The Influence of Fungi Isolated from Peach Twigs on the Pathogenicity of Cytospora cincta

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ABSTRACT

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Four species of fungi consistently isolated from twig elements (bud, leaf scars, and internodes) of peach (*Prunus persica* 'Redhaven') were studied in combination with *Cytospora cincta,* causal agent of perennial peach canker. *Alternaria alternata, Epicoccum purpurascens,* and *Coniothyrium olivaceum* when grown on potato-dextrose agar (PDA) produced substances or caused nutrient depletion which adversely affected the germination and germ-tube growth from conidia of *C. cincta.* In contrast, *Aureobasidium pullulans* produced diffusible products on PDA that stimulated germ-tube growth from *C. cincta* conidia. *Epicoccum purpurascens* produced the largest zones of inhibition when grown in dual culture with *C. cincta* and produced at least two antifungal compounds inhibitory to conidial germination of *C. cincta. Epicoccum purpurascens, C. olivaceum,* and *A. alternata* wound-inoculated singly or in various combinations with *C. cincta,* inhibited canker development on peach twigs. *Epicoccum purpurascens* and *C. olivaceum* were most effective in reducing disease severity when inoculated with *C. cincta* in the field, whereas *A. pullulans* did not significantly ($P = 0.05$) reduce canker enlargement.

Additional key words: *Valsa* spp.

Colonization of tree bark by saprophytic microorganisms was reported by Bier and Rowat (2,4) and others (7,11), who suggested that the bark of a healthy tree or of an individual cutting supports a complex microbiological community. It also has been demonstrated that some saprophytes occurring on and within the bark of trees were efficient, either individually or collectively, in preventing diseases caused by canker pathogens (2, 4, 5, 7, 11). Modification of nutritional properties of living bark may result from the growth of microbiological floras either in or on bark tissues (2, 15). Bier (2, 3) and Wensley (14) proposed that the incidence and severity of the canker diseases among individual host plants was influenced by bark microflora.

The role of saprophytic bark fungi in the perennial peach-canker complex, caused by *Cytospora cincta* and *C. leucostoma* is not clear. We report on the interactions of four fungi isolated from peach bark tissue on the growth and pathogenicity of *C. cincta* (IMI 194892).

MATERIALS AND METHODS

Four fungi [*Aureobasidium pullulans* (de Bary) Arnaud. (IMI 199373), *Epicoccum purpurascens* Ehrenb. ex Schlecht. (IMI 199370), *Alternaria alternata* (Fr.) Keissler (IMI 199372), and *Coniothyrium olivaceum* Bon. (IMI 197848)] were consistently isolated from buds, leaf scars, and internodes (twig elements = TE) of 1-yr-old peach [*Prunus persica* (L.) Batsch 'Redhaven'] twigs that had been surface disinfested by soaking in a 1% NaOCl solution for 5 min, in 70% ethanol for 2 min, and then rinsed in sterile distilled water. The TE were plated on potato-dextrose agar and incubated for 2 wk at room temperature (25 ± 2 C). The IMI numbers were assigned to subcultures of our isolates that were deposited at the Commonwealth Mycological Institute, Kew, Surrey, England.

Media preparation.—Five kinds of media were used: Difco potato-dextrose agar (PDA); malt-extract agar (MEA), and malt-extract broth (MEB); and laboratory-prepared media including peach-bark-extract agar (PBA) and potato-dextrose broth (PDB). Peach-bark-extract agar was prepared by mixing 125 g of thinly shredded powder obtained by grinding field-grown, 1-yr-old peach twigs in a Fitz Mill (9) in 1,000 ml of distilled water, followed by blending in a food blender, and squeezing the slurry through two folds of cheesecloth. The extract was centrifuged at 10,000 *g* for 20 min. Twenty g of agar was added to 1,000 ml of the extract prior to autoclaving at 121 C for 15 min. The pH varied from 5.6 to 5.8.

Potato-dextrose broth (PDB) was made by autoclaving 200 g of peeled, sliced potatoes in 1,000 ml of water for 30 min at 121 C. The resulting extract was strained through

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four layers of cheesecloth and adjusted to 1,000 ml. Fifteen g of anhydrous D-glucose were added to the extract and 50 ml of the solution was dispensed into 250-ml Erlenmeyer flasks prior to autoclaving for 15 min at 121 C.

