POLYKETIDE EXOMETABOLITES OF THE CAUSATIVE AGENT OF RICE BLAST AND THEIR ROLE IN PATHOGENESIS

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A polyketide exometabolite – 3,4,8-trihydroxy-3,4-dihydronaphthalen-1(2H)-one (3,4,8-TDH) – has been isolated from the culture liquids of four isolates of the phytopathogenic fungus Piricularia oryzae Cavara. It has been shown that all the natural isolates investigated, and also nonpathogenic rose mutants and a series of dwarf mutants synthesize 3,4,8-TDH. This substance was not detected in filtrates of cultures of albino mutants. One of the natural isolates produces, together with 3,4,8-TDH, another polyketide – piriculol. The role in pathogenesis of the exometabolites isolated is discussed.

Biologically active exometabolites of the phytopathogenic fungus Piricularia oryzae Cavara were first reported in 1954 by Tamari and Kaji [1]. They detected the pathogen α-picolinic acid in diseased rice plants and isolated it from culture filtrates. These compounds, on introduction into rice leaf blades, caused the same symptoms of disease as are produced in the natural infection of the plants by the pathogen [2]. Furthermore, they inhibited the growth of rice seeds. However, it was subsequently impossible to isolate these compounds from filtrates of other strains of the fungus cultivated both under stationary conditions and with aeration [3, 4]. As the authors suggested, on repeated resowing the fungus lost its capacity for producing these metabolites because of its high variability.

At the present time, various authors have isolated and identified the following metabolites of the pathogen mentioned: 3,4,8-trihydroxy-3,4-dihydronaphthalen-1(2H)-one (3,4,8-TDH), 3,4,6,8-tetrahydroxy-3,4-dihydronaphthalen-1(2H)-one (3,4,6,8-TDH), 4,6,8-trihydroxy-3,4-dihydronaphthalen-1(2H)one (4,6,8-TDH), piriculol, tenuazonic acid, and piriculariol [5-8]. As reported by Iwasaki et al. [6], at high concentrations 3,4,8-TDH suppressed the growth of rice shoots and at low concentrations it slightly stimulated their growth; 3,4,6,8-TDH had no appreciable influence whatever on the growth of rice shoots; and at a concentration of 500 μg/ml 4,6,8-TDH weakly stimulated the growth of the shoots [7].

Piriculol, and also piriculariol, in a concentration of 300 μg/ml suppressed the growth of rice shoots by a factor of approximately 5 [5, 8]. The injection both of piriculol and of piriculariol into leaf tissue caused the development of dark necrotic spots in 24 h [8]. Furthermore, as Bousquet [9] has reported, the action of piriculol was shown in an inhibition of rice roots and their necrosis, while the growth of the coleoptiles remained normal.

Tenuazonic acid in a concentration of 50 μg/ml suppressed the growth of rise shoots completely. The injection of this compound into rice leaf blades led to the appearance of necrotic spots similar to those that developed when the plant was infected with the pathogen naturally [3].

The metabolites of the fungus considered above have been studied mainly from the point of view of their chemical nature and biological activity. However, hitherto there has been practically no information on their role in the process of the manifestation of the pathogenic properties of the fungus. It is not known at what stages of development of the plant–parasite interrelationships their biological activity is realized. One of the approaches to answering these questions could be a comparative analysis of the interaction with the plant

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TABLE 1. Comparative Characteristics of the Isolates and Mutants

Strain	Exometabolites*		Infectivity of the conidia, %	
	3,4,8-TDH	piriculol	dusting	injection
H5-3	+	—	94,0	91,0
H-9	+	+	0,0	2,5
H-1	+	—	95,0	96,0
U-43	+	—	56,0	—
alb-1	—	—	1,1	0,6
alb-2	—	—	0,0	3,0
alb-5	—	—	0,0	0,0
alb-6	—	—	0,0	0,0
alb-12	—	—	0,1	0,5
ros-1	+	—	0,0	0,1
ros-2	+	—	1,0	7,0
ros-6a-1	+	—	0,0	0,2
col-1	+	—	0,0	0,1

*The sign + or − in the "exometabolites" column denotes, respectively, the capacity or incapacity of the given isolate for secreting the substances indicated into the culture liquid.

host of a natural isolate of the fungus producing a definite metabolite and of mutants obtained from this strain with different disturbances in the biosynthesis of this metabolite.

