Phytochemistry, 1970, Vol. 9, pp. 249 to 252. Pergamon Press. Printed in England.

IDENTIFICATION AND ACTIVITY OF WYERONE ACID AS A PHYTOALEXIN IN BROAD BEAN (*VICIA FABA*) AFTER INFECTION BY *BOTRYTIS*

R. M. LETCHER, D. A. WIDDOWSON, B. J. DEVERALL and J. W. MANSFIELD

Departments of Chemistry and Botany, Imperial College, London, S.W.7

(*Received* 12 *July* 1969)

Abstract—An antifungal compound from infected broad bean has been identified as wyerone acid (I). Yields of from 3·5 to 30 μg per g tissue were obtained. Lowest concentrations which prevented all germination of *Botrytis fabae*, *B. cinerea* and *B. allii* were 45, 18 and 9 μg/ml nutrient solution respectively.

INTRODUCTION

A PHYTOALEXIN[1] formed in infection droplets containing *Botrytis cinerea* after incubation in pods of broad bean behaved as a single substance on paper chromatograms in four solvent systems.[2] The compound was extracted as an acid from infection droplets and diseased leaves, and was shown to form in response to infection of leaves by the virulent parasite *B. fabae* as well as the avirulent *B. cinerea*. The different virulences of the fungi are based on their sensitivities to the phytoalexin and abilities to degrade the compound.[3] Several antifungal compounds have been detected in broad bean;[1,4] the structure of one has been determined as an acetylenic furanoid keto-ester (wyerone) (II).[4,5]

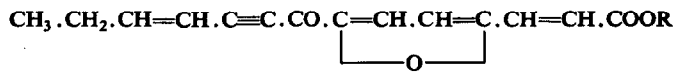
RESULTS

Identification

The phytoalexin was separated from other extracted acids as a narrow band at R_f 0·3 by TLC in methanol/CH_2Cl_2. No other bands nor the chromatographic solvents and supports possessed antifungal activity. The active band had a pale blue fluorescence in u.v. light. The active eluate in ethanol absorbed light strongly at 350 nm, and had an identical u.v. spectrum to that of wyerone. Further chromatography of the active eluate on washed Whatman 3 MM paper in *n*-propanol/water[3] yielded a single antifungal band at R_f 0·9 with the same properties of fluorescence and u.v. absorbance.

Methylation of the active fraction, extracted from TLC plates, yielded a substance which had an R_f of 0·43 on silica gel plates in benzene/ether and of 0·95–1·0 in methanol/CH_2Cl_2. Wyerone migrated in an identical way and had the same intense blue fluorescence under u.v. light.

[1] I. A. M. CRUICKSHANK, *Ann. Rev. Phytopath.* **1**, 351 (1963).
[2] B. J. DEVERALL, *Ann. Appl. Biol.* **59**, 375 (1967).
[3] B. J. DEVERALL and J. C. VESSEY, *Ann. Appl. Biol.* **63**, 449 (1969).
[4] C. H. FAWCETT, D. M. SPENCER, R. L. WAIN, A. G. FALLIS, E. R. H. JONES, M. LE QUAN, C. B. PAGE, V. THALLER, D. C. SHUBROOK and P. M. WHITHAM, *J. Chem. Soc.* (C), 2455 (1968).
[5] C. H. FAWCETT, D. M. SPENCER and R. L. WAIN, *Neth. J. Plant Path.* **75**, 72 (1969).



Wyerone acid (I); R = H
Wyerone (II); R = Me

Identification of the phytoalexin as wyerone acid (I) was confirmed after treating the extracted acid with tritiated diazomethane. The separated product was mixed with wyerone (II) and then recrystallized five times from *n*-hexane at −20°. Table 1 shows the radioactivity of samples removed after each crystallization.

TABLE 1. CRYSTALLIZATION OF METHYLATED ACID AND WYERONE TO CONSTANT RADIOACTIVITY

Successive crystallization	cpm/mg
1st	520
2nd	420
3rd	334
4th	344
5th	368

Yield

Assuming a similar extinction coefficient to that of wyerone,[4] yields of I from different sources were calculated as in Table 2. Wyerone acid was not detected in healthy tissue.

TABLE 2. YIELD OF WYERONE ACID FROM DIFFERENT SOURCES

Source	μg/g fresh material
Leaves infected* by *B. cinerea*	3·5
Leaves infected* by *B. fabae*	12·0
Infection droplets†* containing *B. cinerea*	9·0–30·0‡
Infection droplets†* containing *B. fabae*	1·0

* Bearing six 0·5-cm diam. lesions per leaflet.
† Containing 5×10^5 spores/ml incubated in pods for 24 hr.[2]
‡ Depending upon variety and age of pods.

Wyerone acid was not found on a few occasions when batches of leaves bearing large lesions were extracted after storage for several months at −20°. On these occasions, an antifungal acid absorbing u.v. light at 310 and/or 333 nm was eluted from R_f 0·6 on plates developed in methanol/CH_2Cl_2. This compound may be related to wyerone acid, and may be formed in heavily diseased leaves or by prolonged storage.

Antifungal Activity

Table 3 shows the effects on spore germination of different concentrations of wyerone acid prepared on the basis of the extinction coefficient of wyerone.

