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METABOLISM OF AROMATIC COMPOUNDS BY AN *ALTERNARIA* SPECIES

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Abstract—Washed mycelial felts of an *Alternaria* sp. isolated from soil, when grown on a malt extract medium, rapidly metabolized various cinnamic and benzoic acids. The intermediates in the degradation of *trans*-cinnamic, *m*-coumaric, *p*-coumaric, ferulic and sinapic acids have been identified. *In vivo* studies with ^{14}C-labelled cinnamic acid, *p*-coumaric acid and benzoic acid have shown that these compounds are converted to *p*-hydroxybenzoic acid and protocatechuic acid. When the organism was fed with cinnamic acid-2-^{14}C, a part of the radioactivity after 6 hr was trapped in glutamic and aspartic acids. These results strongly suggest that *Alternaria* shortens the cinnamic acid side-chain by β-oxidation. The organism did not have any detectable levels of phenylalanine and tryosine ammonia–lyase activities.

INTRODUCTION

CINNAMIC acids are known to occur naturally and they either serve as precursors for the formation of flavonoids and lignin in higher plants,[1,2] or get oxidized to the corresponding benzoic acids.[1] Even though the metabolism of cinnamic acids in vascular plants has been well documented, the microbial degradation of these compounds is not clearly understood. Basidiomycete fungi have been known to degrade phenylalanine and tyrosine by a pathway involving an initial deamination to cinnamic acid and *p*-coumaric acid respectively.[3,4] Recently, Bocks[5] identified *p*-coumaric acid, melilotic acid, *o*-coumaric acid and *p*-hydroxybenzoic acid as metabolites of *trans*-cinnamic acid in *Aspergillus niger*. The present paper describes the metabolism of cinnamic and hydroxybenzoic acids by an *Alternaria* sp.

RESULTS AND DISCUSSION

The time-course of disappearance of various aromatic compounds by the washed mycelial felts of *Alternaria* sp. are shown in Figs. 1 and 2. Cinnamic acids like *trans*-cinnamic acid, *m*-coumaric acid, *p*-coumaric acid and caffeic acid were readily metabolized by the organism. The rate of utilization of these compounds was in the order: *trans*-cinnamic acid > *p*-coumaric acid > caffeic acid > *m*-coumaric acid. Sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid), ferulic acid (3-methoxy- 4-hydroxycinnamic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid), benzoic acid, *p*-hydroxybenzoic acid and protocatechuic acid were also utilized by *Alternaria*.

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[2] S. A. BROWN, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 361, Academic Press, New York (1964).
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[4] K. MOORE, P. V. SUBBA RAO and G. H. N. TOWERS, *Biochem. J.* **106**, 507 (1968).
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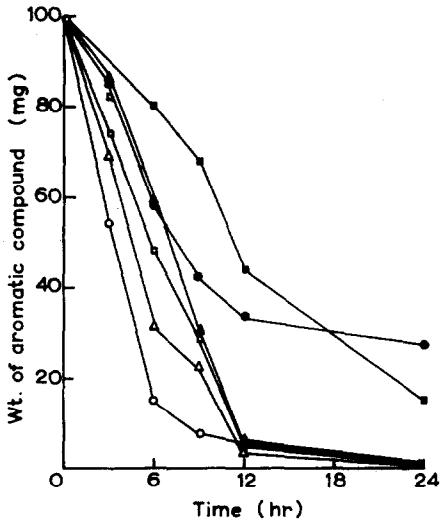

FIG. 1. THE TIME-COURSE DISAPPEARANCE OF THE SUBSTRATES IN THE PRESENCE OF WASHED MYCELIAL FELTS OF *Alternaria* SP.

The substrates used were:

(—●—) *m*-Coumaric acid (—○—) *trans*-Cinnamic acid
(—▲—) Caffeic acid (—△—) *p*-Coumaric acid
(—■—) Sinapic acid. (—□—) Ferulic acid.