The aim of our work was to study two polyketide exometabolites of natural isolates of P. oryzae and mutants defective with respect to their biosynthesis in order to elucidate the role of these compounds in the interrelationships between the pathogen and the plant host.

We investigated filtrates of cultures of four natural isolates with a gray coloration of the mycelium and of nine mutants obtained from one and the same natural isolate H5-3 by induced mutagenesis. Among the mutants studied there were five albinos, and of them alb-1, alb-2, alb-5, and alb-6 arose when a suspension of H5-3 conidia was irradiated with ultraviolet (UV) and alb-12 when H5-3 was cultivated on a solid nutrient medium at 34°C. The rose mutants ros-1 and ros-6a-1 were also obtained by UV irradiation, and ros-2 when a suspension of H5-3 protoplasts was sown on solid nutrient medium. The dwarf mutant col-1 with a gray coloration of the mycelium, differing from H5-3 by a low rate of growth, was isolated when conidia of the initial strain were treated with N-methyl-N-nitro-N-nitrosoguanidine [10].

An extract of filtrates of the culture liquid of the natural strain H5-3 was separated with the aid of thin-layer chromatography in a solvent system into four fractions fluorescing in UV. A substance with a chromtographic mobility of R_f 0.58 inhibited the growth of rice seeds in a concentration of 1 mg/ml. At the same time, when aqueous ethanolic solutions of it were injected into the central veins of rice leaves, no external disturbances of the leaf blade resembling the characteristics of the development of the disease were observed. The other fractions did not affect the growth of rice seeds or the leaf blades of rice at all. Moreover, the absorption spectra of these fractions in the UV did not agree with those of the polyketides known for this fungus.

To determine the chemical nature of the compound with R_f 0.58 we studied its spectral properties. A comparison of the spectral characteristics obtained with literature information enabled the compound isolated to be identified as 3,4,8-TDH [5, 6].

In an investigation of other natural isolates – H-9, H-1, and U-43 – and also a mutant with a gray pigmentation of the mycelium but distinguished by a low rate of growth – col-1 – it was found that they produced 3,4,8-TDH (Table 1). No 3,4,8-TDH was detected in extracts of filtrates of the culture liquids of any of the albino mutants, obtained from the natural isolate H5-3, that were studied. At the same time, it was established that all three mutants with a rose coloration of the mycelium secreted 3,4,8-TDH into the medium in a considerably

larger amount than the natural isolates. Thus, in a culture liquid of the strain H5-3, 3,4,8-TDH was detected in an amount of 0.06 mg/100 ml of medium, while in a filtrate of a culture of the rose mutant ros-1 of the same age it was present in an amount of 0.30 mg/100 ml of medium. This feature of rose mutants may probably be connected with a disturbance in the chain of the biosynthesis of melanin with which the biosynthesis of the metabolite under consideration is obviously connected. It is known [11] that melaninogenesis in the fungus P. oryzae takes place by the acetate pathway with the formation of intermediate compounds of the polyketide type [12].

The fact that no vermelone was detected in the filtrate from any of the cultures investigated gives grounds for the assumption that in the rose mutants melanogenesis is apparently blocked at the stage of the reductive conversion of 1,3,8-trihydroxynaphthalene into vermelone, as a result of which this intermediate may be converted into 3,4,8-TDH through a stage of the formation of 2-hydroxyjuglone, with an accumulation of the latter. In the case of the albino mutants, the biosynthesis of melanin and of 3,4,8-TDH is probably blocked in the earlier stages preceding the formation of scytalone because of which such isolates could lose the capacity for synthesizing 3,4,8-TDH and the melanin pigment.

It must be mentioned that in some of the reisolates obtained after the injection of the conidia of the corresponding mutants into leaf tissue of susceptible varieties of rice the normal gray coloration of the mycelium, pathogenicity, and the capacity for producing 3,4,8-TDH were restored.

