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Hargreaves, JA

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THE ROLE OF PHYTOALEXINS IN THE DISEASE RESISTANCE OF *V. FABA*.L TO INFECTION BY *BOTRYTIS*.

A thesis presented for the degree of

Doctor of Philosophy

in the University of Stirling

by

J.A.HARGREAVES, B.Sc.(Wales)

Department of Biology, University of Stirling.

August, 1976.

Ph.D awarded ~~March 1977~~ November 1976
conferred March 1977

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The work presented in this thesis is the result of my own investigations and has neither been accepted nor is being submitted for any other degrees.

J.A. Hargreaves .... Candidate

J.W. Mansfield .... Supervisor

25th August 1976 .... Date

ABSTRACT

Wyerone, wyerone acid and four other phytoalexins were isolated from broad bean tissues infected by *Botrytis*. Three of the new phytoalexins were shown to be wyerone epoxide, wyerol and medicarpin, the fourth was tentatively identified as hydrohydroxy - Keto - wyerone. All six phytoalexins accumulated in pod endocarp infected with *B. cinerea* and in cotyledons infected with *B. cinerea* or *B. fabae*. In pod tissue *B. fabae* appeared to be able to metabolize the phytoalexins and prevent their accumulation to antifungal concentrations.

Wyerone was the predominant phytoalexin in cotyledons infected with both fungi, whereas wyerone acid was the major component of the phytoalexin response in pods and leaves infected with *B. cinerea*. The other phytoalexins seem unlikely to contribute significantly to the restriction of *Botrytis* in invaded tissue. The role of wyerone in preventing fungal growth *in vivo* is not clear. It may have a direct role in providing an antifungal environment in the lesion, by being deposited on cell walls adjacent to the invading fungal hyphae. Alternatively, it may act indirectly, serving as a precursor for wyerone acid.

Wyerone acid and wyerone epoxide had similar activities against germ tube growth of *B. cinerea* and *B. fabae*. Wyerone, however, was less antifungal than these two phytoalexins but more active than wyerol, *B. cinerea* was more sensitive than *B. fabae* to all of the phytoalexins.

In vitro both *B. fabae* and *B. cinerea* were able to metabolize the phytoalexins wyerone, wyerone epoxide and wyerone acid. The products of the metabolism of these phytoalexins by *Botrytis* were identified. The metabolites produced by *B. fabae* from wyerone epoxide and wyerone acid but not wyerone

were shown to accumulate in spreading lesions caused by *B. fabae* on pods. The detection of wyerol, the metabolite produced by both fungi from wyerone *in vitro*, in restricted *B. cinerea* lesions but not in spreading lesions caused by *B. fabae* in pods suggested that wyerol is more likely to be a precursor for the other furano-acetylene phytoalexins, than a fungal metabolite. The inability to detect wyerol in spreading lesions caused by *B. fabae* in pods also indicates that *in vivo* wyerone is not metabolized by the same pathways as detected *in vitro*. Circumstantial evidence indicated a conversion of wyerone to reduced wyerone acid via wyerone acid in *B. fabae* lesions. A model illustrating the possible pathways of synthesis and of metabolism of the phytoalexins is presented.

Results suggest that the differential pathogenicities of *B. fabae* and *B. cinerea* towards the broad bean can not be explained in terms of differential rates of phytoalexin metabolism alone. Other factors, such as the rate of phytoalexin accumulation and the sensitivity of the fungi to the phytoalexins are probably also involved. The pathogenicity of *B. fabae* towards the broad bean is discussed in terms of induction and metabolism of the phytoalexins and of sensitivity of the fungus to the inhibitors.

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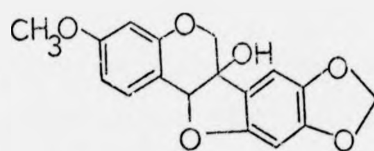
INTRODUCTION

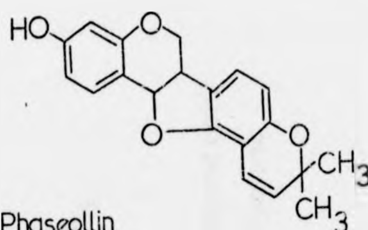
The mechanisms underlying the failure of fungi to infect plants which are not their natural hosts and the specialization of fungal pathogens to a particular host plant, have long intrigued plant pathologists. In recent years the possibility that the outcome of host/parasite interactions is determined by antibiotic agents (phytoalexins) of host origin has received increasing attention. It is well established that many host plants respond to microbial invasion by the *de novo* producion of phytoalexins (Cruickshank, 1963; Cruickshank *et al.*, 1971; Deverall, 1972; Ingham, 1972; Kuc, 1972). These compounds, which inhibit the growth of micro-organisms, are absent from healthy plants and are induced by microbial invasion. Even though the precise role of phytoalexins in disease resistance is still not entirely clear, considerable evidence indicates their involvement in the protection of plants from fungal colonization.

The *Leguminosae* has been one of the most studied families of plants with respect to the involv ment of phytoalexins in disease resistance. A number of leguminous plants produce phytoalexins which are closely related to each other having a pterocarpanoid structure (Fig.1) for example, pisatin from *Pisum sativum* (Perrin and Bottomley, 1962), phaseollin from *Phaseolus vulgaris* (Perrin, 1964), glyceollin (formerly hydroxyphaseollin, Sims *et al.*, 1971) from *Glycine max* (Burden and Bailey, 1975) and medicarpin from *Medicago sativa* (Smith *et al.*,1971). In this respect the broad bean plant appears to be anomalous since the phytoalexin characterized from *Vicia faba* is a furano-acetylenic compound, wyerone acid (Letcher *et al.*, 1970). Another antifungal compound, wyerone, the methyl ester of wyerone acid (Fig.2) has also been detected in broad bean seedlings (Fawcett *et al.*, 1968, 1969.) Although this