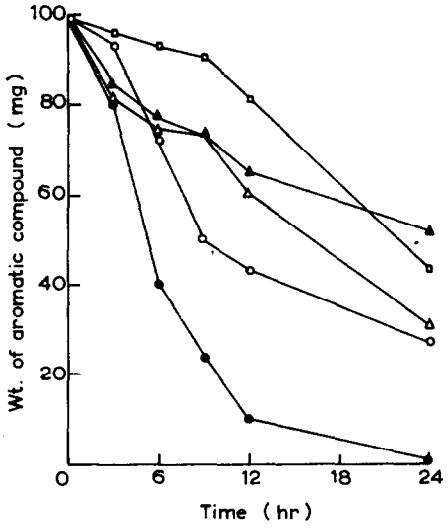

FIG. 2. THE TIME-COURSE DISAPPEARANCE OF THE SUBSTRATES IN THE PRESENCE OF WASHED MYCELIAL FELTS OF *Alternaria* SP.

The substrates used were:

(—●—) *p*-Hydroxybenzoic acid (—△—) Protocatechuic acid
(—▲—) Vanillic acid (—□—) Syringic acid
(—○—) Benzoic acid.

In Table 1 are listed the phenolic acids which accumulated in the culture filtrates when washed cells of *Alternaria* were incubated aseptically with various cinnamic and benzoic acids. In the presence of *trans*-cinnamic acid, the organism accumulated benzoic, *p*-hydroxybenzoic and protocatechuic acids; the latter two compounds were also identified as metabolites of *p*-coumaric acid. *m*-Coumaric acid was oxidized to *m*-hydroxybenzoic acid, but the latter compound accumulated in the medium as it was not further degraded. Both benzoic acid and *p*-hydroxybenzoic acid were hydroxylated to protocatechuic acid but salicylic acid, *m*-hydroxybenzoic acid and 2,3-dihydroxybenzoic acid were not metabolized.

Sinapic acid was converted to syringic acid, the further metabolites of which could not be identified, while ferulic acid was oxidized to vanillic acid. The latter compound was subsequently converted to protocatechuic acid.

TABLE 1. METABOLISM OF AROMATIC COMPOUNDS BY *Alternaria* SP.

Compounds added to the medium	Metabolites identified											
	Cinamic acid	*m*-Coumaric acid	*p*-Coumaric acid	Caffeic acid	Ferulic acid	Sinapic acid	Syringic acid	Vanillic acid	Benzoic acid	*m*-Hydroxybenzoic acid	*p*-Hydroxybenzoic acid	Protocatechuic acid
Cinnamic acid	+	—	—	—	—	—	—	—	+	—	+	+
m-Coumaric acid	—	+	—	—	—	—	—	—	—	+	—	—
p-Coumaric acid	—	—	+	—	—	—	—	—	—	—	+	+
Caffeic acid	—	—	—	+	—	—	—	—	—	—	—	+
Ferulic acid	—	—	—	—	+	—	—	+	—	—	+	+
Sinapic acid	—	—	—	—	—	+	+	—	—	—	—	—
Syringic acid	—	—	—	—	—	—	+	—	—	—	—	—
Vanillic acid	—	—	—	—	—	—	—	+	—	—	+	+
Benzoic acid	—	—	—	—	—	—	—	—	+	—	+	+
m-Hydroxy-benzoic acid	—	—	—	—	—	—	—	—	—	+	—	—
p-Hydroxy-benzoic acid	—	—	—	—	—	—	—	—	—	—	+	+
Protocatechuic acid	—	—	—	—	—	—	—	—	—	—	—	+

The results of feeding ^{14}C-labelled cinnamic, *p*-coumaric and benzoic acids (Table 2) further establish that *Alternaria* metabolizes cinnamic acid and *p*-coumaric acid by oxidation to the corresponding benzoic acids and subsequent conversion to protocatechuic acid. A balance sheet for the distribution of the radioactivity of cinnamic acid-2-^{14}C (4 μc) after 6 hr incubation with washed mycelium of *Alternaria*, is presented in Table 3. From the data it is evident that 4·2 per cent of the radioactivity from cinnamic acid-2-^{14}C was recovered in the free amino acid fraction. Radioautography revealed that this radioactivity was confined to glutamic acid and aspartic acid (Table 4).