In an extract of the filtrate of the culture liquid of the natural isolate H-9, in addition to 3,4,8-TDH a compound with a red-brown fluorescence in UV and a chromatographic mobility R_f of 0.71 was detected. In a concentration of 0.2 mg/ml, this substance suppressed the growth of rice seeds, and its injection into the central vein of a rice leaf caused the formation of necrotic spots similar to those that develop on the infection of the plant with the pathogen.

The spectral properties of the compound isolated have been investigated. The identity of the UV absorption spectrum of this compound and the spectral characteristics given below with those found in the literature permit the conclusion that the substance isolated from a culture liquid of isolate H-9 was piriculol [5].

Thus, strains with gray and rose colorations of the mycelium produce 3,4,8-TDH, while unpigmented albino mutants are not producing-agents of this exometabolite. It can be seen from an analysis of information on the infectivity of the isolates that strains incapable of synthesizing 3,4,8-TDH – the albino mutants – are nonpathogenic. On the other hand, the rose mutants filtrates of the culture liquid of which contain several times as much 3,4,8-TDH as strains of the wild type are likewise nonpathogenic. Furthermore, 3,4,8-TDH does not initiate visible symptoms of the disease (formation of necroses) on its injection into the rice leaf. The facts given apparently indicate that 3,4,8-TDH is a necessary but not the determining factor in the development of the infectious process caused by P. oryzae.

In spite of its normal pigmentation and its capacity for synthesizing two phototoxic metabolites, isolate H-9 was nonpathogenic. At the same time, three pathogenic natural isolates did not produce piriculol at all. It obviously follows from this that the capacity for synthesizing piriculol is not a necessary condition for the realization of the pathogenic properties of P. oryzae.

EXPERIMENTAL

Mass spectra were taken on a Varian MAT-311 instrument at a cathode emission current of 1000 mA and an energy of the ionizing electrons of 10 eV. The temperature of the ion source was 70°C.

IR spectra were recorded on a Specord 75 IR spectrometer in the range of frequencies of 400-3800 cm^{-1} with slit program 4 at a rate of scanning of 60 cm^{-1}/min. The compounds were studied in KBr tablets.

^1H NMR spectra were recorded on a Varian XL-100-15 spectrometer in the Fourier regime at 30°C using 30% solutions of the substances in $(CD_3)_2CO$ and $CDCl_3$ with a working frequency of 100 MHz. HMDS was used as an internal standard. The chemical shifts are expressed in the δ scale. The accuracy of measurement was ±0.15 Hz.

UV spectra were taken on a Hitachi EPS-3T spectrometer (with ethanol as solvent).

The natural P. oryzae isolates U-43, H-1, H-9, and H5-3 and mutants with defects in the chain of biosynthesis of a pigment with a rose and white coloration of mycelium obtained as described previously [10, 13] were used. The fungus was cultivated in Erlenmeyer flasks in a decoction of carrots (50 g/liter of distilled water) at 28°C on a shaking machine (200 cycles/min) for two weeks. Then the mycelium was separated off by filtration through a calico filter, and the culture liquid from 50 flasks was combined and used for the experiments.

The polyketide exometabolites from filtrates of fungal cultures were extracted twice with ethyl acetate at a ratio of solvent to culture liquid of 1:3. The extracts were combined and evaporated to dryness in a rotary evaporator at 40°C. The dry residue was dissolved in a small volume of ethyl acetate and was chromatographed in a thin layer of silica gel (60G, Merck) 1 mm thick or on Silufol plates in the benzene—ethylacetate (10:90) solvent system [4]. The fraction present on the plates after separation were revealed by their characteristic fluorescence in UV. They were eluted with methanol. Where necessary, the individual fractions after elution were rechromatographed.

The biological activities of the substances isolated were determined in the following way. Pieces of filter paper were impregnated with ethanolic solutions of the substances under investigation and were dried and placed in Petri dishes, after which they were moistened and then rice seeds were grown on them. In control samples, the filter paper was impregnated with ethanol. The lengths of the shoots in the control and the samples treated with the isolated exometabolites were compared. In addition, the activity of a particular metabolite was evaluated from the development of necrotic spots on rice leaf blades after the injection of aqueous ethanolic solutions of the given substance into the central veins of leaves of susceptible varieties of rice.

