Phytochemistry, Vol 27, No 11, pp 3481–3482, 1988
Printed in Great Britain

0031-9422/88 $3.00+0.00

ACCUMULATION OF FLAVIOLIN, 4-HYDROXYSCYTALONE AND 2-HYDROXYJUGLONE IN TRICYCLAZOLE-TREATED CULTURES OF *LEPTOSPHAERIA MACULANS**

JAGROOP S. DAHIYA and S. ROGER RIMMER

Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

(*Received in revised form* 4 *May* 1988)

Key Word Index—*Leptosphaeria maculans*; flaviolin; 2-hydroxyjuglone, 4-hydroxyscytalone; UV/vis, IR, ^{1}H NMR and mass spectral analysis, melanin

Abstract—*Leptosphaeria maculans*, the causal fungus of blackleg disease of crucifers, accumulated three metabolites following incorporation of tricyclazole into the growth medium Based on UV/vis, IR, NMR and mass spectral analysis, these metabolites were identified as flaviolin, 4-hydroxyscytalone and 2-hydroxyjuglone, known shunt products of the pentaketide pathway of melanin synthesis.

INTRODUCTION

Leptosphaeria maculans (Desm.) CeS. and de Not., cause of blackleg disease, is a major worldwide pathogen of crucifers [1]. *Leptosphaeria maculans* is a heterothallic pseudothecial loculoascomycete and produces enormous numbers of melanoid pycnidia when cultured on V8 media [2, 3]. Melanin synthesis in *Phoma wasabiae* has been reported to involve the pentaketide biosynthetic pathway [4] Tricyclazole, a systemic fungicide, inhibits melanin synthesis from pentaketide in *Pyricularia oryzae*, *Thielaviopsis basicola* and *Verticillium dahliae* causing accumulation of flaviolin and 2-hydroxyjuglone as shunt products [5–8]. In the present investigation, we report accumulation of flaviolin, 2-hydroxyjuglone and 4-hydroxyscytalone in tricyclazole-treated agar cultures of *Leptosphaeria maculans*, the pathogenic fungus causing blackleg of rapeseed.

RESULTS AND DISCUSSION

The major pigment **1** was obtained as orange-red crystals from ethyl ether. High resolution mass measurements indicated the formula $C_{10}H_6O_5$ (found 206.020; calcd. 206 021) with *m/z* (rel. int.): 206 (100), 178 [M $-CO]^+$ (33), 150 $[M-2CO]^+$ (23), 137 (49), 136 [M $-C_3M_2O_2]^+$ (13), 109 (13), 108 (14), 69 (37). Acidic ethanolic solution of the purified pigment gave UV absorption maxima $\lambda_{max}^{EtOH-HCl}$ nm (ε):214 (21 500), 264 (15 000) 309 (7500), 399 (2100) and 450 (2400). On changing to alkaline condition, it gave UV absorption maxima $\lambda_{max}^{EtOH-NaOH}$ nm (ε) 285 (24 100), 363 (6500) and 554 (2200).

Infra red spectral analysis of the pigment gave ν_{max}^{KBr} cm^{-1}: 1590, 1386, 1242 and 1175. ^{1}H NMR spectral analysis of the pigment in deuterated acetone $[(CD_3)_2$ CO] gave doublets at δ 6.68 and 7.15 confirming the aromatic protons, whereas presence of hydroxyl protons was indicated by singlets at δ 6.15 and 12.65 facilitated due to slow exchange with D_2O. All the foregoing data are in accord with the structure assigned to flaviolin (Fig. 1) The final confirmation of the structure was accomplished by comparing the spectral data with that of an authentic sample.

Compound **2** was isolated as white crystals from ethyl ether (mp 98–108°) with resolidification to give two types of crystals, mp 158–160° and 178–181° On mass spectral analysis it gave *m/z* (rel int.) 210 (64), 192 $[M-H_2O]^+$ (16), 166 $[M-CH_2{=}CHOH]^+$ (26), 138 (10), 130 (8) and 81 (12). Ethanolic solution of the compound gave UV absorption maxima λ_{max}^{EtOH} nm (ε) 218 (13,700), 234 (8300), 284 (13 100) and 312 (6500). Alkaline solution of the compound in ethanol gave UV absorption maxima $\lambda_{max}^{EtOH-NaOH}$ nm (ε):256 (58 000) and 337 (28 500) Infra red spectral analysis of the KBr disc of the compound gave ν_{max}^{KBr} cm^{-1} 1620 confirming the carbonyl group, C=O ^{1}H NMR spectral analysis of the compound in deuterated acetone $[(CD_3)_2$ CO] gave a doublet at δ 2.87 (2H, *J* = 4.8 Hz), a multiplet at δ 4.37 (1H) and a broad doublet at δ 4.81 (1H, *J* = 2.5 Hz). The presence of the aromatic protons in the structure was confirmed by a double doublet at δ 6.70 (IH, *J* = 2.1 and 1.0 Hz) and a doublet at δ 6.29 (1H, *J* = 2.0 Hz). Rapid exchange with D_2O revealed a singlet at δ 12.89 due to a hydroxyl proton, other hydroxyl protons gave broad bands at δ 3.07 and 7.01. Compound **2** was identified as *cis*-4-hydroxyscytalone (Fig 1) when its spectral data were compared with that of an authentic sample.

