Phytochemistry, 1976, Vol. 15, pp. 651–653 Pergamon Press. Printed in England.

CONVERSION OF WYERONE TO WYEROL BY *BOTRYTIS CINEREA* AND *B. FABAE IN VITRO*

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(*Received* 11 *November* 1975)

Key Word Index—*Vicia faba*; Leguminosae; broad bean; phytoalexin; wyerone; detoxification; *Botrytis cinerea*; *Botrytis fabae*; wyerol.

Abstract—The phytoalexin wyerone was induced to accumulate in cotyledons of *Vicia faba* infected with *Botrytis cinerea* or *B. fabae*. The acetylenic keto ester, wyerone, was converted to the less antifungal corresponding hydroxy ester, wyerol, by both species of *Botrytis in vitro*.

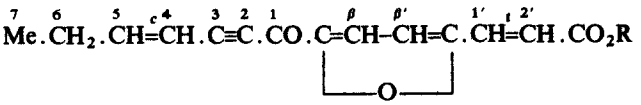
INTRODUCTION

The antifungal compound wyerone (**1**) was first isolated and characterized from broad bean seeds allowed to germinate in the dark for 8 days and was considered to be present in healthy tissues of *Vicia faba* L [1]. Subsequent studies have shown that the inhibitor is only present in trace amounts if at all in healthy bean leaves [2] or stems [3] but accumulates following fungal infection and like wyerone acid (**2**) may be considered to be a phytoalexin [4].

It has been suggested [2] that wyerone may be converted to the more antifungal wyerone acid by *Botrytis fabae*, the cause of aggressive lesions of chocolate spot disease in leaves and pods of *Vicia faba*. Here we report the isolation of wyerone from broad bean cotyledons inoculated with *B. cinerea* or *B. fabae* and the conversion of wyerone to the corresponding hydroxy ester, wyerol (**4**) by both species of *Botrytis in vitro*.

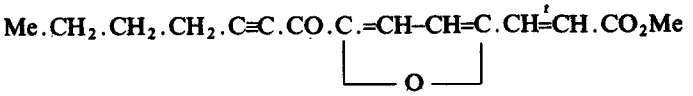
RESULTS AND DISCUSSION

Wyerone isolated from cotyledons infected by *Botrytis fabae* was identified by spectral analyses. The mass spectrum gave M^+258.0915, ($C_{15}H_{14}O_4$ requires M^+258.0892) prominent fragments at *m/e* 227 (27%), 226 (28%), 199 (7%), 179 (19%) and 151 (100%). Diagnostic IR bands were detected at ν_{max} ($CHCl_3$), 2195 (C≡C) 1718 (ester CO), 1634 (ketone CO), 1499, 1026 (furan) and 974 cm^{-1} (CH=CH). NMR signals were observed at δ 1.10 (3H, *t*, 7-H), 2.46 (2H, *m*, 6-H), 3.79 (3H, *s*, –OMe), 5.67 (1H, *dt*, 4-H), 6.36 (1H, *dt*, 5-H), 6.59 (1H, *d*, 2′-H), 6.73 (1H, *d*, β'-H), 7.32 (1H, *d*, β-H) and 7.45 (1H, *d*, 1′-H). The UV spectrum λ_{max} (EtOH or MeOH) 351, 291, 226 nm and other spectra were closely comparable to those published for synthetic wyerone [1]. The presence of a dihydro contaminant (**3**) *m/e* 260 (19%) was indicated in the mass spectrum as previously reported for the natural product [1]. In contrast to

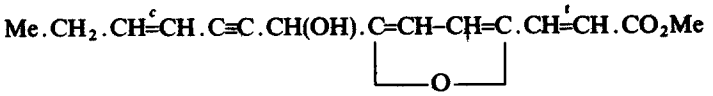


(1) Wyerone, R=Me

(2) Wyerone acid R=H



(3) Dihydrowyerone



(4) Wyerol

leaves and pods [5] cotyledons were resistant to both *B. cinerea* and *B. fabae*, infection being restricted to light brown lesions which were confined to tissue immediately beneath inoculum droplets. Yields of 969–1789, 809–1298 and 14–45 μg wyerone/g.f.w. were obtained from cotyledons six days after inoculation with *B. fabae*, *B. cinerea* or sterile distilled water respectively. Wyerone could not be detected in imbibed cotyledons before inoculation.

