The Effect of Reduced Wyerone Acid on the Antifungal Activity of the Phytoalexin Wyerone Acid Against *Botrytis fabae*

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(Received 15 March 1977; revised 9 May 1977)

INTRODUCTION

The pathogenicity of *Botrytis fabae* Sard. towards leaves of the broad bean plant has been related to the comparative insensitivity of the fungus to the phytoalexins, in particular wyerone acid (1) produced by *Vicia faba* L. in response to fungal infection (Mansfield & Deverall, 1974; Hargreaves & Mansfield, 1975; Hargreaves, Mansfield & Rossall, 1977). Germ tubes and mycelium of *Botrytis fabae* detoxify wyerone acid by reduction to the hexahydro derivative, reduced wyerone acid (2), which accumulates in bean leaf and pod tissue colonized by *B. fabae* (Mansfield & Widdowson, 1973; Mansfield, Porter & Widdowson, 1973; Hargreaves, 1976). Here we report the effect of the reduced derivative on the activity of wyerone acid against *B. fabae*.

METHODS

Pod tissue inoculated with *B. cinerea* Pers. or *B. fabae* (Purkayastha & Deverall, 1965) and extracted with diethyl ether provided wyerone acid or reduced wyerone acid respectively. Wyerone acid was purified by preparative thin-layer chromatography as previously described (Hargreaves, 1976; Hargreaves *et al.*, 1977). Reduced wyerone acid was recovered by gel filtration. A 2.5 × 70 cm column was packed with LH20 Sephadex in methanol. The extract of 250 g tissue collected 6 days after inoculation with *B. fabae* was applied to the column in 5 ml methanol; the flow rate of the same solvent was adjusted to 1 ml min⁻¹ and fractions were collected every 20 min. The contents of each fraction were monitored by thin-layer chromatography on precoated plates (silica gel F254 0.25 mm layers, Merck 5715). Chromatograms were developed in diethyl ether/methanol (8:1 v/v) and examined under ultraviolet (u.v.) light (254 and 366 nm). Reduced wyerone acid was recognized at *R_F* 0.42 as a u.v. absorbing band which quenched the fluorescence of the added fluorochrome at the shorter wavelength. The metabolite was the only compound detected under u.v. light in chromatograms of fractions 14 to 16. Ultraviolet absorption spectra of these fractions were identical to that of authentic reduced wyerone acid (λ_{max} in MeOH, 300 nm). Concentrations of wyerone acid and reduced wyerone acid were adjusted using extinction coefficients published for their methyl esters (Letcher *et al.*, 1970; Mansfield *et al.*, 1973).

Antifungal activity was assayed by adding the test compounds dissolved in synthetic pod nutrients solution at pH 4.0 (SPN; Hargreaves, Mansfield & Coxon, 1976) to preformed germ tubes of *B. fabae*. Spores were germinated in 20 μ l droplets (500 spores per drop of SPN) on glass microscope slides usually for 6 h; at this time a single germ tube had been produced from each spore. The SPN solution was then removed with filter paper and replaced by a droplet of the solution to be assayed. After further incubation, germ tube growth

Fig. 1. Fractional inhibition of *B. fabae* germ tube growth by wyerone acid alone (●), or in the presence of 20 (○) or 40 (□) μg reduced wyerone acid ml^{-1} . For (a), test solutions were added to germ tubes $59.0 \pm \text{s.e.m. } 3.3 \mu\text{m}$ in length and after further incubation for 3 h germ tube lengths in SPN alone or with 20 or 40 μg reduced wyerone acid ml^{-1} were 136.4 ± 8.9 , 98.6 ± 6.5 and $88.0 \pm 5.1 \mu\text{m}$ respectively. For (b), test solutions were added to germ tubes $25.6 \pm 2.1 \mu\text{m}$ long and after 3 h incubation in SPN alone or with 20 μg reduced wyerone acid ml^{-1} were 79.8 ± 6.8 and $64.9 \pm 2.6 \mu\text{m}$ respectively. Regression equations are given in (b).

was measured using an eyepiece graticule or from *camera lucida* drawings of individual germ tubes. The assays were replicated four times and at least 12 randomly selected germ tubes were measured in each replicate droplet.

RESULTS AND DISCUSSION

In the first experiment the activities of wyerone acid and reduced wyerone acid (20 $\mu\text{g ml}^{-1}$) were examined either singly or in combination. The compounds were added to germ tubes of *B. fabae* (mean length $54.4 \pm \text{s.e.m. } 3.3 \mu\text{m}$) and their growth was recorded after a further 18 h incubation. In the absence of these compounds, germ tubes were then $727 \pm 47.9 \mu\text{m}$ in length. In solutions of wyerone acid or reduced wyerone acid, germ tubes were $88.7 \pm 6.0 \mu\text{m}$ and $735 \pm 37.5 \mu\text{m}$ long respectively. The phytoalexin was, therefore, inhibitory at 20 $\mu\text{g ml}^{-1}$ but reduced wyerone acid appeared inactive. In SPN containing both compounds, germ tubes grew to $328 \pm 22.3 \mu\text{m}$ in length showing that reduced wyerone acid decreased the activity of the phytoalexin.