Antagonism on agar plates.—Inhibition of *C. cincta* on MEA or PBA by the four fungi was evaluated in dual culture. A mycelial plug of the potential antagonist was placed at the periphery of four replicate culture plates and incubated for 5 days at 25 C. A mycelial plug of *C. cincta* then was placed 5 cm from the inoculum point of the potential antagonist and the plates were incubated for an additional 7 days at 25 C. The growth, in mm, of the test organism was recorded. Inhibition of the pathogen's development in dual culture was assessed by two parameters (8): the percentage inhibition of radial growth $[100 \times (r_1 - r_2)/r_1]$ and the width of the zone of inhibition (ZI) measured at the smallest distance between both colonies (Fig. 1). Zones of inhibition and inhibition of radial growth of the test organism by the potential antagonist were analyzed using a randomized complete block (split-plot) design with four replications. The potential antagonists were assigned at random to the whole unit within each complete block; test organisms were assigned as subunits within each whole unit.

In a second experiment, the effect of diffusion products from various test organisms on the germination and germ-tube length (in μm) of *C. cincta* was recorded. The method, described by Jefferys (10), utilizes Van Tieghem cells (4 cm in diameter and 1 cm deep) placed in culture plates and the cells filled with PDA. The upper surface of the Van Tieghem cell was streaked with spores of the test organism on a flamed loop and the culture plates were incubated for 5 days at 25 ± 1 C. The Van Tieghem cells with medium, then were inverted with a sterile spatula and the new upper surface was seeded with a water suspension (0.03 ml) of *C. cincta* conidia containing approximately 1.3×10^5 conidia/ml. After incubation for 26 hr the percent germination was determined and the germ-tube lengths of 20 randomly selected *C. cincta* conidia were measured from each cell with a stage micrometer. Five replicates of each organism were examined.

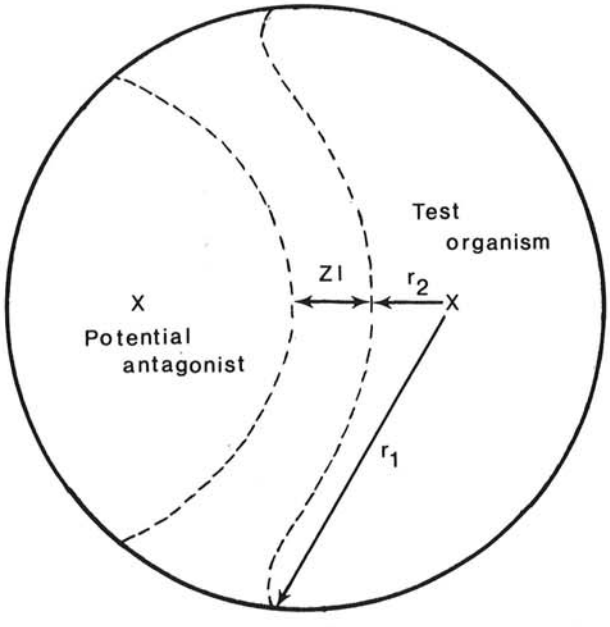

Fig. 1. Diagram of the mode of inoculation of agar plates with the test organism and potential antagonist. Parameters for inhibition are the width of the zone of inhibition (ZI) and the percentage inhibition of radial growth $[100 \times (r_1 - r_2)/r_1]$.

Production of antibiotics by Epicoccum purpurascens.—To test for production of antibiotics by two isolates of *E. purpurascens* (IMI 199370 and IMI 199371), 200-ml aliquots of MEB contained in 1-liter flasks were seeded separately with the different isolates and incubated for 12 days at 25 C. Aliquots (25 μliter) of fermentation broth from the fungal cultures were chromatographed on Eastman 6061 silica gel plates (20 × 20 cm) without fluorescent indicator. Twenty-five μliters of nonseeded fermentation broth and 25 μliters of a 50 μg/ml solution of flavipin (3,4,5-trihydroxy-6-methyl-pthalaldehyde) (Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, England) served as controls. The plates were replicated three times in a mixture of benzene:dioxane:glacial acetic acid (90:25:4, v/v). When solvent fronts reached 12-14 cm, the plates were dried in a constant air flow and developed either with an aqueous solution of $FeCl_3$ (13) or by using the bioautography technique of Peterson and Edgington (12). The bioautography technique consisted of spraying PDA seeded with *C. cincta* conidia (3×10^6/ml) onto the plates, which were incubated in a moist chamber for 48 hr at 25 C. Zones of inhibition were noted as clear areas where *C. cincta* did not grow due to the presence of antifungal compounds. The data were analyzed by analysis of variance.