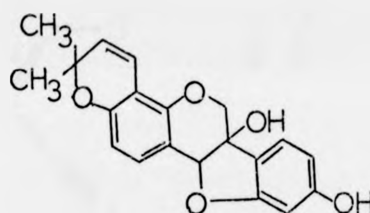
Fig. 1



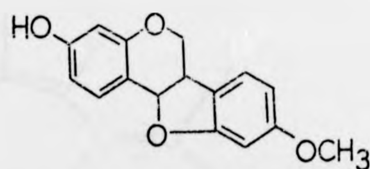
Pisatin



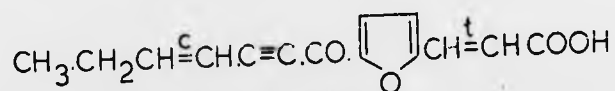
Phaseollin



Glyceollin

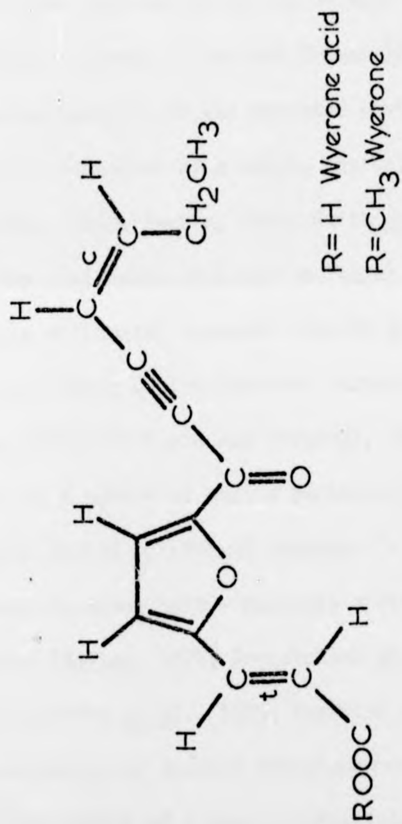


Medicarpin



Wyerone acid

Fig.2



compound was originally believed to be a preformed inhibitor (Deverall, 1972; Smith, 1973) it has since been shown that wyerone, like wyerone acid may be considered to be a phytoalexin (Fawcett et al, 1971; Keen 1972).

Only one other plant has been reported to produce acetylenic phytoalexins in response to fungal invasion. Safflower (*Carthamus tinctorus*) a member of the *Compositae* produces the polyacetylenic phytoalexins Safynol (Allen and Thomas, 1971 c) and dihydrosafynol (Allen and Thomas 1971 a).

In earlier investigations it was reported that some host plant species were characterized by the formation of a single phytoalexin (Letcher et al, 1970; Perrin and Bottomley, 1962; Perrin, 1964; Smith et al, 1971;) and attempts were made to explain the resistance of a host to fungal invasion in terms of the accumulation of a single antifungal compound (Bailey and Deverall, 1971; Christenson and Hadwiger, 1973; Cruickshank and Perrin, 1963; Cruickshank and Perrin, 1971; Higgins, 1972; Mansfield and Deverall, 1974b). Recently, however, it has been shown that in a number of host - parasite interactions more than one post - infectionally formed antifungal compound is involved (Table 1) and that the phytoalexin response in these host - parasite systems is more complicated than originally reported (Bailey, 1974; Cruickshank et al., 1974; Fuenpke and VanEtten 1975, 1976; Smith et al., 1975; VanEtten and Smith, 1975). Consequently any investigation of disease resistance mechanisms must take into account the possible involvement of a post - infectionally formed multi-component phytoalexin response.

The essence of the phytoalexin theory is that during a resistant reaction the inhibitors accumulate to antifungal concentrations around invading fungal hyphae. During a susceptible response inhibitory concentrations are not achieved and consequently the pathogenic fungus is able to colonize infected tissue. In a number of host/fungus interactions much lower concentrations

TABLE 1 Phytoalexins produced by plants characterized by more than one post infectional antifungal inhibitor.

PLANT SPECIES	PHYTOALEXIN	REFERENCE
ALFALFA (Medicargo sativa L.)	Medicarpin	Smith *et al.*, 1971
	Sativan	Ingham and Millar, 1973
BIRDS FOOT TREFOIL (Lotus corniculatus L.)	Sativin	Bonde *et al.*, 1973
	Vestitol	" " " "
BROADBEAN (Vicia faba L.)	Wyerone	Keen, 1972
	Wyerone acid	Letcher *et al.*, 197[illegible]
COTTON (Gossypium hirsutum L.)	Vergosin	Zaki *et al.*, 1972
	Hemigossypol	" " " "
	Gossypol	Bell, 1967
French Bean (Phaseolus vulgaris L.) also COWPEA (Vigna sinensis L.)*	Phaseollin	Perrin, 1964
	Phaseollidin	Perrin *et al.*, 1972
	Phaseollinisoflavan	Burden *et al.*, 1972
	Kievitone	** Smith *et al.*, 1975
	2'-Methoxyphaseollinisoflavan	Van Etten and Smith, 1975
PEA (Pisum sativum L.)	Pisatin	Perrin and Bottomley, 1962
	Maackiain	Stoessel, 1972
	Hydroxytrimethoxy)-pterocarpan	Pueppke and Van Etten, 1975
	Hydroxydimethoxy)-pterocarpan	" " " "
	Trimethoxy)-pterocarpan	" " " "
POTATO (Solanum tuberosum)	Rishitin	Tomiyama *et al.*, 1968
	Phytuberin	Varns *et al.*, 1971
	Rishitinol	Katsui *et al.*, 1971
	Lyubimin	Metlitskii *et al.*, 1971
RED CLOVER (Trifolium pratense L.)	Medicarpin	Higgins and Smith, 1972
	Maackiain	" " " "
SAFFLOWER (Carthamus tinctorus L.)	Safynol	Allen and Thomas, 1971c
	Dihydrosafynol	Allen and Thomas, 1971a
TOBACCO (Nicotiana sp.)	Glutinosone	Burden *et al.*, 1975
	Capsidiol	Bailey *et al.*, 1975
Trigonella sp.	Medicarpin	Ingham and Harborne, 1976
	Maackiain	" " "
	Vestitol	" " "
	Sativin	" " "

* - Reference Baily, 1975b

** - only detected in Fusarium solani f.sp. phaseoli infected bean hypocotyl tissue.

of phytoalexins have been recovered from tissues undergoing a susceptible response than a resistant response, at least during the earlier stages of infection (Bailey and Deverall, 1971; Christenson and Hadwiger, 1973; Cruickshank and Perrin, 1965; De Wit - Elshove and Fuchs, 1971; Bank and Paxton, 1970; Hess et al., 1971; Higgins and Millar, 1968; Keen, 1971; Mansfield and Deverall, 1974 b.) On the basis of experimental evidence differential accumulation of phytoalexins has been explained in terms of phytoalexin induction (Keen, 1971; Varns and Kuc, 1971; Bailey and Deverall, 1971) or the differential ability of virulent and avirulent fungi to metabolize the phytoalexins once formed (Higgins, 1972; Higgins and Millar, 1970; Mansfield and Widdowson, 1973; Stoessel et al., 1973; Van Etten and Smith, 1975). Detailed hypotheses concerning differential induction and metabolism are clearly summarized by Wood (1974).