Moore *et al.*[3,4] reported that basidiomycetes like *Sporobolomyces roseus* and *Schizophyllum commune* degrade phenylalanine or tryosine by non-oxidative deamination to the corresponding cinnamic acid and subsequent β-oxidation. The enzymes involved, viz. phenylalanine ammonia–lyase (EC 4.3.1.5) and tyrosine ammonia–lyase, were shown to be

distributed in several genera of basidiomycetes.[6,7] Although the *Alternaria* sp. investigated readily oxidizes cinnamyl compounds, the presence of ammonia–lyases for phenylalanine and tyrosine were not evident in this organism since crude extracts of the mycelium failed to convert these aromatic amino acids to the corresponding cinnamic acids. However, the

TABLE 2. METABOLISM OF RADIOACTIVE CINNAMIC, *p*-COUMARIC AND BENZOIC ACID BY *Alternaria* SP.

Compound administered in replacement media	Radioactive compounds in ether extract				
	Cinnamic acid (μc)	*p*-Coumaric acid (μc)	Benzoic acid (μc)	*p*-Hydroxybenzoic acid (μc)	Protocatechuic acid (μc)
Cinnamic acid-^{14}C (ring-labelled)	0·289	0	0·195	0·126	0·030
p-Coumaric acid-^{14}C (uniformly labelled)	0	0·150	0	0·041	0·021
Benzoic acid-1-^{14}C	0	0	0·270	0·142	0·059

TABLE 3. METABOLISM OF CINNAMIC ACID-2-^{14}C BY *Alternaria**

Radioactivity in ether extract (%)	Radioactivity in aqueous extract		Radioactivity in CO_2‡ (%)
	Amino acid fraction (%)	Non-amino acid fraction† (%)	
39·4	4·2	28·6	27·8

* 4 μc of cinnamic acid-2-^{14}C in 24 ml of 0·01 M sodium phosphate buffer, pH 7, were incubated for 6 hr with the washed mycelium.

† The percentage radioactivity in non-amino acid fraction is expressed as the difference between the radioactivity in the aqueous fraction [before passing through Dowex 50 (H^+ form)] and the radioactivity in the amino acid fraction.

‡ The $^{14}CO_2$ was trapped in a solution of hyamine hydroxide and the radioactivity measured in a liquid scintillation spectrometer.

TABLE 4. INCORPORATION OF RADIOACTIVITY FROM CINNAMIC ACID-2-^{14}C INTO GLUTAMIC AND ASPARTIC ACIDS IN THE PRESENCE OF WASHED MYCELIAL FELT OF *Alternaria*

Amount of radioactivity in cinnamic acid 2-^{14}C administered (μc)	Total radioactivity in amino acid fraction (μc)	Radioactivity in glutamic acid (μc)	Radioactivity in aspartic acid (μc)
4	0·168	0·103	0·065

existence of endogenous inhibitors for ammonia–lyase activities cannot be ruled out. The various pathways for the metabolism of aromatic compounds in *Alternaria* are delineated in Fig. 3.

[6] R. J. BANDONI, K. MOORE, P. V. SUBBA RAO and G. H. N. TOWERS, *Phytochem.* **7**, 205 (1968).
[7] D. M. POWER, G. H. N. TOWERS and A. C. NEISH, *Can. J. Biochem.* **43**, 1397 (1965).