Antagonism in the field.—Suppression of perennial canker development in the orchard was evaluated by inoculation of *C. cincta* either alone, in combination with one, or with more than one of the four fungi. Inoculum suspensions were prepared in sterile-distilled water by fragmenting 7-day-old PDB cultures of the fungi in a Waring Blendor. The final concentration of the fungi

TABLE 1. Percentage germination and mean length of germ-tubes of *Cytospora cincta* spores incubated for 26 hr at 25 C on potato-dextrose agar in Van Tieghem cells containing diffusion products of several fungi consistently isolated from peach (*Prunus persica*) twig tissue

Source of diffusion product	Germination (%)	Germ-tube length[a] (μm)
Aureobasidium pullulans	84	54.1
Control	74	37.7
Epicoccum purpurascens	0	0.0
Alternaria alternata	0	0.0
Coniothyrium olivaceum	0	0.0
FLSD[b]		
$P = 0.05$	20	10.9
$P = 0.01$	30	16.1

[a]Means based on 20 spores per each of five replications.
[b]Fisher's least significant difference, for comparison between any two means.

TABLE 2. Mean percent inhibition of radial growth[a] of test organism by potential antagonist on malt-extract agar (MEA) or peach (*Prunus persica*) bark extract agar (PBS) in dual culture

Potential antagonist[b]	Test organism									
	Alternaria alternata		*Aureobasidium pullulans*		*Coniothyrium olivaceum*		*Cytospora cincta*		*Epicoccum purpurascens*	
	MEA	PBA	MEA	PBA	MEA	PBA	MEA	PBA	MEA	PBA
Alternaria alternata	4	8	56	26	57	47	27	27	55	57
Aureobasidium pullulans	25	26	4[c]	0[c]	2	0	49	21	26	28
Coniothyrium olivaceum	40	18	26	33	33	70	28	21	42	33
Cytospora cincta	40	49	7	18	18	21	28[d]	23[d]	28	31
Epicoccum purpurascens	23	20	11	29	44	41	68	66	29	33
FLSD[e]										
$P = 0.05 = 22$										
$P = 0.01 = 34$										

[a]Percent inhibition of radial growth calculated by $100 \times (r_1 - r_2)/r_1$. See Fig. 1.
[b]Potential antagonists were placed at the periphery of four replicate petri plates and incubated for 5 days; *C. cincta* then was seeded 5 cm from the inoculum point of the potential antagonist and re-incubated for an additional 7 days at 25 C.
[c]Organisms were placed 4 cm apart instead of 5 cm.
[d]The potential antagonist was incubated for 4 days prior to inoculation of the test organism.
[e]Fisher's least significant difference, for comparison between any two means.

varied from 2 to 9×10^6 propagules/ml. *Cytospora cincta* inoculum consisted of mycelial fragments plus about 5×10^6 conidia/ml. Final concentration of *C. cincta* inoculum was approximately 10^7 propagules/ml. Bark plugs (5 mm diameter) were removed with a cork borer from 1-yr-old Redhaven peach twigs (4- to 6-cm girth) that had been surface disinfested with 70% ethanol. The wound was inoculated by placing approximately 0.3 ml of the inoculum suspension with a syringe on the wound and binding the wound with three layers of masking tape. Trees were inoculated in September and lesion length was recorded 7 wk later.

RESULTS

Antagonism on agar culture plates.—Three species of fungi, *E. purpurascens, A. alternata,* and *C. olivaceum,* produced diffusible products or caused nutrient depletion that significantly ($P = 0.01$) reduced germination of *C. cincta* conidia in Van Tieghem cells (Table 1). Even after 48 hr all *C. cincta* conidia had failed to germinate. *Aureobasidium pullulans* produced diffusible metabolites that stimulated germ-tube growth from *C. cincta* conidia significantly ($P = 0.05$) over controls, but did not increase percent germination of conidia.

Epicoccum purpurascens produced ZI of 6.3 mm and 5 mm on MEA and PBA, respectively, in dual culture with *C. cincta.* The ZI produced by *E. purpurascens* was significantly ($P = 0.05$) larger on MEA than on PBA. *Aureobasidium pullulans* produced ZI in dual culture with *C. cincta* of 2.3 mm and 5 mm on MEA and PBA, respectively. *Alternaria alternata* and *C. olivaceum* did not produce ZI in dual culture with *C. cincta.* Both of these fungi, however, did inhibit radial mycelial growth of *C. cincta* (Table 2). *Cytospora cincta* produced ZI against itself of 1.3 mm on MEA and 1 mm on PBA. Inhibition of radial mycelial growth of *C. cincta* by *E. purpurascens* was greater than by the other test fungi (Table 2).

