Changes in phytoalexin concentrations in tissues of the broad bean plant (Vicia faba L.) following inoculation with species of Botrytis

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Six phytoalexins, the furano-acetylenes wyerol, wyerone, wyerone acid and wyerone epoxide, an unidentified wyerone derivative (M+ 290) and the isoflavonoid medicarpin, have been recognized as the major components of the phytoalexin response of Vicia faba. All of the phytoalexins examined were more active against germ tubes of Botrytis cinerea than B. fabae; differential toxicity was much more marked with wyerone derivatives than with medicarpin. The inhibitors were placed in the following order of antifungal activity against B. cinerea at pH 4.0: wyerone epoxide > wyerone acid > wyerone > medicarpin > wyerol. Activity of the acid was less at pH 5.0. Activities of wyerone acid and epoxide were additive. Phytoalexins accumulated in limited lesions caused by Botrytis. Time-course studies showed that the rapid accumulation of wyerone or wyerone acid could alone account for the restriction of fungal growth in cotyledons or leaves and pods respectively. Wyerone appeared to be deposited on pod endocarp cell walls and converted to wyerone acid in vivo. Results suggested that the weakly active wyerol was a precursor of other wyerone derivatives. An initial increase in phytoalexin concentrations in leaves and pods after inoculation with B. fabae was followed by a decrease as tissues became completely blackened and colonized by the pathogen. The biochemical mechanisms underlying the specificity of B. fabae to V. faba are discussed.

INTRODUCTION

Leaves and pod endocarp tissue of Vicia faba L. are susceptible to colonization by Botrytis fabae Sard; the cause of chocolate spot disease of broad and field beans, but resistant to Botrytis cinerea Pers., resistance typically being expressed by the production of limited lesions within which the growth of invading hyphae is inhibited [19, 24]. By contrast, cotyledons are resistant to both fungi, and cotyledon tissue bearing limited lesions produced by Botrytis has proved to be a useful source of some of the phytoalexins from V. faba [11, 13].

The multi-component phytoalexin response of the broad bean plant to fungal infection may be easily demonstrated by thin-layer chromatography (t.l.c.) plate bioassays with Cladosporium herbarum Pers. [10]. In this paper we report further studies on the detection and characterization of phytoalexins produced by V. faba. Identification of the furano-acetylenes, wyerol [7, 11], wyerone [7, 11], wyerone acid [18] and wyerone epoxide [13], and the isoflavonoid medicarpin [12] as the cause of inhibitory zones on t.l.c. plate bioassays has permitted precise measurements of changes in their concentrations in cotyledon, leaf and pod tissues during resistant or susceptible responses to infection by Botrytis. Experiments on the localization of the

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inhibitors within infected tissues and their antifungal activity are also described, and the relative importance of each of the phytoalexins in the inhibition of invading hyphae is discussed.

**MATERIALS AND METHODS**

*Plant and fungal materials*

In the experiments described, broad bean plants cv. The Sutton and Aquadulce, grown in the University gardens, were used as sources of pods and leaves respectively. Leaves were collected in May and June from plants 8 to 10 weeks old. Cotyledons were obtained from undressed seed of cv. The Sutton. Tissues of the two varieties used give similar responses to infection with *Botrytis* and also show similar patterns of phytoalexin accumulation (Mansfield, unpublished observations).

*B. cinerea* and *B. fabae* were isolated at the start of this work from field bean plants grown at Stirling; *B. allii* Munn. was isolated from onions, and cultures of *C. herbarum* were donated by Dr N. J. Dix. Sporulating cultures of all species were grown on Medium X [17] at 18 °C under blacklight (Philips) using a 16 h photoperiod. Conidia were collected from cultures (10 to 12 days old) and suspensions of *Botrytis* (5 x 10^6 spores/ml sterile distilled water) prepared as previously described [2]. Basic procedures for the inoculation of imbibed cotyledons and pod endocarp tissues have already been given [11, 24]; in this work we inoculated cotyledons and pod seed cavities with c. 40 μl and 0.3 ml inoculum droplets respectively. Preparation and inoculation of abaxial leaf surfaces were essentially as described for adaxial surfaces by Mansfield & Deverall [19] except that leaves were supported on plastic mesh and 20 μl inoculum droplets were utilized. Tissues were randomized between treatments before inoculation.

Inoculum droplets were collected from infected tissues with a Pasteur pipette. Underlying leaf discs were cut with a cork borer [20]. Cotyledon tissue not more than 1 mm thick was sliced off with a razor blade and pod endocarp was scraped off to a depth of c. 3 mm beneath inoculum droplets with a spatula. Droplets and tissues were stored at -20 °C before extraction.

*Extraction procedures*

The extraction scheme utilized for the detection of antifungal compounds is summarized in Fig. 1. Precautions were taken to keep illumination to a minimum. Tissues were homogenized in a Sorvall omnimixer. Evaporation was carried out in vacuo at 30 °C. Fractions 1 to 4 were suspended in MeOH (1 ml per 5 g tissue extracted) and 0.2 ml of each extract applied to 2 cm origins on pre-coated t.l.c. plates (Merck 5715, silicagel F254, 0.25 mm thick) and after development in various solvents chromatograms were bioassayed with *C. herbarum* or *Botrytis*.

