Food Quality and Chemical Composition

Natural Antifungal Compounds from the Peel of Yam Tubers

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ABSTRACT

Two compounds were isolated from extracts of the peel of yam tubers (*Dioscorea rotundata*) by thin-layer chromatography and were antifungal to the bioassay organism, *Cladosporium cladosporioides*, and pathogens causing storage rot of yams. The toxic compounds were identified with the aid of ultraviolet and infrared spectroscopy, nuclear magnetic resonance and mass spectrometry as 2,5-dihydroxy-4-methoxy-9, 10-dihydrophenanthrene and 7-hydroxy-2, -4, 6-trimethoxyphenanthrene. Both antifungal compounds were absent in the yam flesh.

Introduction

The resistance of plants to fungus disease has been reported to be due to the presence of chemical substances in the plant. Such compounds may be present in the plant host before attack and prove toxic to the fungus prior to its penetration of living host tissue (2, 9); or may be newly produced in response to infection by pathogens (1, 4, 8, 13, 16). Various antifungal compounds have been isolated from potato tubers (the closest root crop to yam tubers) infected by pathogens (3, 10, 11, 14, 15) but not record exists of any isolation of natural antifungal compounds from yam tubers.

Previous work (12) has shown that the soft rot pathogens of the yam tuber are wound parasites and could not cause infection through the intact peel of the yam tuber. This experiment was therefore undertaken as part of an investigation of the biochemical basis of natural resistance of the yam peel to infection.

Materials and Methods

Preliminary extractions

Yam tubers were thoroughly washed with water to remove soil debris on the surface and left to dry. The peel tissue was obtained by removing 1-mm-thick layer from the outer surface of the intact tubers and the innermost (parenchymatous) tissue obtained and used as the flesh. Extracts of the peel and flesh were separately prepared as follows: 50 grams of fresh weight of each tissue was extracted with peroxide-free diethyl ether, 10 ml/g for 30 min and filtered through a sintered funnel. The filtrate was collected and evaporated to dryness under reduced pressure at approximately 40°C. The filtrate residue was finally taken up in 500 ml of cyclohexane ethyl acetate for chromatography. Ten volumes

of each solution were spotted onto thin-layer plates (t.l.c.) in duplicates and developed in equilibrated tanks in the following systems: (a) methanol: chloroform (4:96 v/v); (b) cyclohexane: ethyl acetate (1:1 v/v); or (c) hexane: ether (2:1 v/v). One set of the developed t.l.c. plates was exposed to iodine vapour to locate spots while duplicates were used for the bioassay tests.

Preparation of spore suspension

The test organism used was Cladosporium cladosporioides (fresen.) de Vries (5). The fungus was cultures on potato dextrose agar (PDA) slopes at 25°C for 7 days. Spores were harvested in about 5 ml/slope of glass distilled water (GDW) + 1% Tween 80 to moisten spore surface and filtered twice through two layers of sterile muslin to remove agar and mycelium. The solution was then centrifuged for 10-15 min to make a paste of the spores and the supernatant solution decanted. Spores were washed in two changes of GDW and finally taken up in potato dextrose broth (PDB) before adjusting the concentration to approximately 10^6/ml.

Bioassay test with spores

The t.l.c. bioassay test employed was similar to that of Homans & Fuchs (7). Developed t.l.c. plates were sprayed with the spore suspensions of C. cladosporioides) (10^6 per ml) in PDB, and any excess moisture dried off with a hair dryer. The plates were then incubated in cake boxes lined with moist filter paper for 4 days in darkness at 25°C, to locate antifungal compounds. The bioassary test was repeated for the soft rot pathogens (12) of yam.

Inhibition of germ tube growth

The percentage inhibition of germ tube growth was estimated by comparing the germ tube growth of Aspergillus niger, Botryodiplodia theobromae, Fusarium moniliforme or Penicillium sclerotigenum, the soft rot pathogens and of the test fungus, C. cladosporioides incubated with and without the antifungal compounds under identical conditions. The ED_{50} values were then obtained by plotting percentage inhibition against log. of the concentration and interpolating.

Isolation and purification of antifungal compounds

Approximately 2 kg of yam peel was soaked in two changes of 5 litres of distilled peroxide-free diethyl ether, each time, at 13°C for 3 days and decanted. The combined extracts were filtered through sintered funnel and evaporated to dryness. The extract was dissolved in minimum amount of cyclohexane: ethyl acetate (1:1 v/v) and subjected to column chromatography on silica gel (1 gm extract/100 g silica gel). Fifteen millilitre fractions were collected and evaporated. The fraction residues were separately taken up in the solvent and spotted onto t.l.c. plates and developed in various solvents. The solvents included 4% methanol in chloroform (containing 2% ethanol), cyclohexane: ethyl acetate (various proportions), chloroform: ether (4:1 v/v), Toluene: methanol: acetic acid (10:2:1) and various proportions of petroleum ether: diethyl ether: acetic acid.

Fractions which gave best separations of antifungal compounds were combined and subjected to a second column chromatography with neat chloroform as the solvent. The resulting fractions were evaporated to dryness and separately taken up in chloroform, filtered and streaked across preparative 0.5 mm t.l.c. plates in a narrow band 2 cm from the edge (about 30 mg streaked per plate) and developed

with petroleum ether: diethyl ether: acetic acid (45:50:5) solvent which gave best separation in earlier experiments.

The compounds were detected by spraying the markers at the side of the streak with iodine solution. Antifungal compounds were then separately eluted from unsprayed bands of same RF values as those of the markers with 40 ml of solvent and evaporated to dryness.