Wyerone (λ_{max} 350 nm) disappeared after incubation for 24 hr with germinating conidia of *B. cinerea* or *B. fabae*, and a substance absorbing at 312 nm appeared instead. Analysis of incubation mixtures by TLC in hexane (bp 60–80°): acetone (2:1), showed that the metabolism of wyerone (R_f 0.44) by both fungi was associated with the appearance of a single band (R_f 0.35) which quenched the fluorescence of Si gel under UV light and after elution in MeOH gave a compound with λ_{max} 312 nm. Milligram quantities of the 312 nm absorbing metabolite were recovered after the addition of wyerone to mycelial cultures of both *B. cinerea* and *B. fabae*, and each subjected to spectral analyses. The metabolites produced by both fungi were found to be identical to the hydroxy ester, wyerol (**4**), prepared by $NaBH_4$ reduction of natural wyerone. MS gave M^+ 260.1066 ($C_{15}H_{16}O_4$ requires M^+ 260.1050) and prominent peaks at *m/e* 260 (100%), 245 (25%), 243 (19%), 242 (15%), 231 (42%), 229 (42%) and 151 (47%). Comparison of IR spectra of the metabolites and wyerone clearly indicated loss of the strong ketone absorption (1634 cm^{-1}) and appearance of the hydroxyl group (3580, 3370 cm^{-1}). Disappearance of the keto-group adjacent to the acetylene function resulted in drastically reduced intensity of the –C≡C– stretching absorption (2210 cm^{-1}) in the IR spectrum of wyerol. The identity of the hydroxy ester was confirmed by NMR spectroscopy. NMR signals were observed at δ 1.03 (3H, *t*, 7-H), 2.31 (2H, *m*, 6-H), 2.5 (1H, broad, –OH), 3.77 (3H, *s*, –OMe), 5.49 (1H, *dt*, 4-H), 5.62 (1H, *d*, 1-H), 6.02 (1H, *dt*, 5-H), 6.34 (1H, *d*, 2′-H), 6.52 (1H, *d*, β-H) 6.58 (1H, *d*, β′-H) and 7.40 (1H, *d*, 1′-H).

Wyerol was less inhibitory than wyerone towards conidia of *Botrytis*. The ED_{50}s for activity of wyerone against germ tube growth by *B. cinerea* and *B. fabae* were 17.0 and 28.2 μg/ml, and for wyerol 75.9 and by extrapolation 1905 μg/ml respectively. Conversion of wyerone to wyerol by both fungi may therefore be considered a detoxification mechanism. It is notable that both compounds were more active against *B. cinerea* than *B. fabae*.

The accumulation of wyerone at inoculation sites is probably causally related to lesion limitation in cotyledons. The presence of the inhibitor in tissues incubated in the absence of any specific fungal inoculum in this and earlier work is probably the result of wyerone biosynthesis in response to infection by contaminating microorganisms. The lack of either quantitative or qualitative differences in the ability of the two species of *Botrytis* to metabolize wyerone suggests that their differing sensitivities to the inhibitor may be due to some mechanism other than detoxification, and contrasts with the metabolism of wyerone acid by these fungi [5]. Germinating conidia of *B. fabae* but not of *B. cinerea* were able to metabolize the acid to a hexahydro derivative (**5**) which accumulated in lesions caused by *B. fabae* [5]. Whether or not wyerone is converted to wyerol by *Botrytis in vivo* remains to be determined.

(**5**) $MeCH_2CH^{c}{=}CH.CH_2CH_2.CH(OH).C{=}CH{-}CH{=}C(O).CH^{t}{=}CH.CO_2H$

EXPERIMENTAL

IR spectra were recorded in $CHCl_3$. NMR spectra in $CDCl_3$. MS were determined with an A.E.I. MS902 spectrometer by use of a direct insertion probe.

