Growth of *Phoma tracheiphila* on Culture Media in Relation to Fungistatic Phenolic Compounds in Exocortis-infected Sour Orange Seedlings

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Studies on interactions between virus and fungus diseases in citrus have increased since metabolic changes induced by virus diseases in citrus were reported (*3*). In Italy, interest is devoted to studies on the influence of virus infections on mal secco disease, induced by *Phoma tracheiphila* Kanc. et Ghik, in which growth of this fungus is reported to be influenced by flavone constituents of citrus (*1, 5*).

Càtara *et al.* (*2*) reported slower development of mal secco disease on virus-inoculated sour orange seedlings. They also found that growth of *Phoma tracheiphila* on extracts prepared from *Citrus aurantium* infected with infectious variegation virus was proportionately reduced as the phenolic content of these tissue extracts increased.

This paper reports investigations on the growth of *Phoma tracheiphila* on culture media containing extracts of exocortis-infected sour orange.

MATERIALS AND METHODS

Media were prepared from midrib and stem extracts of potted sour orange seedlings that had been bud-inoculated four years previously with exocortis citrus virus. Extracts from healthy sour orange seedlings of the same age were used as controls.

In the first experiments, media were prepared as suggested by Rossetti *et al.* (*6*). Citrus tissues were homogenized in a blendor, and the supernatant was added to 3 per cent agar-agar. In other experiments, 10 ml of tissue extract were added to 90 ml of PDA medium. The mixture was heated and shaken to evaporate the solvent. Both media were sterilized at 121° C for 20 minutes. Dilutions of 1:0, 1:1, 1:10, 1:25, and 1:50 were assayed.

After sterilization, 10 ml of tissue extract medium were placed in 9-cm Petri dishes. Young mycelium from the periphery of a *Phoma tracheiphila* colony growing on PDA was used for inoculation. Cultures were kept at 22° C, in the dark, and the radial growth of the fungus, as the mean of two diameters, was measured every second day.

For chromatography, a procedure suggested by Pinkas *et al.* (*5*) was used. Dried midribs and stems of sour orange seedlings were ground and extracted with chloroform for 92 hours. The mixture was chromatographed on a Merck silica gel (0.05 to 0.20 mm) column packed in benzene. Elution was performed with a solvent mixture containing an increasing concentration of ethyl acetate in benzene. Six fractions (A to F) were combined after thin-layer chromatography (TLC), and tested for fungistatic activity. Fungistatic fractions were pooled, evaporated and taken up in chloroform, treated with activated charcoal, and re-evaporated to dryness. The residue was rechromatographed on a Merck silica gel H column with benzene-ethyl acetate (6:4). Fractions of 15 ml were collected and monitored by silica-gel TLC.

The following solvents were used for TLC: benzene-methanol-acetic acid (45:3:2); benzene-ethanol-water-acetic acid (200:47:15:1); and propanol-water (2:1). Spots were located either by spraying plates with 2 per cent $NaHCO_3$ after I_2