Details of the extraction of tissues with Et₂O for the isolation of milligram quantities of the phytoalexins have been given elsewhere [11–13]. Inhibitors were purified by preparative layer chromatography (p.l.c.) using Merck silicagel G layers 1.5 or 2 mm thick. Extracts from 5 to 10 g fresh wt of infected tissue were applied per cm of the origin. The first solvent system used was hexane 60 to 80 °C : acetone (2 : 1)
Phytoalexin concentrations in *Botrytis*-inoculated tissues followed by CHCl₃ : petroleum spirit 60 to 80 °C (2 : 1) and unless otherwise stated compounds were eluted on a preparative scale with both CHCl₃ and Et₂O [13].

Nuclear magnetic resonance (n.m.r.) spectra were usually recorded in deuterochloroform using TMS as an internal standard while i.r. and u.v. spectra were recorded in CHCl₃ and MeOH respectively. Mass spectra were determined with an A.E.I. MS 902 spectrometer by use of a direct insertion probe.

For quantitative analyses, samples of frozen tissue (1 to 6 g) were homogenized in Et₂O (at least 20 ml/g fresh wt). After centrifugation, the supernatant was decanted and the pellet washed 3 times with 50 ml Et₂O. Combined Et₂O solutions were evaporated and the residue dissolved in MeOH. Inoculum droplets were either added to tissue before homogenization or partitioned separately 3 times with twice their volume of Et₂O. Aliquots (equivalent to 0.5 or 1.0 g fresh wt) of the MeOH soluble material were examined by t.l.c. Phytoalexins were detected on t.l.c. plates by their characteristic appearance under ultraviolet (u.v.) light (254 and 366 nm) and eluted in MeOH. Concentrations of medicarpin, wyrerol, wyerone, wyerone acid and wyerone epoxide were determined from u.v. absorption spectra of eluates using published extinction coefficients [7, 12, 13, 18]. It was assumed that the unidentified wyerone derivative named PA4 possessed an extinction coefficient similar to wyerone and its concentration was estimated using the relationship O.D.₅₄₇ mg⁻¹ cm⁻¹ 1.0 = 10 μg PA4/ml. Medicarpin and PA4 were eluted as a mixture from t.l.c. plates. Absorbance of the mixture at 347 nm was considered to be due to PA4; absorbance at 287 nm due to PA4 was estimated from the absorbance ratio of 347 : 287 nm (5.9) for the purified inhibitor and subtraction of this estimate from the total gave a value for medicarpin absorbance at 287 nm. Where it was not

Fig. 1. Extraction scheme for the isolation of antifungal compounds from tissues of *V. faba.*
possible to obtain the phytoalexins in sufficient amounts or to the degree of purity required for spectroscopy their presence was determined by t.i.c. plate bioassays.

Fractionation of pod tissue

Fresh infected tissue (c. 5 g) was washed 3 times with sterile distilled water (20 ml). Water washings and tissue separated after centrifugation were stored at -20 °C. Cell walls were isolated by a modification of the method described by English et al. [6]. The frozen tissue was crushed to a fine powder under liquid nitrogen in a mortar and pestle and then macerated in a glass homogenizer (MSE) at half-speed for 2 min in cold 100 mM phosphate buffer (3 ml, pH 7.0). The macerate was centrifuged for 10 min at 500 g and the supernatant collected as part of the “buffer extract”. The pellet was macerated and centrifuged once more. The final pellet was resuspended in cold buffer (25 ml) and allowed to stand 4 times for 10 min with occasional stirring before vacuum filtration through Whatman No. 1 filter paper. The buffer washings and final water wash were combined as “buffer extract” and adjusted to pH 5.0 with H₃PO₄. Each aqueous fraction collected was partitioned between Et₂O and water 3 times and the ether extracts prepared for t.l.c. The residual cell wall material was extracted 3 times with 50 ml MeOH. All procedures from water washings to solvent extractions were carried out at 4 °C.

Bioassay techniques

Antifungal compounds were detected on t.i.c. plates by variations of the method devised by Klarman & Stanford [15]. Dense suspensions of spores of C. herbarum, B. allii, B. cinerea or B. fabae in Czapek Dox solution (pH 5.0 or 6.8) were sprayed onto developed chromatograms and incubated at 25 °C in moist chambers for 4 days. Zones inhibitory to C. herbarum were recognized as white areas of silica gel where the dark green fungus failed to grow. The presence of compounds active against the Botrytis species was indicated by a lack of aerial mycelium and by the absence of hyphae turning brown after incubation in iodine vapour.