Inoculation of cotyledons. Seeds of broad bean cv. Aquadulce were germinated for 48 hr between wet paper towels at 25°. Testas were removed from imbibed seeds and undamaged cotyledons separated and placed rounded side downwards on moist tissue paper in large plastic boxes. Cotyledons were then inoculated by spraying with suspensions of *Botrytis* conidia (5×10^5/ml) in sterile H_2O of water alone. Inoculated tissues were incubated in closed boxes for 6 days in the dark at 20°. Tissue beneath inoculum droplets was then excised with a razor blade and stored at −20°.

Isolation of wyerone (**1**). Cotyledon tissue (500 g) infected by *B. fabae* was shaken (120 rpm) in Et_2O (250 ml/100 g) for 24 hr at 8° on an orbital incubator. The ethereal supernatant was collected and after soaking in Et_2O for a further 12 hr residual tissue was homogenized (Sorval omnimixer). The homogenate was washed with Et_2O until washings were colourless. Bulked Et_2O extracts were evaporated to dryness *in vacuo* and the residue subjected to PLC (Merck Si gel GF_{254}, 2 mm thick). Plates were developed in hexane (br 60–80°)–Me_2CO (2:1) and after drying in $CHCl_3$–petrol (br 60–80°) (2:1) Wyerone was detected (final R_f 0.86) as a blue fluorescent band under UV light (366 nm). After elution (2×50 ml $CHCl_3$ and 1×50 ml Et_2O) and solvent evaporation wyerone was crystallized 2× from cyclohexane. Triplicate samples (each *ca* 4g) of tissue inoculated with *B. cinerea*, *B. fabae* or H_2O were homogenized 2× in 50 ml Et_2O. Ether extracts were taken to dryness and subjected to TLC (Merck 5715 precoated Si gel 0.25 mm plates, solvents as above). Wyerone eluted in MeOH from final R_f 0.8 gave UV spectra identical to the crystalline product and concentrations were determined using the published extinction coefficient [1].

Production and isolation of wyerol (**4**). Wyerone in 0.1 ml MeOH was added to 12 flasks each containing 20 ml synthetic pod nutrient soln (SPN; 5 g sucrose, 380 mg casamino acids, 100 mg KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 1 l. dist. H_2O adjusted to pH 4.0 with galacturonic acid) to give a final concn of 200 μg wyerone/ml SPN in each flask. One disc (5 mm dia) of medium X agar [7] bearing actively growing mycelium of either *B. cinerea* or *B. fabae* was added to each flask. Six cultures of each fungus were incubated at 18° in an orbital incubator (200 rev/min). Conversion of wyerone to wyerol was monitored by UV spectrophotometry of aliquots of the cultures and appeared complete after 4 days when individual cultures were partitioned with Et_2O (2×20 ml). Et_2O extracts from replicate cultures containing either *B. cinerea* or *B. fabae* were combined and after solvent removal wyerol (*ca* 6 mg from each fungus) was recovered as a pale yellow solid by PLC and elution as described for wyerone. Wyerol was detected under UV light (254 nm) as an absorbing band at R_f 0.63. Reduction of wyerone (10 mg) with $NaBH_4$ in dioxan–MeOH yielded wyerol (8 mg) which was purified by PLC as above.

Bioassays. Antifungal activity was assayed using a modification of the microscope slide bioassay technique [4,8]. Aliquots (50 μl) of different concentrations of wyerone or wyerol in MeOH were added to SPN (10 ml). Droplets (10 μl) of test solns were pipetted on to glass slides and the addition of 5 μl of conidial suspension (1×10^5 conidia/ml SPN) to each drop gave solns containing final concentrations of each compound ranging from 0–100 μg/ml. Three replicates of each bioassay were prepared. After incubation for 18 hr at 18° total germ-tube production was measured from 35 germinated conidia in each replicate droplet. Germ-tube lengths were measured from camera lucida drawings with a map recorder. ED_{50}s were calculated from graphs of mean germ-tube length against log. inhibitor concentration.

Acknowledgements—We wish to thank R. O. Cain for the synthesis of wyerol from wyerone and J. Eagles for mass spectrometry. J.A.H. was supported by the Agricultural Research Council.

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