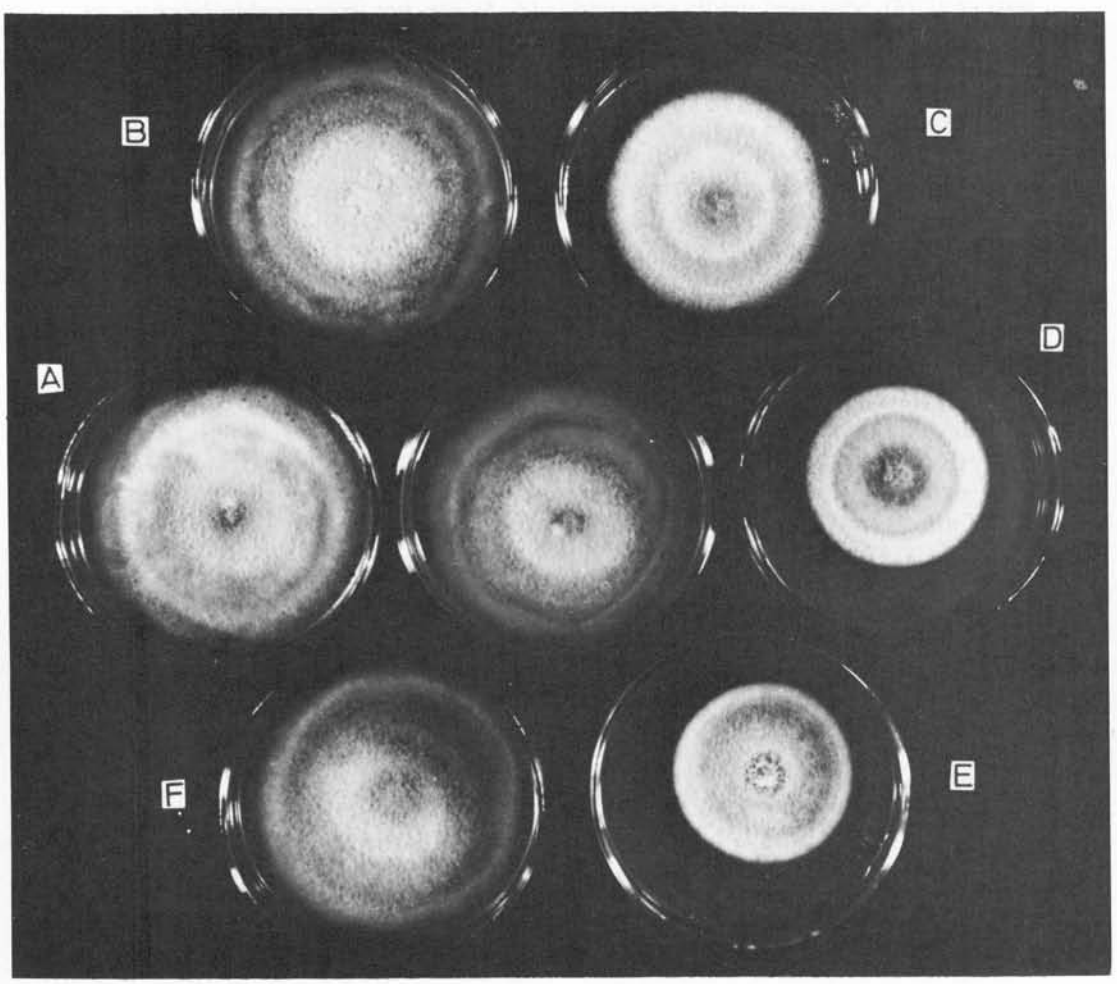
vapor treatment or by heating the plates to 105° C after spraying with anisaldehyde reagent in sulfuric acid and acetic acid.

RESULTS

Bioassays. Growth of *Phoma tracheiphila* was inhibited on media containing citrus extracts regardless of the source. Stem extracts allowed for better growth than did those from midribs. No difference was observed between colonies of the fungus growing on media containing extracts of healthy midribs and those from exocortis-infected midribs, whereas growth was markedly reduced on media containing extracts from affected stems, as compared with growth on those from healthy stems.

An experiment was performed aimed at delineating the fungistatic compounds present in extracts from exocortis-affected tissue. Fractions eluted from column chromatography were

Fig. 1. Distinctive morphological characters of colonies of *Phoma tracheiphila* growing on PDA containing different fractions (A–F) of exocortis-infected stems as compared with a colony growing on PDA without extracts (center).



pooled and added to PDA for assays. Stem extracts were more fungistatic than were those from midribs. Thirty days after inoculation, colonies growing on media with added C, D, and E fractions showed 19.0, 38.8, and 38.1 per cent less growth, respectively, as compared with those growing on media without extract. Fractions A and F reduced fungus growth about 10 per cent, while fraction B induced a slight increase in growth. The morphological characters of the growing colonies are shown in figure 1. When these cultures were transferred to PDA media, differences disappeared. Fungistatic activity was not affected by dilution of 1:1, but declined about 50 per cent upon dilution of 1:10. The 1:50-diluted fractions allowed a growth almost comparable with that of the check media.

Chromatography. Extracts of exocortis-infected seedlings, chromatographed on TLC plates, revealed many different bands in the fungistatic fractions. Nobiletin was identified in the E fraction, and tangeritin in the D fraction. Healthy sour orange seedlings had only a small amount of these flavones in comparison with the exocortis-affected ones.

DISCUSSION

Our results differ from those of Rossetti *et al.* (*6*), who used media prepared from extracts of exocortis-infected leaves of Etrog citron. Since *Phoma tracheiphila* is a vascular fungus, it seemed preferable to use stems in these experiments. The results confirm findings in other studies (*2*) that extracts from stem tissues are more satisfactory for the fungus growth.

Tangeritin and nobiletin could account for the high fungistatic activity of fractions D and E. Both of these flavones, together with other fungistatic flavones, have been reported in citrus cultivars resistant to mal secco disease (*1, 5*). Conversely, both compounds have been detected in cultivars susceptible to the disease, such as sour orange and chinotto (*4*). Results from other authors (*4, 5*) are not comparable because extracts came from different tissues and because plants used were not tested for exocortis.

Salerno *et al.* (*7*) observed a faster development of mal secco on sour orange seedlings inoculated with exocortis citrus virus which did not appear to be related to phenol content of the tissue.

Results reported herein suggest that flavones could possibly influence the evolution of exocortis disease in the field through their influence on growth of the fungus. Since virus infections induce many chemical changes in citrus, it is important to investigate the relationships of different citrus pathogens in mixed as well as in single infections.

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