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METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*.

IX. PENTAKETIDES OF MUTANTS AND THEIR ROLE IN THE BIOSYNTHESIS OF MELANIN

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UDC 576.656+576.809.8+632.428

From mutants of the fungus *Verticillium dahliae* Kleb. having different genetic blocks in the biosynthesis of melanin we have isolated and identified scytalone, flaviolin, 4-hydroxyscytalone, and 2-hydroxyjuglone and have detected 3,4,8-trihydroxytetralone and 4,8-dihydroxytetralone, which, in combination with the results of complementation analysis, have confirmed the scheme of melaninogenesis in *V. dahliae* put forward previously. The participation of 1,3,6,8-tetrahydroxynaphthalene in the basic pathway of the biosynthesis of melanin has been shown.

Recently, mutants have been widely used in investigations on the biosynthesis of antibiotics, toxins, growth regulators, and other metabolites produced by microorganisms [1]. A number of melanin-deficient mutants has been used in the study of melaninogenesis in the fungus *Verticillium dahliae* Kleb. [2]. We have already reported on some compounds (flaviolin, scytalone) participating in this process [3, 4].

In the present paper we consider the pentaketides of various mutants of the fungus in connection with their role in the biosynthesis of melanin in *V. dahliae*.

We used melanin-deficient mutants from the collection of the Department of General Genetics of the Cotton Plant of the Academy of Sciences of the TadzhSSR, which were induced by ultraviolet irradiation (index UV), γ rays (index R), or nitrosomethylurea (index X), and also spontaneous mutants (index S). Among the 27 mutants that we studied, according to the literature [5], eight were from the group of chm mutants (UV-130, X-145, X-172, X-262, R-196, R-420, S-1, and X-272), possessing red-brown microsclerotia, 13 were from the group of alm mutants (R-177, R-503, R-550, X-146, X-127, X-222, UV-172, UV-117, UV-142, UV-160, UV-131, UV-128, and S-2), having white microsclerotia, and six were from the group of brm mutants with dark brown microsclerotia (X-171, X-269, R-841, R-83, S-3, and UV-233).

From acetone extracts of cultures of all the chm mutants apart from X-272 by column chromatography (CC) and preparative thin-layer chromatography (PTLC) on silica gel in system 1 we isolated and identified 3,6,8-trihydroxy-3,4-dihydro-1(2H)-naphthalenone (scytalone) (II) and 2,5,7-trihydroxy-1,4-naphthoquinone (flaviolin) (III) (Scheme 1) and also a substance with mp 98-103°C which on the basis of a comparison of R_f values and mass spectrum with literature information was identified as 4-hydroxyscytalone (IV) [6].

Only compounds (III) and (IV) were isolated from a culture of the mutant X-272, and no compound (II) was detected.

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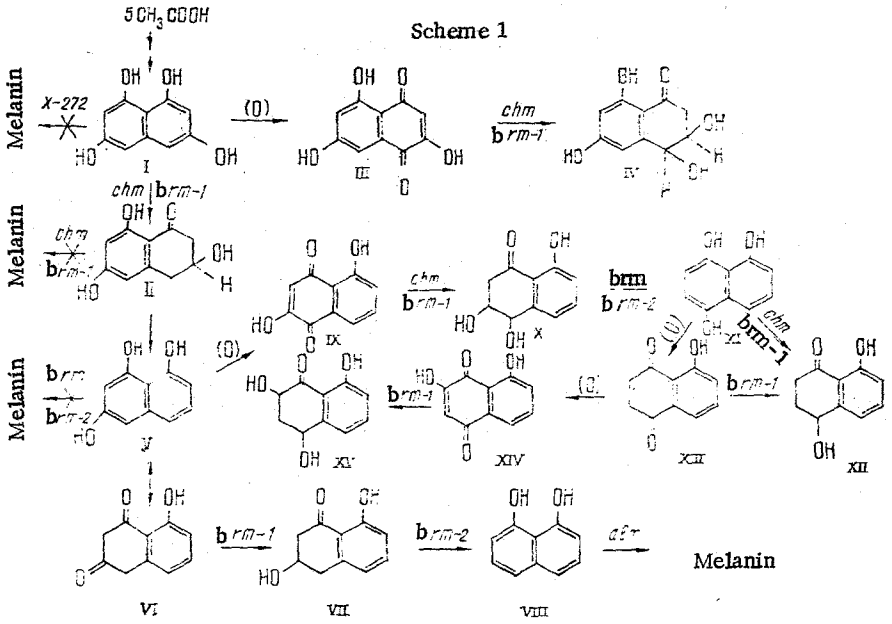
 0009-3130/80/1603-0298$07.50