The antifungal activity of solutions of the phytoalexins in synthetic pod nutrients (SPN) [11] was assayed by modifications of the microscope-slide bioassay technique. Germ tube growth from spores was recorded after incubation for 18 h at 18 °C [11, 19]. The direct effect of phytoalexins on germ tubes was examined by allowing conidia to germinate in 20 µl droplets of SPN solution (2.5 x 10⁴ spores/ml) for 6 h. Germ tubes 50 to 60 µm in length were produced by this time and they adhered strongly to glass slides. The nutrient solution was removed with filter paper and replaced by SPN containing phytoalexins. Controls were replaced with SPN alone. Germ tube lengths were recorded after further incubation for 18 h at 18 °C.

The activity of wysterone when deposited on cellulose was also investigated. Solutions of the phytoalexin in CHCl₃ were added to discs (4 mm diameter) of chromatography paper (Whatman No. 1) on glass slides to give concentrations ranging from 3 to 15 µg wysterone/cm² filter paper. The solvent was evaporated by an air stream and, after drying, discs were covered with 10 µl of SPN and 5 µl of spore suspension (1 x 10⁶ spores/ml SPN). After incubating the slides for 18 h the lengths of the germ tubes produced on the paper were recorded [11].
RESULTS

Infection development following inoculation with B. cinerea or B. fabae

Limited lesions. Limited lesions were caused by B. cinerea in each of the tissues examined and by B. fabae only in cotyledons. In resistant tissues the first symptoms were apparent by 12 h after inoculation when isolated necrotic flecks appeared beneath inoculum droplets. In pods and leaves browning increased until lesions reached their final size about 4 days after inoculation remaining confined to the tissue beneath inoculum droplets. Lesions developed at all sites inoculated with B. cinerea; infection development on the lower surface of field-grown leaves was therefore much less variable than on greenhouse-grown material [19]. Similar restricted lesions developed in cotyledons but they were orange/brick red in colour and reached their final appearance 7 days after inoculation. Limited lesions produced by B. fabae in cotyledons were darker than those produced by B. cinerea and more often completely covered the area of tissue beneath inoculum droplets.

Microscopical observation of epidermal strips and free-hand sections showed that, during limited lesion development, growth of invading hyphae ceased by the third day after inoculation. In cotyledons and leaves intracellular hyphae had been produced which were restricted to one or two epidermal cells. By contrast in pods, little penetration of endocarp cells was observed, short germ tubes produced were often branched and swollen containing granular cytoplasm. Growth abnormalities were most pronounced at hyphal tips which were in contact with host cell walls.

Spreading lesions. B. fabae caused spreading lesions in leaf and pod tissue. Dark brown/black flecks were visible 12 h after inoculation and lesions developed rapidly until, after 3 and 4 days in leaves and pods respectively, the tissue beneath inoculum droplets was completely blackened and colonized by the virulent pathogen, and lesions had begun to spread into surrounding tissues. In pods, fungal growth was mainly intercellular, more intracellular hyphae were observed in leaves, but in both tissues necrosis and blackening was caused typically two or three cells in advance of invading hyphae.

Detection and characterization of phytoalexins

The presence of antifungal compounds in cotyledon, leaf and pod endocarp tissues before and 2 days after inoculation with B. cinerea was investigated. No inhibitors were detected in healthy uninoculated tissues, antifungal compounds were recovered only in the Et₂O soluble fraction of 80% MeOH extracts of tissue bearing limited lesions (Fraction 2, Fig. 1). Subsequent experiments showed that all of the phytoalexins could be extracted from infected tissues with either MeOH or Et₂O.

Plate 1 shows a typical t.l.c. plate bioassay and clearly indicates that the number of inhibitors detected by this technique depended on the amount of tissue extract applied to the chromatogram. The major inhibitory bands (1 to 5, Plate 1) were detected in extracts from each tissue examined and in bioassays carried out using species of Botrytis rather than Cladosporium herbarum. The latter was preferred for routine bioassays as inhibition zones produced were more distinct and the fungus was much more sensitive to all of the phytoalexins.

Following elution from the chromatogram of an Et₂O extract of pod endocarp, the inhibitors present in bands 1, 2, 3 and 5 could not be separated into more than
one antifungal component by t.l.c. However, band 4 was resolved into two inhibitors by two-dimensional t.l.c. in dichloromethane followed by hexane : acetone (2:1).

Milligram quantities of the phytoalexins were recovered from pod (2.5 kg) or cotyledon tissues (15 kg) bearing limited lesions produced by *B. cinerea* or *B. fabae* respectively 6 days after inoculation. The identification of compounds corresponding to inhibition zones on t.l.c. plate bioassays is given in Table 1. Details of the isolation and identification of medicarpin, wyerone and wyerone epoxide have been given elsewhere [11-13].