Detailed comparison of the phytoalexin response in tissue undergoing resistant or susceptible reactions allows the critical evaluation of the role of phytoalexins in disease resistance. Chocolate spot disease of the broad and field bean (Vicia faba L.) caused by Botrytis cinerea Pers. and B. fabae Sard. lends itself to such a comparative investigation. Both fungi cause dark brown lesions on leaves and pods; lesions formed by B. cinerea are essentially confined to the epidermis and remain restricted to the site of inoculation, where as those caused by B. fabae spread through leaves and pods killing and blackening the tissue. (Deverall and Wood, 1961; Leach, 1955; Mansfield and Deverall, 1974a; Purkahastha and Deverall 1965a; Mastie, 1962;)

Wyerone acid which is not present in healthy pods or leaves accumulates in these tissues after infection by B. cinerea (Mansfield, 1972 Mansfield and Deverall 1974b). At B. cinerea inoculation sites in leaves, wyerone acid levels increase rapidly, at the time of fungal invasion of the epidermis, to levels

greater than that completely inhibitory to mycelial growth thus, it appears that the accumulation of wyerone acid can account for the inhibition of fungal growth in restricted lesions caused by B. cinerea.

In contrast, low levels of wyerone acid (or antifungal activity due to this phytoalexin) have been consistently detected in B. fabae lesions in leaves and pods (Deverall, 1967; Deverall et al., 1968; Deverall and Vessey, 1969; Mansfield, 1972; Mansfield and Deverall, 1974 b). Since wyerone acid appears to be induced at equal or even greater rates in lesions caused by B. fabae than B. cinerea in leaves (Deverall et al., 1968; Deverall and Vessey, 1969.) it is unlikely that lack of induction or suppression of synthesis of the inhibitor can account for the low levels of wyerone acid in B. fabae infected tissue.

Mansfield and Deverall (1974b) showed that, in leaves, the partial blackening of B. fabae inoculation sites and surrounding peripheral tissue was accompanied by an increase followed by a decrease in wyerone acid content as the tissue became completely blackened and invaded by B. fabae, suggesting that B. fabae prevented the accumulation of wyerone acid in invaded tissue by metabolizing it to an inactive form.

In bioassays B. fabae is more tolerant than B. cinerea of wyerone acid (Deverall and Vessey, 1969; Mansfield, 1972; Mansfield and Deverall, 1974b) and also detoxifies wyerone acid in vitro at an apparently greater rate than B. cinerea (Deverall and Vessey, 1969; Mansfield and Widdowson, 1973).

The accumulation of a less inhibitory 310nm absorbing substance, instead of wyerone acid, which absorbs light at 360nm, in inoculum droplets from pod seed cavities infected with B. fabae led Deverall (1967) to conclude that this substance may be a metabolite of wyerone acid produced by B. fabae. Subsequently, Mansfield and Widdowson (1973) showed that B. fabae but not B. cinerea metabolized wyerone acid in vitro to a substance identical to that

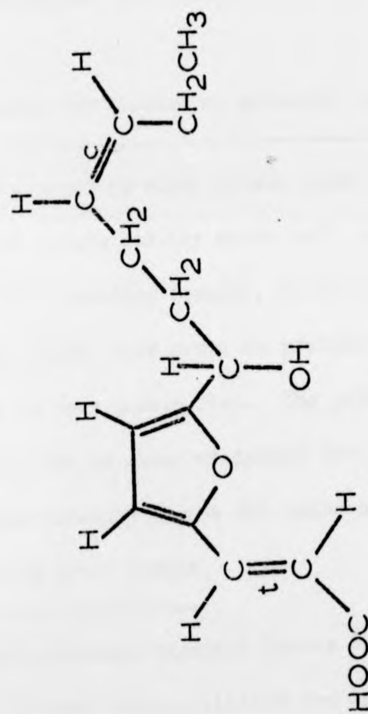
detected by Deverall (1967). This substance was isolated from inoculum droplets containing *B. fabae* spores incubated in pod seed cavities and identified as reduced wyerone acid (Fig.3), a derivative of wyerone acid in which the acetylenic bond has been saturated and the ketone group reduced to an alcohol (Mansfield and Widdowson, 1973; Mansfield *et al.*, 1973). This substance was shown to be less antifungal than wyerone acid, hence the conversion can be considered as a detoxification mechanism.

Thus, it appears that the pathogenicity of *B. fabae* towards the broad bean can be explained by the lack of wyerone acid accumulation at *B. fabae* infection sites due to the ability of this fungus to degrade the phytoalexin as rapidly as it is produced. By contrast, *B. cinerea*, perhaps due to its sensitivity to wyerone acid, is unable to degrade the phytoalexin rapidly and therefore wyerone acid accumulates in lesions to concentrations which prevent the growth of *B. cinerea*.

The role of wyerone as a phytoalexin in the broad bean/*Botrytis* interactions has received little attention. In contrast to wyerone acid, relatively high levels of wyerone accumulated in leaves after infection with *B. fabae* (Fawcett *et al.*, 1971).

The initial aim of this project was to define the phytoalexin response of *V. faba* to infection by *Botrytis* in terms of a multicomponent phytoalexin system, and to determine the relative importance of each component in providing an antifungal environment in restricted lesions caused by *B. cinerea*. Subsequently the specificity of parasitism of *B. fabae* towards *V. faba* has re-examined in relation to the multicomponent phytoalexin response.

Fig.3



Reduced wyerone acid

MATERIALS AND METHODS

1 Plant material.

A Source of seeds.

Two commercial varieties of Vicia faba L. were used throughout the project. The variety "The Sutton" was used in all quantitative studies and the variety "Aquadulce" for the characterization of the compounds reported.

B Growth of plants for studies on detached leaves.

Seeds were wrapped in moist tissue paper and germinated in the dark at 20°c. After 4 days healthy seeds with an emergent radicle were sown in John Innes No.1 potting compost, in 5½" diameter plastic pots, two seeds per pot. The plants were grown in greenhouse kept at a temperature of 20-25°c, with a 16 hour photoperiod. The pots were watered daily at soil level and care taken not to water or damage the emerging leaves. The plants developed four expanded leaves 3-4 weeks after planting.

C Source of field grown leaves.

Healthy and undamaged expanded leaves from 8-10 week old plants grown in the University gardens, were collected and transported to the laboratory in plastic sandwich boxes lined with moist tissue paper. The leaves were used immediately after collection.

D Source of pods.

When the seeds had swollen, pods were harvested from plants grown at the University gardens and used either the same day or stored at 4°C and

used the following day at the latest.

2 Fungi

A Origin and maintenance of stock cultures.

Cultures of species of Botrytis, Cladosporium herbarum and Colletotrichum lindemuthianium (Race gamma) were originally obtained from Dr. J. W. Mansfield. The cultures of Botrytis were maintained on slants of medium X (Last and Hamley, 1956), C. herbarum on V_8 juice agar and C. lindemuthianium on the medium suggested by Mathur et al.(1950). All cultures were stored at 4^oC under sterile liquid paraffin. Periodically throughout the project both B. cinerea and B. fabae were reisolated from naturally or experimentally infected tissue, since the pathogenicity of both these fungi decreased with time and subculturing.