The infectivity of the conidia of the fungal isolates studied was determined by a procedure described previously [10].

The exometabolites were identified from their UV, IR, mass, and PMR spectra.

3,4,8-Trihydroxy-3,4-dihydronaphthalen-1(2H)-one. $C_{10}H_{10}O_4$, $\lambda_{max}^{CH_3OH}$: 259 and 335 nm; ν_{max}^{KBr}: 3600-2400, 1640, 1580, 1050, 960 cm^{-1}.

Mass spectrum (70 eV) (m/z, %): M^+ 194(69), 169(31), 150(61), 121(100), 109(64), 95(78), 84(98), 81(96), 66(79), 58(58), 44(98).

PMR spectrum (CD_3COD_3C, ppm): 7.5 (t), 7.1 (d), 6.7 (d), 4.6 (d), 4.0 (d), 3.1 (m), and 2.7 (m).

Piriculol. $C_{14}H_{16}O_4$, $\lambda_{max}^{CH_3OH}$: 230, 277, and 354 nm; ν_{max}^{KBr}: 3600-2400, 1640, 1610, 1560, 970, and 790 cm^{-1}.

Mass spectrum (70 eV) (m/z, %): M^+ 248(32), 194(15), 178(55), 147(80), 132(100), 121(71), 109(52), 103(81), 97(42).

SUMMARY

A polyketide exometabolite — 3,4,8-trihydroxy-3,4-dihydronaphthlen-1(2H)-one — has been isolated from filtrates of the culture liquid of natural isolates, rose mutants, and a gray dwarf mutant of the phytopathogenic fungus P. oryzae. The natural isolate H-9 also produces another polyketide — piriculol. It has been found that albino mutants do not produce these metabolites.

On the basis of results on infectivity, it has been shown that the production of 3,4,8-TDH may be a necessary factor in the realization of the pathogenic properties of this fungus, while the production of piriculol is not a necessary condition for the development of the infectious process.

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^{13}C and ^{1}H NMR SPECTRA OF BIOLOGICALLY ACTIVE COMPOUNDS.

VII. DIASTEREOMERS OF 2,2-DIALKYLSPIRO[CYCLOPROPANE-3,3'-INDENE]-1-CARBOXYLIC ACIDS OF THE PYRETHROID SERIES

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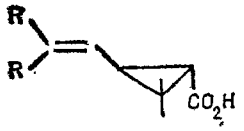
The structures have been established and the stereochemical assignments have been made of eight pairs of diastereomers and three quartets of stereoisomers of esters of 2,2-dialkylspiro[cyclopropane-3,3'-indene]-1-carboxylic acids of the pyrethroid series by the methods of ^{13}C NMR spectroscopy using the shift reagent $Eu(fod)_3$. Criteria have been found for assigning the stereoisomers on the basis of the characteristic values of the ^{13}C NMR chemical shifts of the signals of the methyl groups at C-2 for determining configuration of the substituents of the cyclopropane moiety of the molecule and of the C-2 signal itself for identifying isomers with respect to the side chain of the pyrethroid molecule. Criteria are proposed for identifying stereoisomers from the chemical shifts of the protons of the gem-dimethyl groups at C-2 in the ^{1}H NMR spectra.

In recent years, highly effective synthetic pyrethroids with a low toxicity for warm-blooded mammals that do not pollute the environment, consisting of esters of chrysanthemic (A) or permethric (B) acids, have found wide use in a system of protecting plants from harmful insects [1, 2]. Extremely promising pyrethroids are esters of 2,2-dimethylspiro-[cyclopropane-3,3'-indene]-1-carboxylic acid [3, 4].



R = Me(A)
R = Cl(B)

By ^{13}C NMR spectroscopy using the shift reagent $Eu(fod)_3$ and also by ^{1}H NMR we have shown the structures and have established the stereochemistry of isomers of a series of new synthesized derivatives of 2,2-dialkylspiro[cyclopropane-3,3'-indene]-1-carboxylic acid (I-XI).

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