Following the initial p.l.c. of ether extracts in hexane : acetone (2:1) and CHCl₃ : petrol (2:1), wyerol, medicarpin/PA4 mixture and wyerone acid were recovered from *Rₓ* 0.59, 0.34 and 0.13 respectively. After further purification by p.l.c. developed twice in Et₂O : petrol (2:1), a total yield of 8 mg of wyerol was obtained. Ultraviolet, i.r., n.m.r. and mass spectra of the purified compound were

**Table 1**

Identification of phytoalexins causing inhibition zones on t.l.c. plate bioassays

<table>
<thead>
<tr>
<th>Inhibition zone</th>
<th><em>Rₓ</em></th>
<th>Identity</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.65</td>
<td>Wyerone</td>
<td>CH₂·CH₂·CH=CH·C=C·CO &lt;CH=CH·COOMe</td>
</tr>
<tr>
<td>2</td>
<td>0.53</td>
<td>Wyerone epoxide</td>
<td>CH₂·CH₂·CH=CH·C=C·CO &lt;CH=CH·COOMe</td>
</tr>
<tr>
<td>3</td>
<td>0.46</td>
<td>Wyerol</td>
<td>CH₂·CH₂·CH=CH·C=C·CH(OH) &lt;CH=CH·COOMe</td>
</tr>
<tr>
<td>4</td>
<td>0.38</td>
<td>Wyerone derivative</td>
<td>Unconfirmed (M⁺ 290)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medicarpin</td>
<td>IIO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><img src="image" alt="Medicarpin Structure" /></td>
</tr>
<tr>
<td>5</td>
<td>0.08</td>
<td>Wyerone acid</td>
<td>CH₂·CH₂·CH=CH·C=C·CO &lt;CH=CH·COOH</td>
</tr>
</tbody>
</table>

*After development of chromatograms for 15 cm in hexane : acetone (2:1), followed by CHCl₃ : petrol (2:1).*
virtually identical to those recorded for the metabolite of wyerone produced by both
*B. cinerea* and *B. fabae* [I].

Further p.l.c. of the mixture from *R*<sub>F</sub> 0.34 by development twice in CHCl<sub>3</sub>
allowed the separation of medicarpin (*R*<sub>F</sub> 0.35) and PA4 (*R*<sub>F</sub> 0.28) from extracts of
infected pods. Purified PA4 (1 mg) was recovered after further p.l.c. in CHCl<sub>3</sub>. The deep blue fluorescence of PA4 under u.v. light (366 nm), u.v. spectrum (*λ*<sub>max</sub>
347 nm EtOH or MeOH) and the fragmentation pattern observed in the mass
spectrum M+ 290, *m/e* 261 (53%), 259 (5%), 179 (39%), 151 (38%) and 43 (100%)
strongly indicated that PA4 was a wyerone derivative with hydroxylation of the
acetylenic side chain.

Wyerone acid (band 5) was eluted in MeOH after the first p.l.c. step and sub-
jected to further p.l.c. (1.5 mm layers) in Et<sub>2</sub>O : MeOH (8:1) using low loading
rates. The acid ran as a broad band between *R*<sub>F</sub> 0.3 and 0.4 and after elution from
the centre of this band was concentrated into a small volume of warm EtOH. Et<sub>2</sub>O
was added dropwise as the solution was cooled on ice and the phytoalexin precipitated
out as a pale yellow solid which was collected after centrifugation and washed twice
with Et<sub>2</sub>O. The identity of wyerone acid (17 and 55 mg isolated from cotyledons
and pods respectively) was confirmed by n.m.r. spectroscopy of the compound in
deuteromethanol. Diagnostic signals obtained were identical to those for wyerone
[I] except that no signal due to the methyl ester protons (δ 3.79) was detected.

The *R*<sub>F</sub> values of the phytoalexins after t.l.c. in six solvent systems and their
appearance under u.v. light are recorded in Table 2. Medicarpin was also recog-

<table>
<thead>
<tr>
<th>Properties</th>
<th>Wyerone</th>
<th>Wyerone epoxide</th>
<th>Wyerol</th>
<th>PA₄</th>
<th>Medicarpin</th>
<th>Wyerone acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour under u.v. light (366 nm)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Blue</td>
<td>Blue</td>
<td>—</td>
<td>Blue</td>
<td>—</td>
<td>Blue</td>
</tr>
<tr>
<td><em>R</em>&lt;sub&gt;F&lt;/sub&gt; values in t.l.c.&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvents:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt; (2% EtOH)</td>
<td>0.60</td>
<td>0.53</td>
<td>0.32</td>
<td>0.15</td>
<td>0.19</td>
<td>0.00</td>
</tr>
<tr>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt; : MeOH (10 : 1)</td>
<td>0.90</td>
<td>0.90</td>
<td>0.75</td>
<td>0.62</td>
<td>0.69</td>
<td>0.11</td>
</tr>
<tr>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt; : petrol (2 : 1)</td>
<td>0.42</td>
<td>0.34</td>
<td>0.17</td>
<td>0.05</td>
<td>0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>Hexane : acetone (1 : 1)</td>
<td>0.74</td>
<td>0.68</td>
<td>0.68</td>
<td>0.60</td>
<td>0.61</td>
<td>0.03</td>
</tr>
<tr>
<td>Hexane : acetone (3 : 1)</td>
<td>0.35</td>
<td>0.30</td>
<td>0.28</td>
<td>0.18</td>
<td>0.20</td>
<td>0.00</td>
</tr>
<tr>
<td>Hexane : ethyl acetate : acetic acid (60 : 40 : 1)</td>
<td>0.49</td>
<td>0.39</td>
<td>0.44</td>
<td>0.40</td>
<td>0.41</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> Camag Universal Lamp.