From an extract of a culture of each of the brm mutants we isolated, and identified on the basis of a comparison of R_f values, PMR and mass spectra with literature information, 2,5-dihydroxy-1,4-naphthoquinone (2-hydroxyjuglone) (IX). In the same extracts, we detected another two substances which, from the nature of their behavior on Silufol UV-254 plates and their R_f values in systems 1-4, were probably 3,4,8-trihydroxytetralone (X) and 4,8-dihydroxytetralone (XII) [7].

In none of the alm mutants that we studied were the metabolites given in Scheme 1 identified.

The absence of the pentaketide metabolites formed by the acetate route [8] from all the alm mutants indicates the presence in them of a genetic block at the stages preceding the formation of 1,3,6,8-tetrahydroxynaphthalene (I) and (II). However, in the course of the experiments we established that when scytalone was added to seven-day cultures of the mutants X-146, UV-117, UV-142, UV-160, UV-131, UV-128, and S-2 (first group) they synthesized normal melanin while the others did not possess this capacity. Consequently, in spite of their phenotypic homogeneity, the alm mutants differ from one another and the differentiation of them that we have performed with respect to their capacity for using (II) to form melanin granules similar to those from the wild strain of *V. dahliae* confirms, and in some cases, refines the results of complementation analysis given previously [5].

Scheme 1



The chm mutants that we studied, with the exception of X-272, were identical so far as concerns the composition of the pentaketides with the known mutants brm-1 and brm-3 described in the literature [2, 6]. The isolation from extracts of cultures of these organisms of compounds (II), (III), and (IV) shows, as can be seen from Scheme 1, their presence in a genetic block at the stage of the conversion of (II) into 1,3,8-trihydroxynaphthalene (V), which leads to the accumulation of (II) in the medium and its subsequent autooxidation to (III) [6], which, in its turn, is reduced by these mutants, which possess a high hydrogenase activity, to (IV).

Of the metabolites shown, the addition of (III) or (IV), but not (II), to the alm mutants from the first group does not lead to the formation of normal melanin. This confirms the conclusion made in the literature [2, 6, 9] that (II) is a natural precursor in the biosynthesis of melanin.

In the composition of their pentaketides, just as in phenotypic characteristics, the brm mutants that we studied are identical with the mutant brm-2 described previously [7] and have a genetic block at the stage of the conversion of (V) into 3,8-dihydroxy-3,4-dihydro-1(2H)-naphthalenone (vermelone (VII)). The naphthol (V) does not accumulate in the medium, since it is readily autooxidized to (IX), which is the main product isolatable from cultures of brm mutants. Compound (V) can be isolated if a solution of (II) and sucrose at pH 6.0 is added to a culture of brm-2, as described previously [7].

Neither the 2-hydroxyjuglone (IX) that we isolated nor synthetic juglone (III) led to the formation of melanin when they were added to a culture of alm mutants of the first group. This is in harmony with the results of previous work [2, 7] where it was shown that of all the pentaketides produced by a brm-2 culture (V, IX-XV), only (V) can act as a substrate for the synthesis of melanin, and, just like (II), it is a natural precursor of the latter in *V. dahliae*.

The hypothesis of the participation of (I) in the biosynthesis of melanin in *V. dahliae* was made on the basis of the fact that at a concentration of 10 μg/ml the fungicide tricyclazole inhibits the production of (II) and increases the accumulation of (III) in a culture of the mutant brm-1 [10], while (I) is the main intermediate in the biosynthesis of (II) and (III), as has been established for the fungus *Phialophora lagerbergii* [11, 12]. The absence of (II) and the detection of compounds (III) and (IV) in a culture of the mutant X-272 that we investigated permitted the conclusion that it contained a genetic block at the stage of the conversion of (I) into (II) and enables us therefore to obtain an experimental proof of the participation of (I) in the main route of the biosynthesis of melanin. We are the first to have obtained and studied a mutant of this type.