Identification procedures

The spectrum was determined in grade alcohol with a pye unicam S.P. 800 spectrophotometer. The infrared spectrum was obtained on a thin film between NaCl plates using a Perkin Elmer 297 spectrophotometer. For mass spectrometry an A.E.I. MS 902 was employed and the 90 MHZ proton magnetic resonance spectrum in $CDCl_3$ was determined on a Perkin Elmer R.32 instrument.

Results

Identifications

No antifungal compounds were detected in the yam flesh. The bioassay of the peel extracts showed three antifungal regions at R_F's 0.30, 0.43 and 0.68 in methanol/chloroform solvent. Only the two R_F components with strong antifungal activity were examined in the present study.

High resolution mass spectomentry of more polar compound 1 (R_F 0.43) showed a molecular ion of m/e 242.0908 indicating the formula $C_{15}H_{14}O_3$. The less polar compound 2 (R_F 0.68) gave a molecular ion at m/e 284.1052 corresponding to the formula $C_{17}H_{16}O_6$.

Antifungal activity

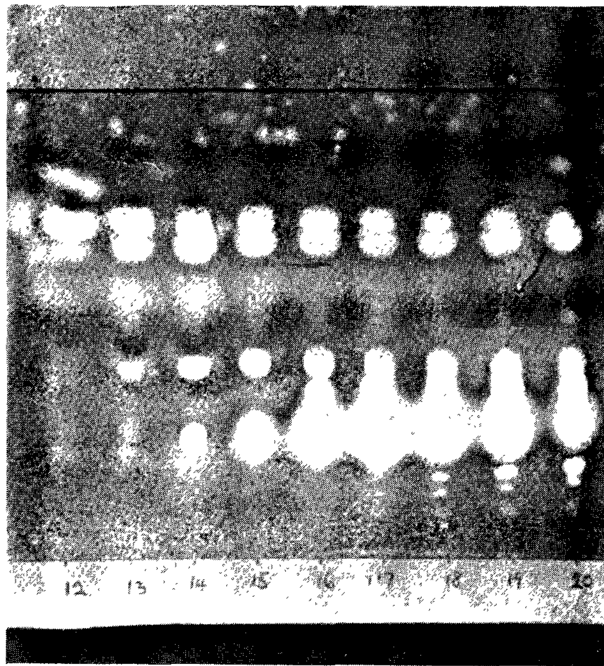
The t.l.c. plates sprayed with spore suspensions of the test fungus and of the soft rot pathogens of yam gave rise to germination inhibition zones. Both compounds affected germ tube growth of all the fungi employed and the ED_{50} values are shown in Table 1.

Table 1. ED_{50} values (ug. ml^{-1}) for compounds 1 and 2 against germ tube growth.

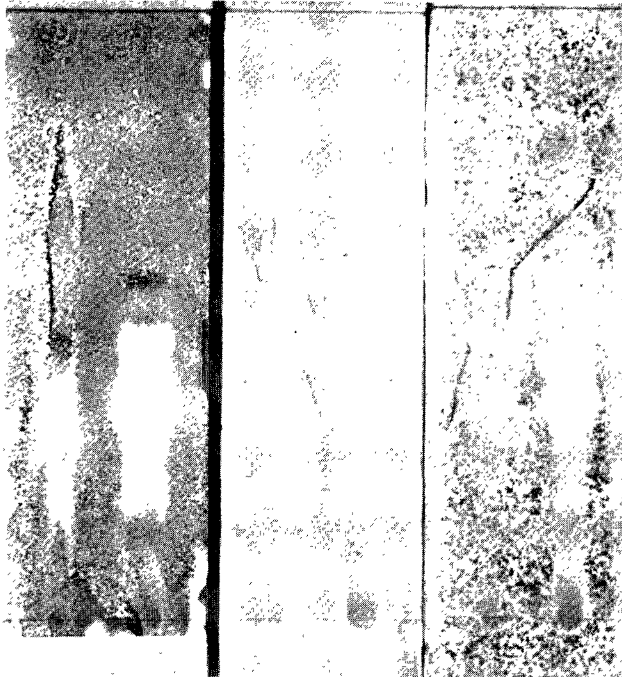
	A. niger	B. theobromae	F. moniliforme	P. sclerotigenum	C. cladosporioides
Compound 1	42	16	65	41	26
Compound 2	14	79	38	32	59

Discussion

We record here for the first time the presence of naturally occurring antifungal compounds in the peel of yam tubers. The spectrum of our compound 1 (Ca $87_{15}H_{14}O_3$) appeared virtually identical to that reported for hircinol (15), a



TLC plate sprayed with <u>C. cladosporioides</u>.



TLC plate sprayed with spore suspensions of the soft rot pathogens of yam.

well known orchid phytoalexin. Both the uv and mass spectra of compound 2 ($C_{17}H_{16}O_6$) correspond closely with the spectra reported for batatasin 1 (6).

The direct spraying of the thin layer chromatograms with spore suspension is a technique that has been successfully used in detection of fungitoxic compounds against Aspergillus niger, Ascochyta pisi, Botrytis cinera, Colletotrichum lindemuthianum, Fusarium culmorum and Penicillium expansum (7). Hircinol, similar to our compound 1, had been shown to exhibit in vitro antimicrobial activity against a range of microorganisms (15) where it was found that concentrations greater than 500 mg. ml^{-1} were necessary to completely inhibit Aspergillus niger to which our compound 1 became lethal at 42 mg. ml^{-1} (Table 1). Similarly, our compound 2 demonstrated antimicrobial activity against the test fungi employed in present study.

The occurrence of these antifungal compounds is of interest since they occur in the peel ("skin") of the yam tubers where they may function as preformed inhibitors in disease resistance apart from the skin acting as a mechanical barrier. Their isolation and synthesis may ultimately be a break through for the control of yam storage rot. Further studies to determine if the compounds also accumulate in response to infection in yam are in progress.

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