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SHORT COMMUNICATION

METABOLISM BY PHYTOPATHOGENIC FUNGI. THE DEGRADATION OF HYDROXYBENZOIC ACIDS BY *GLOMERELLA CINGULATA*

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Abstract—The metabolism of salicylic acid and a number of structural analogues by intact cultures of *Glomerella cingulata* has been studied. Salicylic and 2,3-dihydroxybenzoic acids were unusual in yielding phenol and catechol respectively as metabolites. These conversions represent additional examples of the uncommon non-oxidative enzymatic decarboxylation (E.C. 4.1.1) of aromatic carboxylic acids.

INTRODUCTION

LESS attention has been given to the metabolism of aromatic compounds by fungi than by bacteria.[1] Recent reviews have been published concerning soil fungi[2] and fungal detoxication.[3]

As a contribution to the study of host–parasite relationships involving the phytopathogens *Glomerella cingulata* (Stonem) Spauld. et Schrenk and *Colletotrichum coffeanum* Noack‡ the metabolism of a number of aromatic substrates by these fungi has been studied. In this note the uncommon metabolism of salicylic and 2,3-dihydroxybenzoic acids, in which a non-oxidative decarboxylation process yields phenol and catechol as a terminal and an intermediate product respectively, is reported.

RESULTS AND DISCUSSION

The utilization of salicylic acid and structural analogues as sole carbon source substrates was studied by a stationary culture technique. Aliquot portions of a solution of the substrate in a liquid medium, containing inorganic nutrients only, which had been inoculated with a heavy spore suspension of the phytopathogen were incubated at 23°. The course of the metabolism was followed at 12-hr intervals by ethyl acetate extraction and TLC of the concentrated extract or by u.v. spectroscopy. As the toxicities of the methyl esters to

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‡ *C. coffeanum*[4] is described as the conidial stage of *G. cingulata* and is responsible for the anthracnose of green coffee berries known in East Africa as "coffee berry disease".

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[2] M. E. K. HENDERSON, *Pure Appl. Chem.* **7**, 589 (1963).
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Glomerella cingulata were always considerably less than those of the free acids, it was convenient to use the appropriate ester as the substrate, concentrations of 10^{-3} M generally being used. In cases where the metabolism of the acid was directly studied, concentrations below the respective ED_{50} were employed.

After a short lag phase, methyl salicylate supported an initially vigorous growth of *G. cingulata*. TLC examination, using several solvent systems, indicated that the ester was rapidly utilized, with a small quantity of salicylic acid appearing as an intermediate, to yield phenol as the terminal metabolite. After 48 hr, mycelial growth had declined and all substrate and the bulk of the intermediate acid had been converted to phenol. The identity of the two metabolites was confirmed by isolation from a preparative scale fermentation and the formation of *S*-benzylthiouronium salicylate and the *N*-phenylurethane derivative of phenol.

Similarly, when salicylic acid (2×10^{-4} M) was metabolized by *G. cingulata*, u.v. examination of the culture liquid indicated that the acid was readily utilized and that phenol was the sole product. The non-utilization of phenol by *G. cingulata* was confirmed by the u.v. monitoring of a 14-day incubation in which the substrate concentration remained undiminished.

Examination of a number of analogous methyl hydroxybenzoates revealed that methyl 2,3-dihydroxybenzoate was similarly metabolized via the free acid to yield catechol, the parent phenol. In this case catechol, identified by TLC in several systems, appeared as an intermediate only and was subsequently further metabolized, presumably with ring fission. Of the other substrates studied the methyl esters of 3-hydroxybenzoic, 4-hydroxybenzoic and 3,4-dihydroxybenzoic acids all supported a vigorous growth of *G. cingulata* and were totally utilized. Although the metabolisms again proceeded via the free acids there was no evidence for the formation or accumulation of the parent phenol or an alternative intermediate. A number of miscellaneous substrates, including the methyl esters of 2-methoxybenzoic, 2,4-dihydroxybenzoic, 2,5-dihydroxybenzoic and anthranilic acids, all failed to support a viable growth of *G. cingulata*. Acetylsalicylic acid was fully utilized by the fungus and although salicylic acid was formed as the sole observable intermediate there was no indication of the subsequent formation of phenol, suggesting that the availability of acetate possibly had a modifying influence on the metabolism.