B Production of conidia.

Conical flasks (250ml) containing 40ml of the appropriate medium were inoculated with a disc of medium bearing sporulating mycelium and spread along the surface of the medium in sterile distilled water. The cultures were grown under fluorescent tubes (Philips, blacklight) with an emission spectrum range of 410nm - 310nm and maximum at 360nm at 25 + 2^oc with a 16 hour photoperiod.

C Preparation of conidial suspensions.

Ten to 14 days old cultures of the fungi were used to prepare suspensions of conidia. Approximately 20ml of sterile distilled water was added to flasks containing the sporulating culture and conidia were released into suspension by shaking the water on the culture surface. Mycelial debris was removed by filtering through 4 layers of muslin. The suspension

used the following day at the latest.

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Cultures of species of *Botrytis*, *Cladosporium herbarum* and *Colletotrichum lindemuthianium* (Race gamma) were originally obtained from Dr. J. W. Mansfield. The cultures of Botrytis were maintained on slants of medium X (Last and Hamley, 1956), *C. herbarum* on V_8 juice agar and *C. lindemuthianium* on the medium suggested by Mathur *et al.* (1950). All cultures were stored at 4°C under sterile liquid paraffin. Periodically throughout the project both *B. cinerea* and *B. fabae* were reisolated from naturally or experimentally infected tissue, since the pathogenicity of both these fungi decreased with time and subculturing.

B Production of conidia.

Conical flasks (250ml) containing 40ml of the appropriate medium were inoculated with a disc of medium bearing sporulating mycelium and spread along the surface of the medium in sterile distilled water. The cultures were grown under fluorescent tubes (Philips, blacklight) with an emission spectrum range of 410nm - 310nm and maximum at 360nm at 25 + 2°c with a 16 hour photoperiod.

C Preparation of conidial suspensions.

Ten to 14 days old cultures of the fungi were used to prepare suspensions of conidia. Approximately 20ml of sterile distilled water was added to flasks containing the sporulating culture and conidia were released into suspension by shaking the water on the culture surface. Mycelial debris was removed by filtering through 4 layers of muslin. The suspension

collected was washed twice with sterile distilled water by centrifugation at 850g for 2 minutes. The concentration of the conidial suspension was estimated by haemocytometer counts and a suspension of the required concentration (normally 5×10^5 conidia /ml) made by dilution. Where conidia were to be suspended in a medium other than water, volumes of standard suspensions were centrifuged and the conidia resuspended in the appropriate medium.

D Isolation of fungi from infected tissue.

Excised pieces of tissue (either pod or leaf) from the edge of lesions were plated out, without being surface sterilized, on to V_8 juice agar. Sporulating mycelium was teased out and replated onto V_8 juice agar then repeatedly subcultured on medium X. The pure cultures were stored on medium X.

3 Culture media.

A Medium X.

Content:

(i)	Glucose	10g	(ii) $MgSO_4 \cdot 7H_2O$	0.5g
	Mycological peptone	2g	Distilled water	100ml
	Casein hydrolysate (acid)	3g	(iii) Oxoid agar No.3	20g
	KH_2PO_4	1.5g	Distilled water	700ml
	$NaNO_3$	6g		
	KCl	0.5g		
	Yeast nucleic acid	0.5g		
	Distilled water	200ml		

Preparation:

The solutions (i) and (ii) were prepared while (iii) was placed

in a water bath. When the agar had dissolved (i) (ii) and (iii) were mixed. Aliquots (40ml) were dispensed into 250ml conical flasks and autoclaved for 20 minutes at $1Kg/cm^2$.

B V_8 juice (acid) agar.

Content:

V_8 Juice (Campbell's soups Ltd.)	300ml
Distilled water	700ml
Oxoid agar No.3	30g

Preparation:

The agar and distilled water was placed in a steamer until the agar had dissolved, then the V_8 juice was added and the pH of the mixture adjusted to about 5.0 with NaOH. Aliquots were dispensed into medical flats or conical flasks and autoclaved as described above. Plates were poured when the bottles had cooled.

C *Colletotrichum* medium.

Content:

(i)	Glucose	2.8g	(ii) Oxoid agar No3	20g
	Mycological peptone	2g	Distilled water	900ml
	KH_2PO_4	2.7g		
	$MgSO_4 : 7H_2O$	1.3g		
	Distilled water	100ml		

Preparation:

The solution (i) was prepared while (ii) was placed in a water bath. When the agar had dissolved (i) and (ii) were mixed and autoclaved as described above.

D Synthetic pod nutrients (SPN).

Content:

Sucrose	5g
Casamino acids	380mg

KH_2PO_4	100mg
$MgSO_4$ $7H_2O$	50mg
Distilled water	1000ml

Preparation:

The pH of the mixture was adjusted with galacturonic acid to 4.0 (unless stated in the text) dispensed into appropriate vessels and autoclaved for 20 minutes at 1 Kg/cm^2.

4 Chemicals.

A Solvents for tissue extraction.

Analar grade chemicals were used throughout, however some batches of Et_2O were found to contain antifungal substances, thus the Et_2O was redistilled before use.

B Solvents for Thin Layer Chromatography (TLC).

All solvents were analar except dichloromethane and hexane (br. 60-80°) which were of Laboratory reagent grade.

Chloroform ($CHCl_3$) contained 2% EtOH.

C TLC spray reagents.

Unless other wise stated reagents were prepared according to the methods described in the booklet 'Dyeing reagents for Thin Layer and Paper Chromatography' produced by E.Merck, Darmstadt, Germany. Preparation of all reagents is summarized below for ease of reference.

(i) 2,4 - Dinitrophenylhydrazine (2,4 - DNP).

Spray solution : Solution (0.4%) of 2,4 - DNP in 2N HCl

Treatment: For distinction of the 2,4 dinitrophenylhydra zones

(DNPH) formed the above solution was sprayed consecutively with 0.2% solution of potassium hexacyano ferrate (III) in 2N HCl. After spraying saturated Keto DNPH show a blue color immediately, saturated aldehyde DNPH show an olive green color more slowly. Unsaturated carbonyl derivatives change only slowly or not at all.

(ii) Iodine vapour.

The chromatogram was placed into a chamber containing some crystals of iodine. Many organic compounds show brown spots after exposure to iodine.

(iii) Isatin - sulphuric acid.

Spray solution : 0.4g isatin in 100ml 97% sulphuric acid.

Treatment: After spraying chromatograms, they were heated to 120°c Acetylenes appear as brown or green spots (Harborne, 1973)

(iv) Lead (IV) acetate - ros aniline.

Spray solution I : 3g lead (II,IV) oxide was dissolved in 100ml acetic acid with occasional stirring until complete solution.

