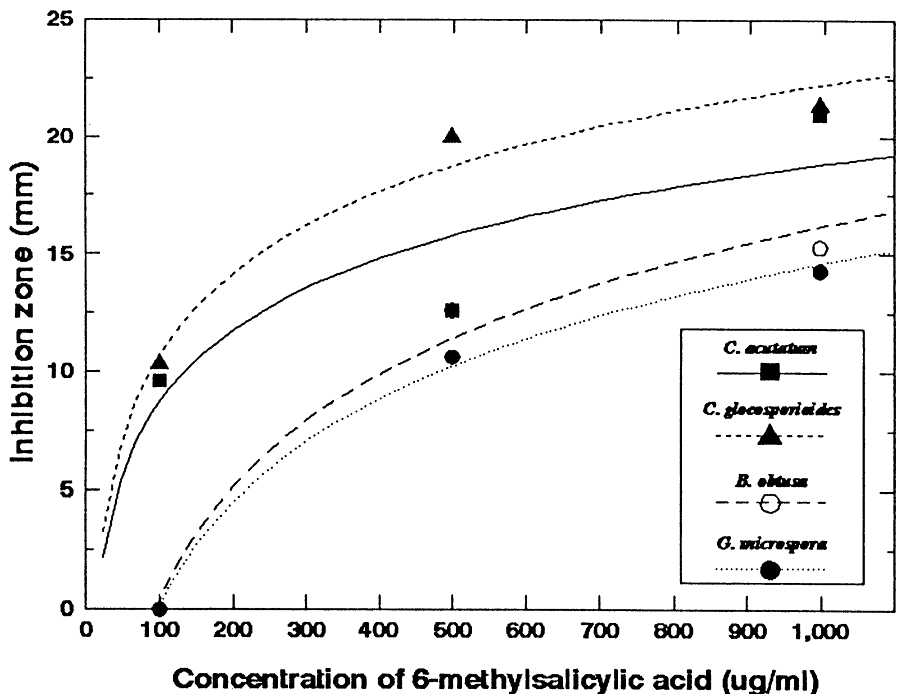

Fig. 1. Antifungal activity of 6-methylsalicylic acid against apple fungal pathogens in vitro. Responses of fungi to increasing rates of 6-methylsalicylic acid described by following equations: *Peltaster fructicola*, $z = -1.17 + 0.023c - 0.0000137c^2$ ($R^2 = 0.95$); *B. obtusa*, $z = -1.45 + 0.0366c^2 - 0.00000196c^2$ ($R = 0.96$); *C. gloeosporioides*, $z = 2.106 + 0.568c - 0.000037c^2$ ($R^2 = 0.95$); and *C. acutatum*, $z = 3.75 + 0.0176c^2$ ($R^2 = 0.85$); where z = diameter of zone of inhibition and c = toxin concentration.

produced large inhibition zones against all fungal species tested. Spores within 5–10 mm of impregnated disks failed to germinate; germ tube length and subsequent mycelial growth increased with distance from the disks. Trichothecolone acetate was generally more active and produced larger zones of inhibition than 6-methylsalicylic acid against all fungi; average diameter of zones of inhibition with trichothecolone acetate at 400 μg were greater than those of 6-methylsalicylic acid at 1,000 μg. There was no difference ($P = 0.05$) in the diameter of the inhibition zone among fungal species at 200 μg of trichothecolone acetate, but at 400 μg the zone of inhibition was significantly greater for both *Botryosphaeria* spp. than for the *Colletotrichum* spp. (diameters for zones of inhibition for *B. dothidea, B. obtusa, C. acutatum,* and *C. gloeosporiodes* at 400 μg were 32.0, 29.5, 25.0, and 24.0 mm, respectively).

Colletotrichum spp. were more sensitive to 6-methylsalicylic acid than were *Botryosphaeria* spp. There was a significant increase in the zone of inhibition with increasing rates of 6-methylsalicylic acid for each fungal species tested except *B. dothidea,* which was not inhibited at 500 μg/disk (Fig. 1).

Antifungal activity in vivo. There was a decrease in the percent fruit pieces infected by *C. acutatum* with increasing concentrations of 6-methylsalicylic acid ($P = 0.01$). The percent fruit pieces infected at 0, 250, 500, 1,000, and 5,000 μg/ml was 66, 70, 8, 18 and 14, respectively. Some phytotoxicity was observed at 5,000 μg/ml.

DISCUSSION

Isolates of *P. fructicola* produced trichothecolone, trichothecolone acetate, 6-methylsalicylic acid, and dihydroxybenzoic acid in culture broth while an isolate of *L. elatius,* another fungus associated with sooty blotch, did not produce trichothecolones or 6-methylsalicylic acid. Cell-free culture filtrate and all four toxins produced by *P. fructicola* caused necrotic lesions on leaves of several different weed species and eight cultivars or strains of apple. Trichothecolone acetate was the most toxic compound and dihydroxybenzoic acid the least toxic on the weed species tested. However, 6-methylsalicylic acid was more toxic to the apple cultivars and strains than trichothecolone acetate. The possible role of these toxins in the pathogenesis of *P. fructicola* is not known. Several fungi are known to produce 6-methylsalicylic acid in culture (10,11,14). The role of 6-methylsalicylic acid produced by *Stagonospora sacchari* Lo & Ling in pathogenesis has been correlated to its production by germinating spores that result in selective leakage of electrolytes from the susceptible sugarcane leaf tissues (24). Because *P. fructicola* colonizes primarily the cuticle, with little or no penetration of the epidermis, it is unlikely that 6-methylsalicylic acid is involved in pathogenesis. The roles of trichothecolone and trichothecolone acetate in pathogenesis have not been determined. Trichothecolone and its acetate have been isolated from *Trichothecium roseum* (Pers.:Fr.) Link, which infects anise (*Pimpinella anisum* L.) fruit in the field (2).

Our data indicate that if *P. fructicola* produces toxic metabolites on infected apple fruit, it may compete with *Botryosphaeria* spp. and *Colletotrichum* spp. on the apple cuticle. Both trichothecolone acetate and 6-methylsalicylic acid were inhibitory to these fungi in vitro. Further, treatment of fruit with 6-methylsalicylic acid reduced infection by *C. acutatum.* The antimicrobial activity of 6-methylsalicylic acid produced by *Phoma* sp. on assorted bacteria and fungi has recently been reported (23). Detection of the toxins on *P. fructicola*–infected fruit would provide additional evidence for their role in reducing infection by such pathogens as *Botryosphaeria* spp. and *Colletotrichum* spp. in the orchard. We attempted to detect 6-methylsalicylic acid and trichothecolone acetate in a sample of peel from fruit heavily affected with sooty blotch, but were unable to detect them in the presence of a large amount of wax from the apple cuticle.

Trichothecolone and trichothecolone acetate are mycotoxins and have been shown to cause mycoses and related disorders in mammals (20). Although we have not demonstrated the presence of these mycotoxins on *P. fructicola*–infected fruit, their production in vitro suggests they may be a potential problem if sooty blotch is not satisfactorily controlled. Fruit affected with sooty blotch are likely to become more common as fewer fungicides are applied in order to reduce fungicide residues at harvest or as IPM programs are utilized that rely on chlorine dips at harvest to remove sooty blotch or flyspeck symptoms (6).

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