Antibiotic production by Epicoccum purpurascens.—Two antifungal compounds were detected at R_f 0.69 and 0.55 on the bioautographs of the fermentation broth from both *E. purpurascens* isolates. Considerable variation existed in the size of the ZI obtained from the two isolates but the component at R_f 0.69 always produced the larger ZI. No ZI was produced by nonseeded control fermentation broths or flavipin on bioautographs. Chromatographed samples of flavipin gave a blue-black reaction at R_f 0.84 when sprayed with

TABLE 3. Mean canker length on 1-yr-old peach (*Prunus persica* 'Redhaven') twigs wound-inoculated with *Cytospora cincta* alone or in combination with four other fungi

Fungal combinations[a]					Canker length (mm)
Cc	Ap	Ep	Aa	Co[b]	
X					14.83
X	X				12.50
X		X			0.83
X			X		4.67
X				X	2.50
X	X	X			2.67
X	X		X		2.83
X	X			X	2.00
X		X	X		3.00
X		X	X	X	2.33
X			X	X	2.83
X	X		X	X	2.33
X	X	X	X		1.33
X	X	X		X	1.67
X		X	X	X	2.83
X	X	X	X	X	2.83
FLSD[c]					
$P = 0.05$					3.71
$P = 0.01$					5.23

[a]Columns with a X indicate organism(s) used in inoculation.
[b]Abbreviations: Cc = *Cytoposora cincta,* Ap = *Aureobasidium pullulans,* Ep = *Epicoccum purpurascens,* Aa = *Alternaria alternata,* and Co = *Coniothyrium olivaceum.*
[c]Fisher's least significant difference, for comparison between any two means.

an aqueous solution of $FeCl_3$. Fermentation broth of both *E. purpurascens* isolates produced a blue-black reaction with aqueous $FeCl_3$ at R_f 0.69. No visible reaction occurred between nonseeded control broth and aqueous $FeCl_3$.

Antagonism in the field.—Extensive gum production was evident in the wounds that had been inoculated with *C. cincta* alone or in combinations of *C. cincta* and *A. pullulans*. Less gumming was observed when other combinations of fungi were used.

There was no significant ($P = 0.05$) difference in canker development when *C. cincta* was inoculated alone or in combination with *A. pullulans* (Table 3). *Epicoccum purpurascens, C. olivaceum,* and *A. alternata* were effective in reducing canker development when inoculated individually with *C. cincta*. Combinations of two or more of the fungi were equally effective in reducing disease development by *C. cincta*.

DISCUSSION

In vitro fungal interactions resulted in one of the following: production of a ZI, contact inhibition, or no inhibition. Fokkema (8) pointed out that the various growth rates of the test organisms affect the radial growth of the pathogen independent of their antagonistic actions, whereas the ZI remains unaffected. Alternately, the width of the ZI may be affected by the retardation of the growth of the saprophyte.

Epicoccum purpurascens was the most effective fungus tested in reducing *C. cincta* canker development. Interactions with the other fungi in multiple combinations with *C. cincta* did not enhance the ability of *E. purpurascens* to inhibit the pathogen. Results of the in vitro tests show that *E. purpurascens* produced the largest ZI in dual culture and was the most effective fungus in reducing radial mycelial growth of the pathogen. Microscopic examination of cultures of *E. purpurascens* and *C. cincta* in close proximity showed hyphae of *E. purpurascens* coiled around hyphal segments of *C. cincta* (Fig. 2). Campbell (6) reported a similar attack of *E. purpurascens* on *Helminthosporium sativum* spores. Campbell (6) also observed that *E. purpurascens* produced an unidentified diffusible substance which restricted hyphal growth of *H. sativum* when they were grown side by side on agar media. Bamford et al. (1) confirmed this observation and isolated an antifungal compound produced by *E. purpurascens* and *E. andropogonis* which was identical (by melting point and comparison of the infrared spectra) with flavipin previously obtained from *Aspergillus flavipes* and *A. terreus* by Raistrick and Rudman (13). Our results from the bioautographs of flavipin show that this antibiotic at low concentrations apparently did not affect the growth of *C. cincta* and that our isolates of *E. purpurascens* grown in MEB did not produce detectible amounts of flavipin. There were, however, at least two antifungal compounds produced by *E. purpurascens* that were inhibitory to *C. cincta*. These compounds, if isolated and purified, could be of value in controlling perennial canker.