Spray solution II : 0.05g rosaniline base was dissolved in a mixture of 10 parts glacial acetic acid and 90 parts acetone.

Treatment: Spray with I after 4-5 minutes with II, 1,2, diols appear as pink spots on a white background.

(v) 4-nitro-aniline diazotised (p - DNA).

Spray solution: 2ml 4-nitro-aniline (0.5%)in 2NCl, 6-10ml sodium nitrite (0.5%), 8ml 20% sodium acetate. On spraying chromatograms phenolic compounds form products of various colours. (Pascal Ribereau - Gayan, 1972)

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(iii) Isatin - sulphuric acid.

Spray solution : 0.4g isatin in 100ml 97% sulphuric acid.

Treatment: After spraying chromatograms, they were heated to 120°c Acetylenes appear as brown or green spots (Harborne, 1973)

(iv) Lead (IV) acetate - ros aniline.

Spray solution I : 3g lead (II,IV) oxide was dissolved in 100ml acetic acid with occasional stirring until complete solution.

Spray solution II : 0.05g rosaniline base was dissolved in a mixture of 10 parts glacial acetic acid and 90 parts acetone.

Treatment: Spray with I after 4-5 minutes with II, 1,2, diols appear as pink spots on a white background.

(v) 4-nitro-aniline diazotised (p - DNA).

Spray solution: 2ml 4-nitro-aniline (0.5%)in 2HCl, 6-10ml sodium nitrite (0.5%), 8ml 20% sodium acetate. On spraying chromatograms phenolic compounds form products of various colours. (Pascal Ribereau - Gayan, 1972)

(vi) Picric acid.

Spray solution: 0.05m ethanolic picric acid solution

Treatment: The chromatograms were placed into a chamber containing Et_2O/EtOH/glacial acetic acid (80:20:1) for 30 minutes then into a chamber containing ammonia for 1-2 minutes. Epoxides appear as orange spots on a yellow background.

(vii) Potassium hexacyanoferrate (III) - iron (III) chloride.

Spray solution: Aqueous potassium hexacyanoferrate (III) (1%) and aqueous iron (III) chloride (2%) mixed freshly before use in equal parts.

Treatment: colours (Blue) formed were intensified by spraying chromatograms with 2N HCl.

(viii) Potassium permanganate (alkaline) ($KMnO_4$).

Spray solution: An equal volume of 5% aqueous sodium carbonate solution was added to 1% aqueous pottassium permanganate. Compounds form a yellow spot on a pink background (Harborne, 1973).

(ix) Vanillin - sulphuric acid.

Spray solution: vanillin (3g) was dissolved in 100ml absolute alcohol containing 0.5ml conc. sulphuric acid.

(Holloway and Challen, 1966)

Treatment: After spraying chromatograms they were heated to 120°C. Higher alcohols give a blue colouration.

5 Measurement of pH.

Measurements were made with a pH meter (Corning -eel model 7) or narrow range indicator papers (BDH) for small volumes (<10ml).

6 Spectral analysis.

Ultraviolet (UV) spectra were obtained on a Pye Unicam SP 1800 spectrophotometer. Infra red (IR) spectra were recorded in $CHCl_3$ solution or KBr discs with a Pye Unicam SP200G spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained with a Perkin Elmer model R32 90 MHz spectrometer for solutions in deuteriochloroform or deuteriomethanol. Mass spectra (MS) were determined with an AEI MS902 spectrometer by use of a direct insertion probe. IR, NMR and MS spectroscopy was carried out at the A.R.C. Food Research Institute, Norwich, and most spectra were analysed by Dr. D.T. Coxon.

7 Inoculation techniques.

A Cotyledons.

Broad bean seeds were germinated for 36-48 hours between wet paper towels in plastic trays at 25°c. The testas from imbibed seeds were carefully removed and undamaged cotyledons separated and placed rounded side downwards on a moist tissue paper in large plastic boxes. Cotyledons bearing bacterial rots and discoloured tissue were discarded. Cotyledons were then inoculated by flooding the upper surface with conidial suspensions with the aid of a pasteur pipette or a laboratory spray gun (Shandon power pack). Inoculated tissues were incubated in closed boxes in the dark at 18°c.

B Pods.

Pods were washed in tap water immediately after collection to remove residual soil, drained and left to dry. The clean dry pods were opened to expose the seed cavities using a clean razor blade. The seeds were discarded and the half pods cut into pieces bearing 2 or 3 seed cavities.

Before applying the conidial suspensions, seed cavities showing visible blackening due to damage or infection by contaminating organisms, or which had little endocarp tissue were discarded. The half pod pieces were placed on moist tissue in large plastic boxes and inoculated with conidial suspensions using a 10ml pipette or an automatic pipetting syringe (1ml capacity). Approximately 0.3ml conidial suspension was pipetted into each seed cavity without allowing the droplet to touch any cut pod surface. Inoculated tissues were incubated in closed boxes in the dark at 18°c.

C Leaves.

Fully expanded undamaged leaves were cut from plants with a clean sharp razor blade. The cut petioles were immediately wrapped in moist tissue paper. Detached garden grown leaves were washed thoroughly in a jet of distilled water and gently blotted dry with tissue paper. Detached leaves were placed inside transparent plastic sandwich boxes (11.5 x 17.5 x 5 cm) with tight fitting lids. The boxes were lined with tissue paper moistened with tap water. Leaf laminae were supported by plastic mesh raised above the moist tissue paper by bottle tops. This form of support was prefered to test tubes (Mansfield, 1972) since laminae were held horizontal and the number of droplets lost by run off consequently reduced. The petiole wicks were touching the moist box lining. One garden grown leaf and three greenhouse grown leaves were placed in each box.

Droplets (10 µl) of conidial suspension were placed on the adaxial leaf surface of each leaflet by means of a micrometer syringe (Agla) fitted with a hypodermic needle. Conidia tended to settle down in the suspension, so homogeneity was maintained by inverting the syringe. Droplets were applied to areas between prominent veins to prevent 'run off', there was

always at least 5mm between the circumference of each droplet. Leaves were incubated in a growth cabinet (Gallenkamp) at 18 + 1°c and illuminated for 16 hours each day by a bank of fluorescent tubes (Philips, Coolwhite).

8 Measurement of infection on leaves.

The system devised by Mansfield and Deverall (1974 a) was used to measure the degree of infection on leaves by Botrytis. This involved an estimation of the browning or blackening of the leaf tissue. Plant tissue was considered resistant if the inoculated fungus was unable to grow out of the inoculation site into adjacent uninoculated tissue.

9 Preparation and examination of infected pod tissue for light microscopy.

Pieces of infected pod endocarp tissue (c. $2mm^2$) were cut from pod seed cavities with a clean scalpel and placed in a drop of water on a clean glass microscope slide, to which a drop of cotton blue was added. A square cover slip (20 x 20 mm) was carefully placed over the tissue and gently tapped until most of the air bubbles had disappeared from the preparation.