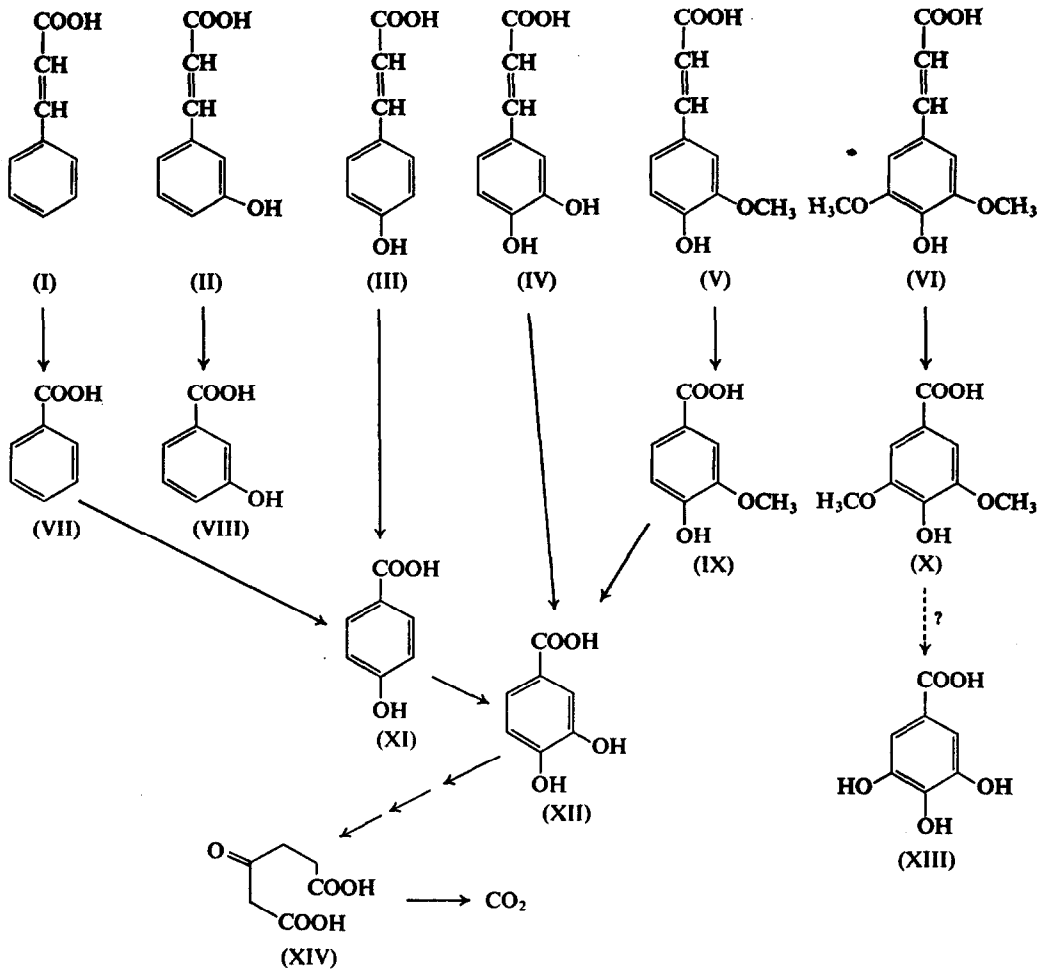

FIG. 3. PATHWAYS FOR THE DEGRADATION OF AROMATIC COMPOUNDS BY *Alternaria* SP.

(I) *trans*-Cinnamic acid
(II) *m*-Coumaric acid
(III) *p*-Coumaric acid
(IV) Caffeic acid
(V) Ferulic acid
(VI) Sinapic acid
(VII) Benzoic acid
(VIII) *m*-Hydroxybenzoic acid
(IX) Vanillic acid
(X) Syringic acid
(XI) *p*-Hydroxybenzoic acid
(XII) Protocatechuic acid
(XIII) Gallic acid
(XIV) β-Ketoadipic acid

It has been suggested by Webley *et al.*[8] and Henderson and Farmer[9] that cinnamic acids are converted to the corresponding C_6–C_1 acids by β-oxidation. Though the latter workers included an *Alternaria* sp. in their investigation and demonstrated its ability to utilize *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin, they did not identify the intermediates formed by this organism. Direct evidence for the conversion of cinnamic acids

[8] D. M. WEBLEY, R. B. DUFF and V. C. FARMER, *J. Gen. Microbiol.* **13**, 361 (1955).
[9] M. E. K. HENDERSON and V. C. FARMER, *J. Gen. Microbiol.* **12**, 37 (1955).