<sup>b</sup> In saturated tanks on precoated silica gel plates (Merck 5715) developed for 15 cm.

...nized by its yellow/orange coloration after spraying chromatograms with *p*-nitro-
aniline reagent [25]. It proved impossible to separate all the inhibitors for quantitative
analysis in a single t.l.c. step. However, providing extracts contained little chlorophyll
all but wyerone acid could be separated by developing chromatograms in hexane :
acetone (2 : 1) followed by CHCl<sub>3</sub> : petrol (2 : 1). Medicarpin and PA₄ although
separated from other compounds were not resolved in this system, but because of
their different *λ*<sub>max</sub> and the negligible absorbance of medicarpin at 347 nm, the
concentrations of each phytoalexin in the mixture eluted from $R_F$ 0·31 to 0·38 could be determined by u.v. spectrophotometry. Wyerone acid was separated from extracts of all tissues by t.l.c. in ether : methanol (6 : 1 or 8 : 1, $R_F$ 0·5 or 0·3 respectively); other phytoalexins ran close to the solvent front in these solvents. In addition to the acid only wyerone and wyerone epoxide could be purified from leaf extracts for quantitative analysis. Thin-layer chromatography development of leaf extracts in chloroform : petrol (1 : 2) 3 times in unsaturated tanks allowed the separation of both wyerone (final $R_F$ c. 0·5) and wyerone epoxide ($R_F$ c. 0·2) from interfering chlorophylls.

Changes in phytoalexin concentrations in tissues inoculated with B. cinerea or B. fabae

**Limited lesions.** In all tissues phytoalexin accumulation was associated with the appearance of symptoms. In cotyledons the pattern of phytoalexin accumulation was qualitatively similar in limited lesions caused by B. cinerea or B. fabae but the latter induced slightly higher levels of all of the inhibitors (Fig. 2). Most striking

![Graph showing changes in phytoalexin concentrations](image)

**Fig. 2.** Changes in concentrations of wyerone (▲), wyerone acid (●), wyerone epoxide (■), wyerol (△), medicarpin (○) and PA4 (□) in cotyledon, leaf and pod tissues inoculated with B. cinerea or B. fabae. Yields include phytoalexins present within overlying inoculum droplets. (a) Cotyledon; (b) leaf; (c) pod.
was the increase in wyerone which reached levels at least 10 times greater than those recorded in pod or leaf tissues (Fig. 2). Medicarpin was present in cotyledons only at low levels during the later stages of the disease reaction.

Wyerone acid accumulated rapidly in leaf and pod endocarp tissues inoculated with *B. cinerea* to reach maximum concentrations within 3 days after inoculation. In leaves wyerone followed an essentially similar pattern of accumulation as the acid, but reached a much lower final concentration (Fig. 2). In pod endocarp wyerone accumulated more slowly than the acid, but reached similarly high levels after 6 days. In both tissues comparatively low yields of wyerone epoxide and other phytoalexins were recovered.

In repeated experiments, examination of the distribution of phytoalexins between inoculum droplets and underlying tissues in pod endocarp inoculated with *B. cinerea* showed that wyerone was confined to infected tissue (Fig. 3). Only wyerone acid and wyerone epoxide accumulated to measurable concentrations in droplets; yields ranging from 29 to 61 and 3·0 to 5·0 µg/ml were recorded for the acid and epoxide respectively 2 to 6 days after inoculation, wyerol; medicarpin and PA4 were present in trace amounts.

Fig. 3. Diagrams of t.l.c. plate bioassays of extracts from pod tissue (0·25 g) and overlying inoculum droplets (equivalent to 0·25 g tissue) collected 12 h, 1, 2, 3, 4 and 6 days after inoculation with (a) *B. cinerea* or (b) *B. fabae*. Extracts were applied to 1·5 cm origins, and chromatograms developed in hexane : acetone (2 : 1) then CHCl₃ : petrol (2 : 1), areas of inhibition are unshaded.
Spreading lesions. In pod and leaf tissue infected with *B. fabae* an initial increase in the concentrations of phytoalexins was followed by a decrease as the susceptible tissues became completely blackened and invaded by the pathogen (Figs 2 and 3). No inhibitors were detected in inoculum droplets collected from pods (Fig. 3).

Localization of wyerone in pod endocarp tissues infected by *B. cinerea*

The absence of wyerone from inoculum droplets despite the accumulation of the phytoalexin to concentrations >100 μg/g fresh wt within underlying pod tissues suggested that this inhibitor was deposited in some way within infected tissue. This possibility was investigated by examining the recovery of wyerone and other phytoalexins at different stages during the fractionation of infected pod endocarp.