The conversion of (V) into 1,8-dihydroxynaphthalene (VIII) from which, according to the literature [9, 13, 14], melanin is formed directly, takes place through (VII), as has been established by Stipanovic and Bell [15].

The results that we have obtained on the composition of the pentaketide metabolites of *V. dahliae* mutants in combination with the information given previously with respect to complementation analysis [5] have permitted the scheme of the biosynthesis of melanin by the fungus *V. dahliae* (the main steps of which were proposed in various publications [2, 6, 7, 9, 10, 15]) to be confirmed and supplemented. The biosynthesis of melanin by the fungus *V. dahliae* and the formation of pentaketide metabolites by the mutants studied can be represented by Scheme 1.

The use of *V. dahliae* mutants with various genetic blocks of the biosynthesis of melanin permits the breakdown of the complex process of synthesis into elementary components and the creation of a convenient model for studying the mechanism of the genetic regulation of the route of the synthesis of biopolymers in eukaryotes.

It is known that the causative agent of verticillium wilt of the cotton plant — the fungus *V. dahliae* — survives the seasonal absence of the host plant in the soil in the form of microsclerotia which are protected from unfavorable factors of the environment (solar radiation, low temperatures, desiccation, lytic action of biologically active microflora) with the aid of melanin [16-18]. Consequently, the results obtained open up a possibility for the directed search for agents blocking the synthesis of melanin and thereby lowering the viability of the microsclerotia in the soil.

EXPERIMENTAL

The conditions for recording the UV, IR, NMR, and mass spectra were similar to those described previously [3].

For CC we used Chemapol silica gel L 100/160 and Reanal alumina (activity grade II), and for PTLC Chemapol silica gel L 5/40 and also Silufol and Silufol UV-254 plates with the following solvent systems: 1) ether–benzene–formic acid (50:50:1); 2) chloroform–acetone (9:1); 3) acetone–methanol (9:1); 4) ether–benzene (9:1).

The cultures of the mutants of the fungus *V. dahliae* were grown in Petri dishes on solid Czapek medium in the dark at 23-25°C for 7-10 days.

Preparation of Acetone Extracts of Cultures of the alm, chm, and brm Mutants. The agar in the Petri dishes was cut into small pieces, placed in flasks, covered with acetone (5 volumes of solvent per volume of agar), and placed in a shaking machine for 6 h. Then the mixture was filtered, and the acetone was distilled off in vacuum in a IR-1M evaporator. The aqueous residues formed were acidified with dilute HCl to pH 5.0 and were extracted twice with ethyl acetate; the extracts were dried over anhydrous Na_2SO_4, the solvent was distilled off in the IR-1M, and the dry residues obtained were used for the isolation of the metabolites.

Isolation of Scytalone (II), Flaviolin (III), and 4-Hydroxyscytalone (VI) from Extracts of Cultures of the chm Mutants. The dry residue was dissolved in ether and deposited on a

column with dimensions of 2 × 25 cm containing silica gel L 100/160. Elution was performed first with ether and then with methanol. The ethereal eluate, on standing, formed gray crystals which were separated off, dissolved in methanol, and purified by CC on a 1 × 10 cm column of alumina with elution by methanol. The substance obtained after the solvent had been distilled off was recrystallized from ether to give pure (II). The spectral characteristics and melting point of (II) were identical with those which we have described previously [4].

To isolate the (IV), the ethereal eluate remaining after the separation of the crystals of (II) was separated by PTLC on silica gel in system 1 and the zone with R_f 0.09 was collected. The substance was eluted from the silica gel with ether, and after recrystallization from the same solvent crystals of (IV) with mp 98-103°C were obtained.

Mass spectrum (120°C): m/e (%) 210 (M^+, 61), 192 (10), 166 (23), 138 (61), 137 (100), 131 (10), 81 (10).

Compound (III) was isolated from the methanolic eluate by TLC on Silufol plates in system 1 (zone with R_f 0.50). The substance was eluted from the silica gel with ether, the solution was concentrated, and then hexane was poured into it, which led to the precipitation of (III), the melting point and spectral characteristics of which were identical with those which we have given previously [3].