to the corresponding C_6–C_1 acids in higher plants has been obtained recently by Zenk,[10,11] Zenk and Muller[12] and Vollmer *et al.*[13] Moore *et al.*[4] reported that during the metabolism of cinnamic acid by *Sporobolomyces roseus*, the radioactivity from the propanoid side-chain was incorporated into glutamic acid. Our present studies with *Alternaria* show that the radioactivity from cinnamic acid-2-^{14}C is trapped in glutamic acid as well as aspartic acid and these results strongly suggest that in micro-organisms cinnamyl compounds are converted to the corresponding benzoic acids by β-oxidation leading to the removal of the side-chain as acetate in a manner analogous to fatty acid oxidation.[13]

During her studies on the transformation of coumarin, *o*-coumaric acid and *trans*-cinnamic acid by *Aspergillus niger*, Bocks[5] identified melilotic acid, *p*-coumaric acid, *o*-coumaric acid and *p*-hydroxybenzoic acid as metabolites of *trans*-cinnamic acid. Investigations with *Alternaria*, however, provided no evidence for an initial hydroxylation of cinnamic acid to *p*-coumaric acid. Instead, this compound was readily degraded with the intermediary formation of benzoic acid, *p*-hydroxybenzoic acid and protocatechuic acid. Blakley and Simpson[14] reported that in *Pseudomonas*, cinnamic acid was first reduced to phenylpropionic acid which underwent successive hydroxylations leading to the formation of 2,3-dihydroxyphenylpropionic acid. The latter compound was shown to be further degraded by *Achromobacter*.[15] In the case of *Alternaria*, protocatechuic acid was the terminal aromatic acid that underwent ring cleavage. Cell-free extracts of this fungus contained very high protocatechuic acid oxygenase activity and the product of dissimilation of the aromatic ring was β-ketoadipic acid as identified by Rothera reaction.[16] The crude preparations, however, were devoid of any catechol oxygenase activity.

EXPERIMENTAL

The Organism and Conditions of Growth

A strain of *Alternaria* sp. isolated from the soil by enrichment culture[17] was grown on a nutrient medium containing malt extract, 30 g, bacto-peptone, 5 g, and yeast extract, 1 g, per litre. Stock cultures were maintained on the same medium solidified with 1·5% agar. The flasks (500 ml) containing 60 ml of medium in each were autoclaved for 20 min at 120° and inoculated with a heavy suspension of spores, each flask receiving identical amount of inoculum. Incubation was carried out for 5 days at 30° without shaking. The mycelial felts were washed several times aseptically with distilled water and used for replacement experiments.

Analysis of the Replacement Medium

The growth medium above was replaced with 100 ml 0·01 M sodium phosphate buffer, pH 7, containing 100 mg of each substrate and the flasks were incubated at 30° with gentle agitation for 24 hr. Aliquots from the medium were removed aseptically at 6-hr intervals, acidified to pH 2 with 1 N HCl and extracted thrice with equal volumes of peroxide-free ether. The solvent was removed under vacuum and the residue was dissolved in 1 ml of ethyl acetate. Suitable aliquots were chromatographed on Whatman No. 1 filter paper and the compounds were identified by spraying with various reagents.[18–20] The identity of each compound was further established by elution from paper chromatograms with ethanol and comparison of the u.v. spectra with those of authentic samples.

[10] M. H. ZENK, *Proc. 2nd Meet. Europ. Biochem. Soc. Vienna* (edited by G. Billek), Vol. 3, p. 45, Pergamon Press, Oxford (1965).
[11] M. H. ZENK, *Phytochem.* **6**, 245 (1967).
[12] M. H. ZENK and G. MULLER, *Z. Naturf.* **19b**, 398 (1964).
[13] K. O. VOLLMER, H. J. REISENER and H. GRISEBACH, *Biochem. Biophys. Res. Commun.* **21**, 221 (1965).
[14] E. R. BLAKLEY and F. J. SIMPSON, *Can. J. Microbiol.* **10**, 175 (1964).
[15] S. DAGLEY, P. J. CHAPMAN and D. T. GIBSON, *Biochem. J.* **97**, 643 (1965).
[16] B. A. KILBY, *Biochem. J.* **43**, v (1948).
[17] A. M. D. NAMBOODIRI and J. V. BHAT (unpublished).
[18] R. K. IBRAHIM and G. H. N. TOWERS, *Arch. Biochem. Biophys.* **87**, 125 (1960).
[19] L. REIO, *J. Chromatog.* **1**, 338 (1958).
[20] L. REIO, *J. Chromatog.* **4**, 458 (1960).