The results of these tests points out that several fungi can inhibit canker development in the field. The fungi isolated from peach tree tissues used in this study were effective either alone or in combination in reducing canker development by *C. cincta*. These results suggest that part of the variation in canker development and severity may be attributable to the presence or absence of these or other microorganisms.

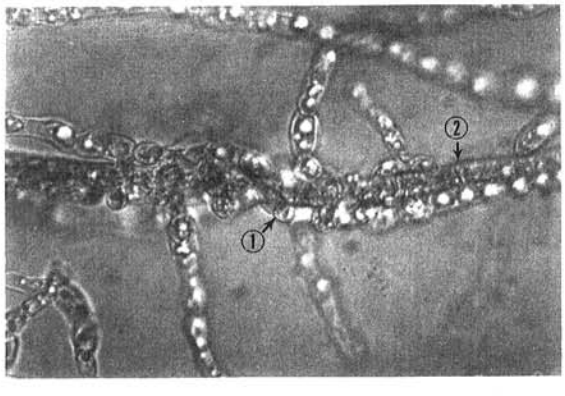

Fig. 2. Mycelium of *Epicoccum purpurascens* (1) coiled around a hyphal segment of *Cytospora cincta* (2).

LITERATURE CITED

1. BAMFORD, P. C., G. L. F. NORRIS, and G. WARD. 1961. Flavipin production by Epicoccum spp. Trans. Br. Mycol. Soc. 44(3):354-356.
2. BIER, J. E. 1963. Tissue saprophytes and the possibility of biological control of some tree diseases. For. Chron. 39:82-84.
3. BIER, J. E. 1964. The relationship of some bark factors to canker susceptibility. Phytopathology 54:250-253.
4. BIER, J. E., and M. H. ROWAT. 1962. The relation of bark moisture development of canker diseases caused by native, facultative parasites. VII. Some effects of the saprophytes on the bark of poplar and willow on the incidence of Hypoxylon canker. Can. J. Bot. 40:61-69.
5. BIER, J. E., and M. H. ROWAT. 1963. Further effects of bark saprophytes on Hypoxylon canker. For. Sci. 9:263-269.
6. CAMPBELL, W. P. 1956. The influence of associated microorganisms on the pathogenicity of Helminthosporium sativum. Can. J. Bot. 34:865-874.
7. CHUDJAKOW, J. P. 1961. Epiphytische Mikroorganismen und die möglichkeit ihrer Verwendung zum Schutze der Pflanzen gegen Krankheiten. Hommung und Förderung phytopathogener Mikroorganismen im Boden. Dtsch. Akad. Landwirt schaftwiss. Berl. Tagungsber. 41:135-144. (In Russian, English summary).
8. FOKKEMA, N. J. 1973. The role of saprophytic fungi in antagonism against Drechslera sorokiniana (Helminthosporium sativum) on agar plates and on rye leaves with pollen. Physiol. Plant Pathol. 3:195-205.
9. GAIROLA, G., and D. POWELL. 1971. Extracellular enzymes and pathogenesis by peach Cytosporas. Phytopathol. Z. 72:305-314.
10. JEFFERYS, E. G. 1948. A technique for rapid demonstration of antifungal substances by fungi or other microorganisms. Trans. Br. Mycol. Soc. 31:246-248.
11. KYSTIC, M., and S. HOCEVAR. 1959. Uticaj nekih antagonistickih mikroorganizama na infeckeife pitomag kestena od Endothia parasitica Anders. Zast. Bilja 54:41-52.
12. PETERSON, C. A., and L. V. EDGINGTON. 1969.

Quantitative estimations of the fungicide benomyl using a bioautograph technique. J. Agric. Food Chem. 17:898-899.

13. RAISTRICK, H., and P. RUDMAN. 1956. Studies in the biochemistry of microorganisms. 97. Flavipin, a crystalline metabolite of Aspergillis flavipes and A. terreus. Biochem. J. 63:395-406.

14. WENSLEY, R. N. 1970. The microflora of peach bark and its possible relation to perennial canker [Leucostoma cincta (Fr.) v. Hohnel (Valsa cincta)]. Can J. Microbiol. 17:333-337.

15. ZABEL, R. A. 1964. Summary with emphasis on trends and needs in research. Symposium on forest trees. Phytopathology 54:275-278.