Compound **3** was recovered as yellow orange crystals from benzene On mass spectral analysis it gave *m/z* (rel. int) 190 (100), 162 $[M-CO]^+$, 134 $[M-2CO]^+$ (28), 121 (86), 120 (17), 105 (14) 93 (24) and 92 (26) Methanolic solution of the compound gave UV absorption maxima λ_{max}^{MeOH} nm (ε): 284 (12 100) and 429 (3500). Acidic solution of the compound in methanol had an absorption $\lambda_{max}^{MeOH-HCl}$ nm (ε)·283 (6900) and 411 (2300), and alkaline solution gave absorption $\lambda_{max}^{MeOH-HCl}$ nm (ε):261 (14 100), 386 (1600) and 470 (2100). ^{1}H NMR of the compound in

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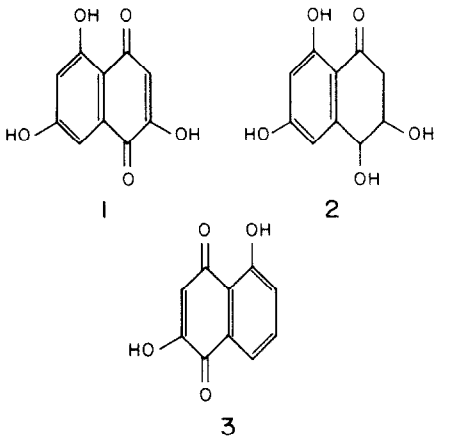

Fig 1 Chemical structure of the metabolites from *Leptosphaeria maculans* (**1**) flaviolin, (**2**) *cis*-4-hydroxyscytalone, (**3**) 2-hydroxyjuglone

deuterated acetone [$(CD_3)_2CO$] gave a broad singlet at δ 4.36 facilitated by exchange with D_2O, and singlets at δ 6.29 and 12.72 due to hydroxyl protons revealed by slow exchange with D_2O. Multiplets at δ 7.25–7.45 and 7.60–7.81 confirmed the aromatic protons in the structure (Fig 1). All the foregoing spectral data are in accord with the spectral data of authentic 2-hydroxyjuglone.

Melanin synthesis in blackleg fungus (*L. maculans*) was completely blocked in the agar media supplemented with tricyclazole (10 μg/ml). The fungus only developed a light brown colour in the presence of tricyclazole, and the underlying media turned reddish-brown. The major pigment identified in the agar medium extract was flaviolin whereas accumulation of 2-hydroxyjuglone and 4-hydroxyscytalone was also recorded. Flaviolin, 2-hydroxyjuglone and 4-hydroxyscytalone are the shunt products of the pentaketide melanin synthetic pathway [5–9]. Accumulation of such metabolites has been reported in *Verticillium dahliae* and *Thielaviopsis basicola*. Tricyclazole also inhibits melanin synthesis in *T. basicola*, and *Phoma wasabiae* causing the accumulation of flaviolin and 2-hydroxyjuglone [5, 9].

Melanin biosynthesis in *Verticillium dahliae* has been partially elucidated [5, 6]. 1,3,6,8 Tetrahydroxynapthalene, (+)-scytalone, 1,3,8 trihydroxynapthalene, (−)-vermelone and 1,8-dihydroxynapthalene are successive intermediates in the biosynthetic pathway. Tricyclazole, inhibits melanin synthesis at two sites in *V. dahliae*, i.e. the enzymatic reductions of 1,3,6,8-tetrahydroxynaphthalene and 1,3,8-trihydroxynaphthalene. Blockage at these sites causes accumulation of flaviolin and 2-hydroxyjuglone, the auto-oxidative products of 1,3,6,8-tetrahydroxynaphthalene and 1,3,8-trihydroxynaphthalene, respectively [6]. It is obvious from experimental observations that the same pentaketide pathway reported in the case of *Verticillium dahliae*, *Thielaviopsis basicola* and *Phoma wasabiae* may be involved in melanin synthesis in *L. maculans*.

EXPERIMENTAL

Fungal culture. *Leptosphaeria maculans*, the fungal culture used in the present studies, was isolated from the infected stem samples of *Brassica napus* collected from a blackleg-infested field near Elgin, Manitoba, Canada.

Extraction and isolation of pigments. The fungus was grown on potato dextrose agar medium supplemented with tricyclazole (*ca* 10 μg/ml) for 28 days at 25°. Agar cultures were chopped and pigments were extracted with Me_2CO. Me_2CO was removed *in vacuo* to leave an aq. phase. The pH of the aq. phase was adjusted to 5 with H_3PO_4, satd. with NaCl, and extracted with Et_2OAc. The organic solvent was evapd. and the concentrate was used for chromatography. The crude pigments were dissolved in Et_2O and applied to a column (2 × 20 cm) of alternate 2 cm layers of silica gel and polyamide. The column was eluted with Et_2O. Further purification of the eluates was done by using silica gel TLC plates (250 μm thickness) and a solvent system of $CHCl_3$ and MeOH (9:1).

Spectral analysis of the purified pigments. Mass spectra were measured with a VG-analytical 11-250J by direct probe insertion. NMR spectra were recorded with TMS as an internal standard. Hydroxyl protons were determined by rapid exchange with D_2O. UV/vis spectra were determined in 95% EtOH plus 0.1 M NaOH (EtONa) and 95% EtOH plus 0.1 M HCl (EtOH–HCl).

Acknowledgement We would like to thank Mr Wayne Taylor, Mass Spectral and NMR Services, Chemistry Department, University of Manitoba for technical assistance. The financial support of Continental Grain Company Limited and the National Research Council of Canada IRAP program is greatly appreciated. Special thanks to Lillian Buckler for typing the manuscript.

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