Wyerone was recovered from the crude cell wall fraction (Table 3, Plate 2). Moreover, analysis of the yields of wyerone and wyerone acid from different fractions indicated that wyerone→wyerone acid conversion was taking place during the fractionation procedure. In repeated experiments, t.l.c. plate bioassays (Plate 2) showed that wyerone was the only major inhibitor closely associated with the cell wall fraction. Fractionation of 3-day-old *B. cinerea* lesions gave similar results.

### Table 3

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Wyerone</th>
<th>Wyerone acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original tissue</td>
<td>159</td>
<td>85</td>
</tr>
<tr>
<td>Water washings (1)</td>
<td>2</td>
<td>143</td>
</tr>
<tr>
<td>Buffer extract (2)</td>
<td>8</td>
<td>48</td>
</tr>
<tr>
<td>Residual cell walls (3)</td>
<td>51</td>
<td>8</td>
</tr>
<tr>
<td>Total yield from fractions 1, 2 and 3</td>
<td>61</td>
<td>199</td>
</tr>
</tbody>
</table>

* Expressed in μg/g fresh wt of original tissue.

**Antifungal activity of phytoalexins against *B. cinerea* and *B. fabae***

All of the inhibitors assayed were more active against *B. cinerea* than *B. fabae* and differential sensitivity was much more apparent with wyerone derivatives than medicarpin (Table 4). Wyerone epoxide was the most active compound tested, whereas wyerol possessed comparatively little antifungal activity. The effect of wyerone acid on both fungi was greatly reduced at pH 5.0, but pH had little effect on the activities of the other inhibitors. Germ tubes often appeared distorted after direct exposure to concentrations of wyerone derivatives slightly less than those causing complete inhibition, swelling and bursting of hyphal tips was occasionally observed.
PLATE 1. Thin-layer chromatography plate *C. herbarum* bioassays of extracts from 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125 g of infected leaf tissue collected 3 days after inoculation with *B. cinerea*. Extracts were applied to 1 cm origins and the chromatogram developed in hexane : acetone (2 : 1) then CHCl₃ : petrol (2 : 1).
PLATE 2. Thin layer chromatography plate *C. herbarum* bioassays of fractions of 1 g pod endocarp tissue collected 6 days after inoculation with *B. cinerea*. Fractions obtained from water washings of whole tissue (water), phosphate buffer extract of washed cells (buffer) and MeOH extraction of residual cell walls were compared with a MeOH extract of the original tissue (whole tissue). Solvents as for Plate 1.
In view of the sparing solubility of wyerone [8] and the apparent deposition of this inhibitor in cell walls in infected tissue, the antifungal activity of wyerone deposited on filter paper was investigated. It was interesting that in this bioassay system, germ tubes of both fungi were inhibited when their tips came into contact with cellulose fibrils coated with wyerone. *B. fabae* was again found to be less sensitive

### Table 4

<table>
<thead>
<tr>
<th>Phytoalexin</th>
<th>B. <em>cinerea</em></th>
<th>pH 4.0</th>
<th>B. <em>faba</em></th>
<th>pH 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>ED&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td><strong>MLD</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td><strong>ED&lt;sub&gt;50&lt;/sub&gt;</strong></td>
<td><strong>MLD</strong></td>
</tr>
<tr>
<td>Wyerone</td>
<td>10-1</td>
<td>20-25</td>
<td>25-0</td>
<td>40-50</td>
</tr>
<tr>
<td>Wyerone acid&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.5 (3.3)</td>
<td>10-15</td>
<td>6.8 (7.0)</td>
<td>20-25</td>
</tr>
<tr>
<td>Wyerone epoxide</td>
<td>2.7</td>
<td>4.0-6.0</td>
<td>5-6</td>
<td>10-15</td>
</tr>
<tr>
<td>Wyerol</td>
<td>85.0</td>
<td>150-200</td>
<td>140</td>
<td>250-300</td>
</tr>
<tr>
<td>Medicarpin</td>
<td>14.0</td>
<td>50-75</td>
<td>17.5</td>
<td>50-75</td>
</tr>
</tbody>
</table>

<sup>a</sup> Concentration of phytoalexin (µg/ml) which reduced germ tube growth by 50%; determined from graphs of germ tube growth against phytoalexin concentration.

<sup>b</sup> Minimum concentration (µg/ml) which completely prevented germ tube growth; expressed as the range of concentrations from the highest at which some growth was measured to the lowest tested at which no growth was recorded.

<sup>c</sup> Figures in parentheses give results of assays of wyerone acid activity obtained in the presence of 2% MeOH; otherwise no solvent was added for tests on this phytoalexin. Other inhibitors were all assayed in SPN containing 2% MeOH.

**Fig. 4.** Antifungal activities of wyerone acid and wyerone epoxide alone or in combination against germ tube growth by conidia of *B. cinerea*. Wyerone acid alone (●), epoxide alone (■), combinations of acid and epoxide (▲), expected reduction in germ tube length if effect of phytoalexins additive (△).
than *B. cinerea* to the phytoalexin. Over the range of wyerone concentrations examined (3 to 15 μg/cm²) percentage reduction in germ tube growth increased linearly from 83 to 90 and 59 to 64% for *B. cinerea* and *B. fabae* respectively.