Colletotrichum coffeanum was able to utilize salicylic acid as a sole carbon source but unlike *G. cingulata* there was no evidence for the production of phenol and no alternative intermediate metabolites were observed.

Modification of the substituents of aromatic compounds often occurs before ring rupture. Although studied in greatest detail for bacteria the same generalizations seem to be applicable to fungi.[1-3, 5] It is generally recognized that hydroxylation to give a 1,2- or 1,4-dihydroxylated intermediate always precedes ring cleavage. With aromatic acids hydroxylation is frequently achieved by the oxidative removal of a carboxyl group and in the case of salicylic acid conversion to catechol has often been demonstrated.[6-11]

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By comparison, the direct non-oxidative removal of a carboxyl group from an aromatic ring is uncommon and this process has been demonstrated for the higher fungi in the following cases only: 2,3-dihydroxybenzoic acid with *Aspergillus niger*;[9, 12] 2,4- and 3,4-dihydroxybenzoic acids with *Aspergillus* spp.;[13, 14] orsellinic acid with *Gliocladium roseum*;[15] and stipitatonic acid with *Penicillium stipitatam*.[16]

The conversion of 2,3-dihydroxybenzoic acid to catechol by *G. cingulata* appears to be analogous to the similar decarboxylation reported for *A. niger*.[9, 12] However, the decarboxylation of salicylic acid by *G. cingulata* to yield phenol is apparently novel and, in differing from the usual metabolic fate of this substrate, the metabolism warrants further detailed examination at the enzymological level.

EXPERIMENTAL

Glomerella cingulata and *Colletotrichum coffeanum*, previously isolated from active lesions on apple fruit and green coffee cherry respectively, were maintained on potato dextrose agar. Spore suspensions (10^8 per ml) were prepared from 2-week-old plates by agitation with sterile water and filtration through muslin. The substrate solutions (10^{-3} M, pH 4·8) were prepared in a sterile mineral salt medium containing NH_4Cl, 1·50 g/l; KH_2PO_4, 1·25 g/l; and $MgSO_4$, H_2O, 0·86 g/l. After inoculation with concentrated spore suspension to give a final spore concentration of 10^6 per ml, the substrate solution was dispensed in aliquot portions (100 ml) and incubated at 23°. The metabolism was monitored at 12-hr intervals by extraction with ethyl acetate (50 + 3 × 25 ml) and TLC examination of the dried (Na_2SO_4), concentrated extract (1·0 ml).

Thin-layer chromatography was performed using silica gel "GF_{254}" plates activated at 105° (1 hr) and the solvent systems, benzene, benzene–dioxane–acetic acid (90:25:4), benzene–methanol–acetic acid (45:8:4), di-*iso*propyl ether–acetic acid (99:1) and di-*iso*propyl ether–hexane–acetic acid (24:24:2). Compounds were detected by fluorescence or fluorescence quenching in u.v. light (254 and 365 nm). Ultraviolet spectra were recorded using a Unicam SP700 spectrophotometer, the utilization of salicylic acid and production of phenol being measured by the change in absorbance at 305 and 273 nm respectively.

For the isolation and characterization of salicylic acid and phenol the dried (Na_2SO_4) ethyl acetate extracts from a methyl salicylate incubation (4 × 1·5 l., 10^{-3} M, 64 hr) were combined and concentrated (10 ml). Salicylic acid was separated by shaking with dilute $NaHCO_3$ (2 × 2·5 ml) and precipitated by the addition of a saturated solution of *S*-benzylthiouronium chloride to the neutralized aqueous extract. Crystallization gave *S*-benzylthiouronium salicylate, m.p. 145°, undepressed on mixing with authentic derivative. The phenolic fraction was evaporated, dissolved in chloroform and chromatographed over a short silicic acid column (22 × 2 cm). Unmetabolized methyl salicylate was eluted first and discarded. The phenol containing eluate was concentrated and refluxed with excess phenyl *iso*-cyanate in petroleum spirit (80–100°) for 1 hr. Recrystallization of the solid that separated on cooling gave the *N*-phenylurethane, m.p. 126°, undepressed on mixing with authentic derivative.

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