Identification of the Metabolites of the brm Mutants. Compound (IX) was isolated from the total extract by TLC on Silufol plates in system 1, an orange-colored band with R_f 0.79 being collected. The substance was eluted from the silica gel with ether and was subjected to rechromatography in system 2 (R_f 0.63). After recrystallization from benzene, (IX) was obtained with mp 219-220°C.

PMR spectrum in $(CD_3)_2CO$: 4.34 (1 H, exchange with D_2O), 6.27 (1 H, singlet, slow exchange with D_2O), 7.25-7.45 (1 H, multiplet), 7.60-7.80 (2 H, multiplet), 12.70 (1 H, singlet, exchange with D_2O).

Mass spectrum (80°C): m/e (%) 190 (M^+, 100), 162 (50), 134 (30), 121 (90), 120 (15), 105 (10), 93 (25), 92 (27).

When the total extract was separated on Silufol UV-254 plates, two substances were detected which appeared in the form of dark spots in UV light, and which assumed a red-brown color after being sprayed with a 1% solution of $FeCl_3$. The first, with R_f 0.38 in system 1 and 0.39 in system 2, was probably the tetralone (XII), and the second, with R_f 0.60 in system 3 and 0.25 in system 4, was probably the tetralone (X), information on which has been given in Stipanovic and Bell [7].

The testing of the metabolites as substrates for the synthesis of melanin was carried out by using the alm mutants X-146, UV-117, UV-142, UV-160, UV-131, UV-128, and S-2 by a method described elsewhere [5].

SUMMARY

Scytalone, flaviolin, 4-hydroxyscytalone, and 2-hydroxyjuglone have been isolated from mutants of the fungus *V. dahliae* having various genetic blocks, and 3,4,8-trihydroxytetralone and 4,8-dihydroxytetralone have been detected among the metabolites. A new experimental proof has been obtained of the participation of 1,3,6,8-tetrahydroxynaphthalene in the main route of the biosynthesis of melanin. The isolation of the metabolites mentioned in association with the results of complementation analysis has enabled the new stages of the scheme of melaninogensis in *V. dahliae* proposed previously to be confirmed and supplemented.

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METABOLITES OF THE PATHOGENIC FUNGUS *Verticillium dahliae*.

X. INDUCTION OF PHYTOALEXINS IN THE COTTON PLANT BY METABOLITES IN THE PATHOGEN

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UDC 576.809.8+547.651+632.428+632.484

The capacity of ten metabolites isolated from the culture liquid and mycelium of the phytopathogenic fungus *Verticillium dahliae* Kleb. and identified by chemical and spectral methods or of known chemical nature for inducing the synthesis and accumulation of phytoalexins in the cotton plant has been investigated for the first time. It has been established that the maximum inducing capacity among the metabolites investigated in the concentrations tried is possessed by elaidic acid and di-2-ethylhexyl phthalate. A considerable amount of phytoalexins is also induced under the action on the cotton plant of PCL-1 and a substance of carbohydrate nature. In view of the inducing capacity of the lipid component the protein-lipoid-polysaccharide complex we have studied its total lipid and fatty acid compositions. It has been shown that the compositions of the classes of neutral lipids of the protein-lipoid-polysaccharide complex of the mycelium and the culture liquid do not differ fundamentally, while there are certain differences in the qualitative composition and quantitative amounts of fatty acids of the protein-lipoid-polysaccharide complex and of the culture liquid, with the exception of the $C_{16:0}$ and $C_{18:0}$ acids, the amounts of which are approximately the same.

The lipids and secondary metabolites (phytotoxins etc.) of the fungus *Verticillium dahliae* Kleb. play an important role in the pathogenesis of cotton wilt. With the aid of metabolites of phytophathogenic microorganisms it is possible to induce resistance of plants to diseases [1-7]. We have established previously that the capacity for evoking protective reactions of the cotton plant to wilt is possessed by a high-molecular-weight protein-lipoid-polysaccharide complex (PLPC) of *V. dahliae* and its individual components [8, 9].

In the present paper we give the results of further investigations of the metabolites of *V. dahliae* as inductors of the synthesis and accumulation of phytoalexins in the cotton plant.

The metabolites (I), (III), (IV) from the mycelium and (VII)-(X) from the culture liquid (CL) of *V. dahliae* were isolated and identified by the method described previously (see the literature to Table 1), and the method of isolation and information on the chemical na-

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 0009-3130/80/1603-0302$07.50