To follow the utilization of various aromatic compounds in the presence of washed mycelial mats of *Alternaria*, suitable aliquots were removed aseptically from the replacement medium at 3-hr intervals and estimated spectrophotometrically.[4]

Experiments with Radioactive Compounds

DL-Phenylalanine-^{14}C (ring-labelled), L-tyrosine-^{14}C (uniformly labelled) and benzoic acid-1-^{14}C were derived from New England Nuclear Corporation. Cinnamic acid-2-^{14}C was obtained from Merck, Sharp and Dohme Inc., Rahway, U.S.A. Cinnamic acid-^{14}C (ring-labelled) and *p*-coumaric acid-^{14}C (uniformly labelled were enzymatically synthesized from DL-phenylalanine-^{14}C (ring-labelled) and L-tyrosine-^{14}C (uniformly labelled), respectively, using a partially purified enzyme preparation from *Sporobolomyces roseus*.[4,21]

The growth medium supporting the mycelial felts was replaced aseptically with 25 ml of 0·01 M sodium phosphate buffer, pH 7, containing 2 μc of the radioactive compound. The flasks were incubated at 30° for 3 hr with gentle agitation. The reaction was arrested by the addition of 2 ml of 1 N HCl. The mycelium was macerated with glass powder in a mortar along with the medium and centrifuged for 10 min at 10,000 *g*. The cell debris was washed twice with distilled water and added to the cell-free extract. The combined washings were extracted thrice with equal volumes of peroxide-free ether. The ethereal layer was shaken with anhydrous Na_2SO_4 and evaporated to dryness. Paper chromatography, radioautography and radioactive determinations were done according to the procedure of Moore *et al.*[4] Radioactivity measurements were carried out in a Nuclear-Chicago 720 series scintillation spectrometer.

To study the metabolic fate of the side-chain of cinnamic acid, 4 μc of cinnamic acid-2-^{14}C in 25 ml of 0·01 M sodium phosphate buffer, pH 7, were incubated for 6 hr with the washed mycelium of *Alternaria*. The phenolic acids were extracted into ether as described above. The aqueous layer which contained the amino acids was passed through a column of Dowex 50 (H^+ form) and washed with 1 l. distilled water. The amino acids were eluted from the resin with 4% NH_4OH. The eluate was concentrated and subjected to paper chromatography and radioautography.[4]

Assay for Phenylalanine and Tyrosine Ammonia Lyases and Protocatechuate Oxygenase

Cell-free extracts were prepared by grinding the freshly harvested mycelium (10 g) at 4° with an equal weight of glass-powder and centrifuging at 12,000 *g* for 20 min following extraction with 0·01 M sodium phosphate buffer, pH 7 (30 ml). The procedure described by Bandoni *et al.*[6] was used to test the non-oxidative deamination of phenylalanine and tyrosine by cell-free extracts of *Altenaria*. Protocatechuic acid oxygenase or catechol oxygenase activity was studied colorimetrically. Cell-free preparations of the mycelium (0·5 ml) were incubated for 30 min at 30° with 1 μmole of protocatechuic acid or catechol (0·1 ml) and 0·1 M tris-HCl buffer, pH 7·2 (0·4 ml), in a total volume of 1 ml. The extent to which the substrate disappeared from the reaction mixture was determined according to the method described by Nair and Vaidyanathan.[22]

Acknowledgements—We thank Dr. R. J. Bandoni for providing the culture of *Sporobolomyces roseus* and Dr. G. H. N. Towers for his interest in this work.

[21] P. V. Subba Rao, K. Moore and G. H. N. Towers, *Can. J. Biochem.* **45**, 1863 (1967).
[22] P. M. Nair and C. S. Vaidyanathan, *Anal. Biochem.* **7**, 315 (1964).