Within inoculum droplets above pod endocarp *B. cinerea* was exposed to a solution containing two major antifungal components, wyerone acid and wyerone epoxide. The possibility that these inhibitors might act synergistically in solution was therefore examined. Bioassays were carried out maintaining the same combined concentration of phytoalexins but varying the proportion of wyerone acid or epoxide in the mixture. The final combined concentration of phytoalexins was selected to give about 50% inhibition of germ tube growth from conidia, thus 5 μg phytoalexin/ml was utilized for *B. cinerea* and 10 μg/ml for *B. fabae*.

Figure 4 illustrates the results obtained with *B. cinerea*. The inhibition of germ tube growth by various combinations of the phytoalexins differed little from that expected if the activities of wyerone acid and epoxide were additive. *B. fabae* gave similar results, and there was no indication of synergistic activity.

**DISCUSSION**

Tissues of *V. faba* respond to infection with *Botrytis* by the production of five furano-acetylenic compounds possessing antifungal activity, wyerol, wyerone, wyerone acid, wyerone epoxide and PA4. The production of these structurally related phytoalexins parallels the multi-component phytoalexin responses reported for other plants, for example the accumulation of isoflavanoid inhibitors in *Phaseolus vulgaris* L. [1, 26] and sesquiterpenoids in *Solanum tuberosum* L. [27]. The production of the isoflavanoid medicarpin in addition to wyerone derivatives is anomalous and to date *V. faba* appears unique in its production of chemically unrelated compounds which act as phytoalexins [16, 28]. Although the accumulation of medicarpin is of particular phylogenetic interest [12, 14], the time-course of its accumulation and low levels reached during resistant reactions suggest that compared to the wyerone derivatives this phytoalexin is not of great significance to the inhibition of growth of invading hyphae.

Different patterns of accumulation of wyerone derivatives were observed in cotyledon, pod and leaf tissues bearing limited lesions. Wyerone acid was the major phytoalexin recovered from leaves and pods during the first 3 days after inoculation. By contrast in cotyledons wyerone was by far the predominant inhibitor. Wyerol which has been identified as a metabolite of wyerone produced by both *B. cinerea* and *B. fabae in vitro* [11], accumulated only within limited lesions which did not contain metabolites of wyerone acid or epoxide [9, 27]. It therefore seems probable that wyerol was of plant origin occurring as a precursor for other wyerone derivatives. Although some caution must be applied to discussion of biogenetic routes based on accumulation data obtained from heterogeneous tissue, the different patterns of wyerone and wyerone acid accumulation in pod endocarp bearing limited lesions suggest that wyerone may act as a precursor for wyerone acid. This idea is supported by the observed conversion of wyerone to wyerone acid during the fractionation of pod tissue. These results indicate that the biogenetic relationship between wyerone derivatives is probably:
The striking accumulation of wyerone in cotyledons may therefore be due to the presence of low levels of plant esterase carrying out reaction (2) in this tissue.

The accumulation of phytoalexins in V. faba was associated with tissue necrosis and cellular browning, but the relationship between necrobiosis and phytoalexin production is far from clear. Microspectro-fluorimetric studies have shown that wyerone acid accumulates in live cells adjacent to dead cells in bean leaves infected with B. cinerea [22]. The other phytoalexins may also accumulate primarily in living cells. Inhibitors may diffuse out of live cells and/or may be released following their death and the associated disruption of plasmalemma and tonoplast. The deposition of wyerone on cell walls observed in our work may therefore occur before or after cell death. In the latter case deposition may merely reflect the precipitation of the phytoalexin from solution in living cytoplasm.

Microscopical observations showed that during the development of limited lesions the events responsible for the initial restriction of invading hyphae must be essentially complete before the third day after inoculation. Wyerone acid would therefore seem to be the most important inhibitor produced by leaf and pod tissues reaching levels 2 days after inoculation several times greater than that completely inhibitory to germ tubes of B. cinerea. Similarly, wyerone accumulation can alone account for the inhibition of hyphae of both B. cinerea and B. fabae in cotyledons. Although individually, other phytoalexins appear less important, they contribute to the production of a potentially highly antifungal environment within infected tissue. If wyerone is localized in cell walls adjacent to invading hyphae it may play a more important role in resistance than is suggested by its low concentrations within leaf and pod tissues during the first 2 days after inoculation. Wyerone deposited on cellulose fibrils effectively inhibited germ tube growth by B. cinerea.

Results presented for the accumulation of wyerone acid and wyerone in pods of V. faba are analogous to those reported for kievitone and phaseollin respectively in hypocotyls of Phaseolus vulgaris L. infected by Rhizoctonia solani Kuhn by Smith et al. [26]. If like wyerone, phaseollin is localized in some way within infected tissue it may be more significant than is at first apparent in the Phaseolus/Rhizoctonia interaction. It is of interest that in the many studies on disease resistance in P. vulgaris the strong absorption of phytoalexins (probably mainly phaseollin) onto healthy tissue reported by Muller [23] has frequently been overlooked.