10 Preparation of plant extracts.

A Preparation of tissues for extraction.

(i) Cotyledons.

Infection sites were excised with a sharp razor blade and collected in a preweighed beaker at -20°c. Where extraction of milligram quantities of the phytoalexins was undertaken, whole cotyledons were collectdd and extracted immediately.

(ii) Pods.

Inoculum droplets were collected from pod seed cavities using

a pasteur pipette. Large volumes were collected under suction and the collection vessel was kept in ice in the dark. After removing inoculum droplets the underlying pod endocarp was collected with a small spatula and stored at -20°c.

(iii) Leaves.

Inoculum droplets, when present, were collected using a pasteur pipette. The underlying tissue was excised with a 5mm cork borer and collected in a preweighed beaker at -20°c. After the weight of the tissue had been determined it was combined with inoculum droplets for extraction.

B Extraction of plant tissue.

Precautions were taken throughout to keep illumination to a minimum. The development of methods for extracting phytoalexins from infected tissues is described in Chapters 1 and 2. Tissue was homogenized in a Sorval omnimixer by three 15 second bursts at half speed and the homogenate centrifuged in 50ml polystyrene tubes for 5 minutes at 850g. Solvent evaporation was carried out on a thin film evaporator (Büchi, Rotavapour R) at less than 25°c *in vacuo*. Where MeOH extracts were made from tissues, the residue, after solvent evaporation, was partitioned between Et_2O and water in 250ml separating funnels. Residual water in the final Et_2O extracts was removed either by centrifuging the Et_2O extract in tapered centrifuge tubes containing a small amount of anhydrous Na_2SO_4 or by adding a small volume of EtOH which was then evaporated *in vacuo*.

Large volumes of inoculum droplets were partitioned with Et_2O in 250ml separating funnels. Smaller volumes were partitioned in test tubes (50ml), the Et_2O and water phases were agitated with a "whirly mixer".

12 Chromatography.

A Analytical chromatography.

All quantitative data was obtained from separation of extracts on

precoated analytical TLC plates (Merck, silica gel 60 F_{254}, 0.25mm thick). Extracts were applied to chromatograms using drawnout pasteur pipettes. After acending chromatography developed chromatograms were dried and examined under 254 or 366nm UV light (Universal lamp, Camag), The bands detected were marked with a pencil. When neccssary bands were eluted in MeOH after being carefully removed from plates with a microspatula. Silica gel recovered was then suspended in MeOH and centrifuged at 850g for 5 minutes. The resultant supernatant was then examined by UV absorption spectroscopy. MeOH was found to be the most efficent solvent for routine elution. Methods of quantifying the phytoalexins and their metabolites are described in the results section.

B Preparative chromatography.

Milligram quantities of the phytoalexins and their metabolies were isolated from extracts by PLC on 1.5 or 2.0mm thick layers of silica gel (Merck, GF_{254} type 60) prepared as follows. Glass plates(20 x 20 cm or 20 x 30 cm) were washed in a solution of Teepol then rinsed in distilled water. The clean dry plates were loaded onto a 'Unoplan' leveler model P(Shandon). Silica gel was weighed into an Erlenmeyer flask and distilled water added (1:2 w/v). The mixture was shaken vigorously for 90 seconds and the slurry spread onto the plates within 15 seconds after shaking. The plates were transferred in a horizontal position to a drying rack at least 5 minutes after spreading the absorbent layer, and the racks placed horizontally in a drying oven at 110°c. The plates were left in this position for not more than 10 minutes. The rack was then turned through 90° so that the final drying and activation process (45 minutes) took place with the plates vertical. The dried plates were used the same day or stored in a dessicator cabinet until required.

Both analytical and preparative chromatography was carried out in chromatography tanks lined with tissue paper soaked in the solvents used.

12 TLC plate bioassays.

Antifungal substances were detected in chromatograms of extracts by the method devised by Karlman and Stanford (1968). Spores of C. herbarum in Czapek dox liquid medium (pH5) were sprayed onto developed chromatograms and incubated at 25°c in moist chambers for 4 days. Inhibitory zones were detected as areas of white silica gel where the dark green fungus failed to grow.

13 Fractionation of pod tissue.

Infected tissue was collected and washed with sterile distilled water (1:3 w/v) three times at 4°c. The water washings and tissue were stored at -20°c. Cell walls were then isolated by a modification of the method used by English et al.(1971). The frozen tissue was crushed to a fine powder in liquid nitrogen with a mortar and pestle. This frozen powder was then macerated in a glass homogeniser (MSE) at half speed for 2 min in 3 ml of cold 100mM potassium phosphate buffer (pH7). The macerate was centrifuged for 5 minutes at 850g and the resultant supernatant collected as part of the 'buffer extract'. The pellet was macerated and centrifuged once more. The final pellet was resuspended in cold buffer solution (25ml) in a buchner funnel. This suspension was allowed to stand for 10 minutes with occasional stirring before the buffer was removed by suction. This procedure was repeated four times.

The buffer washings were followed by one wash with cold distilled water (25ml) to remove salts. The buffer and water washings were combined as 'buffer extract ', adjusted to pH5 with H_3PO_4 and stored at -20°c. This procedure removed all the chlorophyll from the tissue and microscopic observations of the residue showed that it was composed of fragments of cell wall material. The residue was extracted three times with 50ml MeOH.

Each aqueous fraction collected was partitioned between Et_2O and water (2:1 w/v) three times and the Et_2O extracts prepared for TLC as previously described. All the procedures from maceration of the tissue to solvent extraction were carried out at 4°c.

14 Bioassay techniques.

The method used was based on that of Purkaastha and Deverall (1965 b). Antifungal activity was assayed against germ tube growth by *B. cinerea* and *B. fabae*.

Glass slides were cleaned by rubbing in hot water and teepol, then rinsed in tap water and soaked overnight in Decon 90. Slides were then rinsed in tap water and finally with distilled water and dried in an oven at 150°c for 4 hours. This cleaning technique had no deleterious effects on the germination of spores or on the spread of the bioassay droplets.

The required amount of the compound to be assayed was added

in 50μl MeOH to 10ml sterile SPN solution. A control SPN solution containing 0.5% MeOH accompanied each experiment. Ten μl aliquots of the test solution were pipetted onto separate slides. Usually three separate treatments were allocated to each slide. There were three replicates for each treatment. Five μl of conidial suspension (1 x 10^5/ml) was added to each drop (giving a final concentration of 0.33% MeOH). The slides were supported on test tubes, in moist tissue paper lined sandwich boxes, and the bioassays incubated at 18°c for 18 hours in the dark.