In subsequent experiments the activity of a range of wyerone acid concentrations from 1 to 10 $\mu\text{g ml}^{-1}$ was examined in the presence or absence of reduced wyerone acid (20 or 40 $\mu\text{g ml}^{-1}$). Incubation periods in test solutions were reduced to 3 h to allow more accurate measurements to be made of the short germ tubes produced and also to minimize possible metabolism of wyerone acid. In preliminary tests no metabolism of the phytoalexin was detected in solutions of 1 or 2 μg wyerone acid ml^{-1} incubated with *B. fabae* under these conditions (Rossall, unpublished). The antifungal activity of wyerone acid is expressed in Fig. 1(a, b) as fractional inhibition of germ tube growth in solutions lacking the phytoalexin but containing reduced wyerone acid when applicable. Values for fractional inhibition of 1.0 and 0, therefore, indicate complete inhibition and no inhibition due to wyerone acid respectively.

Results presented in Fig. 1(a) clearly show that increasing concentrations of reduced wyerone acid (which under the bioassay conditions of short incubation employed was itself

slightly antifungal) caused an increasing reduction in the activity of wyerone acid. However, the effect of reduced wyerone acid was greatly decreased with increasing concentrations of the phytoalexin.

Results obtained using germ tubes produced from spores after only 4 h incubation allowed a double reciprocal plot to be drawn (Fig. 1*b*). The linear relationships obtained with both lines intercepting the ordinate close to 1.0 are analogous to those describing the binding of ligands to enzymes and other macromolecules (Price & Dwek, 1974). Linearity would be expected if an equilibrium exists between binding sites in *B. fabae*, wyerone acid and the binding site/wyerone acid complex, formation of the complex resulting in the inhibition of growth of *B. fabae*. Fractional inhibition of growth would therefore represent fractional saturation of binding sites by wyerone acid. Furthermore, these results strongly suggest that reduced wyerone acid competitively inhibits the binding of wyerone acid to a receptor site in *B. fabae*, particularly as the effect of the metabolite was overcome at high concentrations of the phytoalexin (Fig. 1*b*).

The binding site may be the site of action of wyerone acid or may be a site involved in the uptake of the compounds (wyerone acid in particular) into fungal germ tubes. However, in view of the lipophilic nature of the undissociated molecules it would seem probable that they enter cells passively, and competition for some receptor site within hyphae is therefore more likely. The greater sensitivity of species of *Botrytis* other than *B. fabae* to wyerone acid (Letcher *et al.*, 1970; Rossall unpublished) may relate to the presence of more binding sites in the sensitive fungi.

These experiments raise interesting topics for further investigation. The presence of wyerone acid receptor sites in *Botrytis* requires confirmation by direct binding studies using radioactively-labelled wyerone acid. In addition, the possible relevance to disease resistance of the inhibition of phytoalexin activity by reduced wyerone acid within infected tissue, merits more detailed examination.

We wish to acknowledge the support of the Agricultural Research Council.

REFERENCES

- HARGREAVES, J. A. (1976). *The role of phytoalexins in the resistance of Vicia faba L. to infection by Botrytis*. Ph.D. thesis, University of Stirling.
- HARGREAVES, J. A. & MANSFIELD, J. W. (1975). Phytoalexin production by *Vicia faba* in response to infection by *Botrytis*. *Annals of Applied Biology* **81**, 271–276.
- HARGREAVES, J. A., MANSFIELD, J. W. & COXON, D. T. (1976). Conversion of wyerone to wyerol by *Botrytis cinerea* and *B. fabae* *in vitro*. *Phytochemistry* **15**, 651–653.
- HARGREAVES, J. A., MANSFIELD, J. W. & ROSSALL, S. (1977). Changes in phytoalexin concentrations in tissues of the broad bean plant (*Vicia faba* L.) following inoculation with species of *Botrytis*. *Physiological Plant Pathology* (in the Press).
- LETCHER, R. M., WIDDOWSON, D. A., DEVERALL, B. J. & MANSFIELD, J. W. (1970). Identification and activity of wyerone acid as a phytoalexin in broad bean (*Vicia faba*) after infection by *Botrytis*. *Phytochemistry* **9**, 249–252.
- MANSFIELD, J. W. & DEVERALL, B. J. (1974). Changes in wyerone acid concentrations in leaves of *Vicia faba* after infection by *Botrytis cinerea* or *B. fabae*. *Annals of Applied Biology* **77**, 227–235.
- MANSFIELD, J. W. & WIDDOWSON, D. A. (1973). The metabolism of wyerone acid (a phytoalexin from *Vicia faba* L.) by *Botrytis fabae* and *B. cinerea*. *Physiological Plant Pathology* **3**, 393–404.
- MANSFIELD, J. W., PORTER, A. E. A. & WIDDOWSON, D. A. (1973). The structure of a fungal metabolite of the phytoalexin wyerone acid from *Vicia faba* L. *Journal of the Chemical Society (Perkin I)*, 2557–2559.
- PRICE, N. C. & DWEK, R. A. (1974). *Principles and Problems in Physical Chemistry for Biochemists*. Oxford: Clarendon Press.
- PURKAYASTHA, R. P. & DEVERALL, B. J. (1965). The detection of antifungal substances before and after infection of beans (*Vicia faba* L.) with *Botrytis* species. *Annals of Applied Biology* **56**, 269–277.