TABLE 3. ANTIFUNGAL ACTIVITY OF WYERONE ACID AGAINST *Botrytis*

Activity of wyerone acid (μg/ml)	*B. fabae*	*B. cinerea*	*B. allii*
ED_{50} against germination	26·0	9·0	2·9
ED_{50} against germ-tube growth	13·5	2·0	0·7
Lowest concentration which prevented all germination	45·0	18·0	9·0

DISCUSSION

The concentrations of wyerone acid in infection droplets and diseased tissues and its relative activity against germ-tube growth of *Botrytis fabae* and *B. cinerea* are consistent with its proposed role as a phytoalexin. The apparent absence of wyerone acid in healthy tissue contrasts with the extraction of wyerone at the rate of 3·5 μg/g from dark-grown "Green Windsor" seedlings.[4] Possibly the acid is formed by hydrolysis of the methyl ester when cells are damaged by infection. However, another source of the acid is suggested because the highest yields of the acid in Table 2 were from varieties of bean found to contain negligible amounts of wyerone compared with the "Green Windsor" variety.[6]

Wyerone is active (ED_{50} – 10 μg/ml) against some fungi, but not (ED_{50} – 100 μg/ml) against *B. cinerea*.[4] Conversion to the free acid greatly enhances its activity against *B. cinerea*, but the related acetylenic compound capillin is even more active ($ED_{50} < 1$ μg/ml).[5]

EXPERIMENTAL

TLC plates were prepared using Merck silica gel G. Fluorescent bands were detected by exposing the plates to u.v. light with maximum intensity at 360 nm. Radioactive samples were counted using a Beckman liquid scintillation counter with an efficiency of 50% for tritium. All operations were carried out below 50° and in the dark where possible. Evaporations were carried out in water pump vacuum on a rotary film evaporator. Light absorptions were measured in ethanol.

Infected Leaves

Plants of "The Sutton" variety of broad bean were grown to the four-leaf stage in a greenhouse. Leaves were rinsed with water, detached and placed on glass supports on moist filter paper in plastic sandwich boxes. Three 20-μl drops of a suspension of 5×10^5 spores of *Botrytis fabae* per ml in water or of *B. cinerea* in orange juice were placed on each half leaflet. Closed boxes were incubated at 17° for at least 2 days when large brown lesions had developed beneath the infection droplets. Leaves were stored at −20° before extraction. For estimates of yield, several varieties of bean including some lines from the Plant Breeding Institute, Cambridge, were used.

Extraction

Frozen leaves (350 g) were macerated in 80% ethanol (1·5 l.), the extract filtered and evaporated to small volume (50 ml). The solution was diluted with 5% Na_2CO_3 (50 ml) and extracted with ether (4 × 500 ml). The aqueous solution was adjusted to pH 4·0 (phosphoric acid), extracted with ether (4 × 500 ml) which was washed with water, dried (Na_2SO_4) and evaporated to a yellow gum, partially soluble in CH_2Cl_2 (3 × 5 ml). The soluble portion was chromatographed using methanol/CH_2Cl_2 (1:5). After drying for 30 min, the developed plates were illuminated with u.v. light and the blue fluorescent band (R_f 0·3) was removed and extracted with warm ethanol (100 ml) (λ_{max} 350 nm with absorbance 1·35).

Methylation

The ethanolic solution was evaporated and the residue treated with ethereal diazomethane, yielding an extract which showed one major component (R_f 0·43) when chromatographed with benzene-ether (9:1).

[6] D. M. SPENCER, personal communication (1968).

Methylation with Tritiated Diazomethane[7]

Ethereal CH_2N_2 (1 ml) from nitrosomethyl urea (0·5 g), dioxan (0·3 ml) and 0·05 ml tritiated water (0·2 C/ml) at 0° was treated with benzoic acid (1 mg) and left at room temperature for 15 min. Dry N_2 was blown through the solution and passed into dioxan (10 ml) containing the purified acidic antifungal principle obtained from 350 g of broad bean leaves. After 15 min, the dioxan was evaporated and the residue chromatographed in benzene/ether (9:1). The blue fluorescent band at R_f 0·43 was extracted with CH_2Cl_2 mixed with wyerone (12·5 mg) and the solid product crystallized five times from *n*-hexane at −20°. After each crystallization approximately 0·5 mg was removed for counting.

Bioassay

Extracts were taken to dryness in small tubes. 0·5 ml of aqueous phase from infection droplets was added to each tube, which was agitated on a "Whirlimixer". Two 20-μl drops of solution were placed on clean microscope slides. Drops were overspotted with 1 μl drops of a suspension of 2×10^5 spores/ml of *Botrytis* in water. Slides were incubated in moist sandwich boxes at 17° for 21 hr. Spores in each drop were examined for production and length of germ-tubes. Percentage germinations and mean germ-tube lengths were plotted against the logarithms of the concentrations of wyerone acid to permit calculation of ED_{50}s.

Acknowledgements—We thank Dr. C. H. Fawcett of Wye College and Professor Sir Ewart Jones for the samples of wyerone, Professor D. H. R. Barton for valuable discussions and Miss B. C. L. Clarke for assistance with the biological work which was aided by an A.R.C. grant.

[7] (a) K. J. v.d. MERWE, P. S. STEYN and S. H. EGGERS, *Tetrahedron Letters* 3923 (1964); (b) G. PAGE, personal communication.