In addition to the problem of localization one of the major difficulties in assessing the significance of phytoalexins to the inhibition of invading fungi is to determine if the inhibitors are active in vivo. The activity of wyerone acid is markedly affected by the composition of media used for in vitro bioassays [4]. In particular, in SPN solution the activity of the acid, unlike the methylated wyerone derivatives, was
dependent on pH. The decreasing activity of the acid at higher pH is probably through increased dissociation of the weak acid and failure of the anion to enter fungal cells [4]. The pH of inoculum droplets containing *B. cinerea* decreased from 4.7 to 3.9 between 1 and 3 days after inoculation onto pod endocarp (unpublished results) indicating that wyerone acid is probably highly active within infected tissues and emphasizing the importance of this inhibitor.

The fungitoxicity of wyerone derivatives has been related to the presence of the keto-acetylenic moiety [7, 21] and the relative inactivity of wyerol would support this argument. The differing activities of wyerone, wyerone acid and wyerone epoxide when compared at low pH are less easily explained. The additive nature of the activities of the acid and epoxide suggests that they may have the same site of action in which case the presence of the epoxide group may enhance binding to a specific receptor site. The differential sensitivities of *B. cinerea* and *B. fabae* to the wyerone derivatives may be related to the presence of fewer receptor sites in the virulent pathogen.

Although phytoalexin accumulation can explain the inhibition of fungal hyphae during the development of limited lesions, the mechanisms underlying the pathogenicity of *B. fabae* to leaf and pod tissues are less well defined. Changes in phytoalexin concentrations during the development of spreading lesions suggest that *B. fabae* is able to metabolize the inhibitors and thereby prevent their accumulation to antifungal concentrations around invading hyphae. Failure to detect inhibitors in inoculum droplets from pods and the low concentrations reported in earlier work [3, 5, 20, 21] support this conclusion. Phytoalexins recovered from tissue bearing developing lesions were probably present within live cells affected but not yet killed by *B. fabae*. Hyphae may therefore not be exposed to the inhibitors until they are released and immediately diluted in surrounding intercellular fluids following host cell death. In addition to time-course data, the detection in spreading lesions of metabolites of wyerone acid [21] and wyerone epoxide produced in vitro [9, 13] indicates that there is a turnover of these phytoalexins. By contrast the absence of wyerol accumulation in tissues colonized by *B. fabae* suggests that wyerone is metabolized by a different route in vivo or that *B. fabae* may not come into direct contact with wyerone within infected tissue. In vivo wyerone may be converted to wyerone acid by the plant and the acid subsequently metabolized by *B. fabae*.

The differential abilities of *B. cinerea* and *B. fabae* to colonize leaf and pod tissues appear to relate primarily to the interaction between these species and wyerone acid accumulation. Although metabolism of phytoalexins by host tissues may occur, the net accumulation of inhibitors within infected bean tissues is probably controlled primarily by a balance between phytoalexin production by the plant and degradation by the fungus. It has been suggested that wyerone acid is metabolized more rapidly by *B. fabae* than by *B. cinerea* [3, 21] and that this may tip the balance in favour of the pathogen. However, apparent differences in the rates of wyerone acid metabolism previously reported may merely reflect the differential sensitivities of the fungi to the phytoalexin. *B. fabae* may be predisposed to metabolize wyerone acid because of its comparative tolerance to the inhibitor. An additional factor contributing to the pathogenicity of *B. fabae* which has been overlooked in the past may be the ability of this species to suppress phytoalexin production. Cells may simply be killed more...
Phytoalexin concentrations in Botrytis-inoculated tissues

rapidly by B. fabae than by B. cinerea and tissues therefore produce less phytoalexin. In consequence B. fabae may be exposed to lower concentrations of inhibitors than B. cinerea. Further characterization of the metabolites of the phytoalexins produced by Botrytis should allow the estimation of gross phytoalexin production necessary to test this hypothesis.

In cotyledons the rates of phytoalexin production (wyerone in particular) are far greater than those in pod and leaf tissues and probably easily outweigh the ability of B. fabae to metabolize the inhibitors produced. As a result phytoalexins accumulate and growth of B. fabae is inhibited. Rapid phytoalexin production by cotyledons may relate to the high density of cells in the tissue and also the presence of large amounts of storage materials such as starch, lipids and proteins which provide potential sources of energy and precursors for rapid phytoalexin biosynthesis.

We suggest that the most useful approach to further investigation of broad bean/Botrytis interactions would be to carry out a genetical analysis of mechanisms of disease resistance and fungal pathogenicity in V. faba. This could be achieved by isolating mutants of B. cinerea and B. fabae differing from “wild types” in sensitivity to and ability to metabolize wyerone derivatives and also ability to kill cells of V. faba. Examination of the pathogenicity of such isolates should allow the role of phytoalexins in disease resistance and the biochemical basis underlying the specificity of B. fabae to be resolved.

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REFERENCES