At the end of the incubation period, the conidia were killed and stained by adding a small drop of cotton blue in lactophenol to each droplet. Percentage germination was estimated by examining 5 x 100 conidia from each treatment. Germination was considered as the production of a germ tube of any length. Germ tube lengths were measured from camera lucida drawings with a map recorder. Germ tube production by thirty-five conidia was measured for each treatment. All results were expressed as replicate means. Variation between replicate droplets was invariably low.

15 Metabolism of phytoalexins by germinating conidia.

Solutions were prepared by adding the phytoalexin in MeOH to SPN solution (final concentration of 0.1% MeOH). Sterile glass ware, equipment and conditions were maintained throughout these experiments.

The system for investigating the metabolism of phytoalexins was designed so that enough solution could be collected to yield accurate quantitative date and that the conditions affecting conidia were similar to those in the bioassay system on glass slides.

Aliquots (4.5ml) of test solutions were dispensed into 100ml conical flasks and 0.5ml of suspensions of either <u>B. cinerea</u> or <u>B. fabae</u> conidia ($5x10^5$/ml) in SPN solutions or sterile SPN solution alone added. The flasks were incubated at 18^oc in the dark without shaking. Under these coniditions, in the absence of phytoalexins both fungi grew uniformly over the surface of the flask in contact with the solution. There was no apparent stimulation of growth at the liquid/air interface suggesting that oxygen tensions were not limiting growth.

After various incubation periods, 20ml Et_2O was added to each flask and the aqueous and water phases agitated for 5 minutes using a magnetic stirrir. The two phases were allowed to settle out and the flasks were then stored at -20^oc. The Et_2O phase was decanted from each frozen aqueous phase into a 100ml evaporating flask. This extraction procedure was repeated, the two Et_2O fractions combined and the solvent removed <u>in vacuo</u>. Et_2O extracts were resuspended in MeOH for TLC. This extraction procedure removed all UV absorbing substances detected in phytoalexin solutions incubated with either fungus.

16 <u>Metabolism of phytoalexins by mycelium.</u>

The phytoalexin solutions were prepared as described in chapter 5. The required volume of SPN solution (20-100ml) was dispensed into 250ml conical flasks and one disc (5mm dia) of medium x agar bearing actively growing mycelium of either <u>B.cinerea</u> or <u>B.fabae</u> added to each flask. The cultures were incubated on an orbital incubator (Gallenkamp) at 200 rev/min. After the incubation period individual cultures were partitioned twice with equal volumes of Et_2O in separating funnels (250ml). Et_2O extracts from replicate treatments were combined, the solvent removed <u>in vacuo</u> and the residue resuspended in MeOH for chromatography.

17 Relationship between fungal growth and metabolism of phytoalexins.

Due to difficulties incurred in measuring fungal growth and phytoalexin metabolism, in phytoalexin solutions containing conidia, in the same incubation vessel a system was developed where metabolism and growth could be measured in the same incubation solutions but in different vessels. Preliminary experiments showed that the rate of phytoalexin metabolism by both B. cinerea and B. fabae in 250 ml conical flasks and in petri dishes (9cm diameter), containing 15ml phytoalexin solution, was similar. Germ tube growth in these petri dishes was virtually identical to that in 'mini' dishes containing 0.3ml phytoalexin solution (1.8cm diameter, 0.5cm deep, made by cutting off the base of thin glass bottles, 10ml capacity). Thus, it appeared that the physical conditions influencing the growth of conidia in the same solutions but in different vessles were identical and that germ tube growth in 'mini' dishes closely reflects that in 250ml conical flasks.

The method used is as follows:

Two ml of suspensions of either B. cinerea or B. fabae conidia (5×10^5 /ml) in SPN solutions were added to 18ml of phytoalexin solution, prepared as described above, in a 250ml conical flask. Aliquots (0.3ml) were removed from this solution and dispensed into 'mini' dishes. These dishes were placed in glass petri dishes on seed test thick filter paper (Whatman) which was flooded with 20ml sterile distilled water, and incubated at $18 \pm 1^{\circ}c$ alongside the conical flasks. The metabolism of the phytoalexins was followed by removing samples of the incubation solution, under sterile conditions at predetermined intervals and recording the UV absorption spectra of these solutions directly in sterile cu vettes. After each reading the sample was replaced in the incubation flask. There were two replicate flasks for each treatment. The calculation of the concentration of the phytoalexins and their

metabolites is described in Chapter 5. At intervals the conidia in the mini dishes were killed and stained by adding a drop of cotton blue in lactophenol. The growth of fungal germ tubes were estimated by camera lucida drawings of the germ tubes, viewed from the underside of the mini dishes. Three replicate dishes accompanied each treatment. Total germ tube production from thirty-five conidia were measured for each treatment. All results are expressed as replicate means.

EXPERIMENTAL WORK AND RESULTS

CHAPTER 1

Detection and characterization of phytoalexins from the broad bean plant

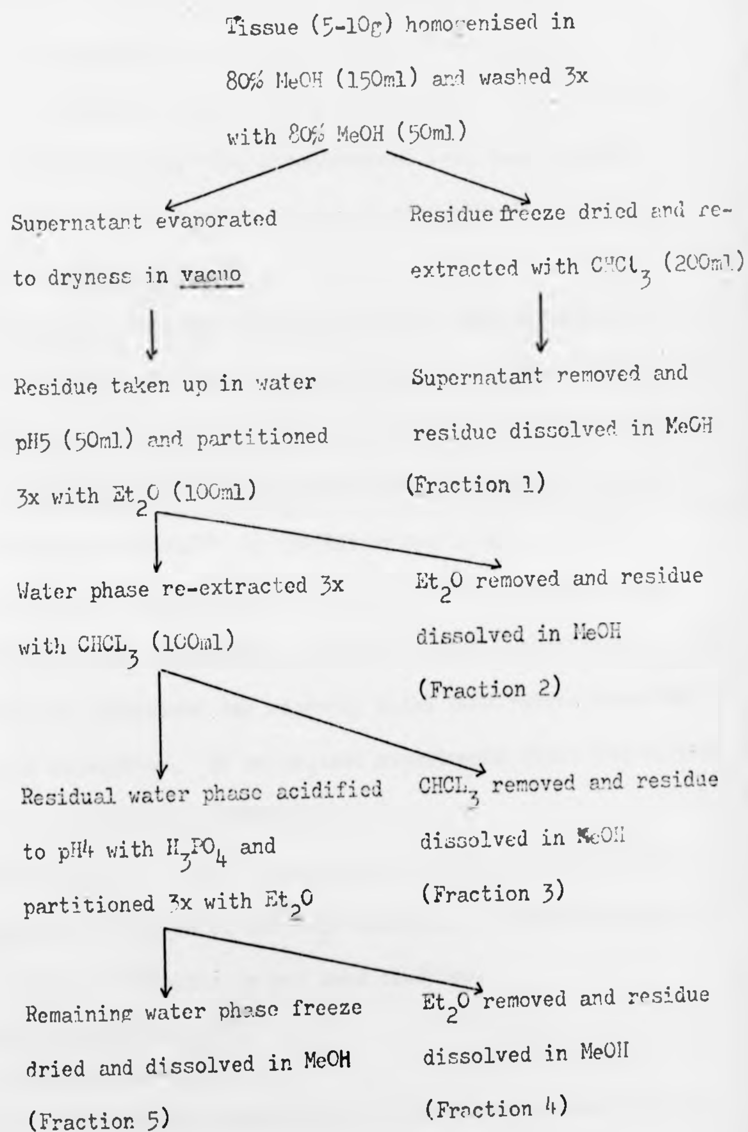
1 Isolation of phytoalexins

Two extraction procedures have been employed for the isolation of antifungal compounds from V. faba. Letcher et al. (1970) and Mansfield and Deverall (1974 b) isolated wyerone acid from the Et_2O soluble acid fraction of 80% EtOH extracts of infected tissue by TLC or PC. Wyerone, however, was prepared by Fawcett et al. (1968; 1969) from germinating broad bean seedlings by mincing and steeping the tissue in benzene. The supernatant was dried and wyerone purified by column chromatography (silica gel) after elution with benzene : Et_2O (95:5).

In order to investigate phytoalexin production by V. faba the extraction scheme outlined in Fig.4 was devised. It was considered that this extraction process would allow recovery of all the inhibitors (no matter what their polarity) present in infected tissue. The presence of inhibitors in inoculum droplets incubated on pod seed cavities was also examined. Droplets were partitioned three times with twice their volume of Et_2O (Fraction 6) then $CHCl_3$ (Fraction 7).

Broad bean seeds and pods were prepared and the exposed cotyledons and pod seed cavities inoculated with a conidial suspension of B. cinerea or sterile distilled water. Two days later inoculated tissue and inoculum

Figure 4 Extraction procedure for isolating antifungal substances from plant tissues.



Final extracts (Fraction 1-5) subjected to TLC plate bioassays

droplets from seed cavities were collected.

Fractions 1-5 from tissues, prepared as described in Fig.4 and Fractions 6 and 7 from droplets were suspended in MeOH (1ml equivalent to 5 g.f.w. tissue or 5ml droplets) and 0.2ml of each extract applied to 2cm origins on analytical TLC plates. The chromatograms were developed in Et_2O : MeOH (6:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm) and after drying bioassayed with C. herbarum.

In tissue extracts, antifungal compounds were detected only in Fraction 2, that is the Et_2O soluble fraction of the initial 80% MeOH extract. Four inhibitory bands were detected in this fraction from infected pod and cotyledon tissue. Two of these inhibitory areas corresponded to wyerone acid (RF 0.37) and wyerone (RF 0.8) No inhibitory bands were detected in pod tissue, and only a faint inhibitory band corresponding to wyerone was detected in cotyledons, after incubation with water alone. Identical results were obtained when this experiment was repeated using 100% rather than 80% MeOH for the initial extraction. In subsequent experiments described in this chapter tissues were extracted in 100% MeOH.

One inhibitor, wyerone acid, was recovered from inoculum droplets containing B. cinerea in Fraction 6, the Et_2O extract. No inhibitors were present in water droplets incubated on pod seed cavities.

2 Partition of inhibitors into Et_2O.

Deverall and Vessey (1969) showed that the compound responsible for the inhibitory activity of inoculum droplets containing B. cinerea conidia recovered from pod seed cavities behaved as an Et_2O soluble acid. This property of the inhibitor was subsequently used to allow the isolation and identification of wyerone acid as the inhibitory principle (Letcher et al., 1970). This experiment was carried out to determine if the other inhibitory

substances detected in infected pod and cotyledon tissues behave as Et_2O soluble acids.

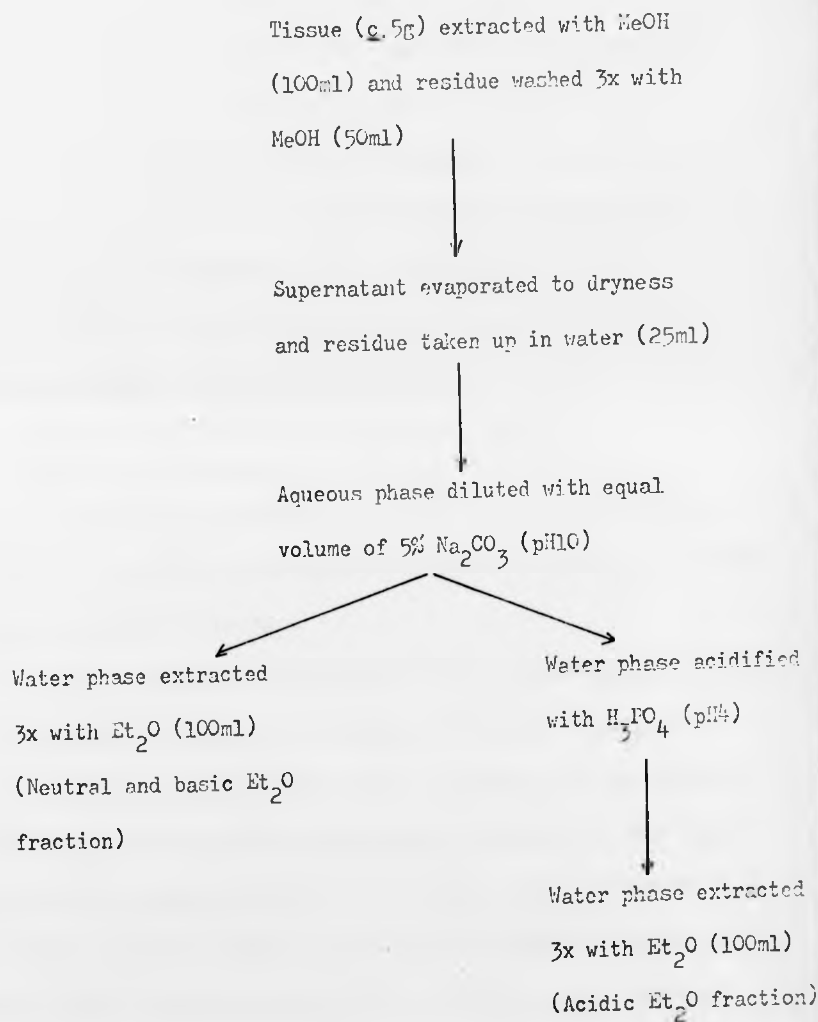
Pod and cotyledon tissue infected with B. cinerea, prepared as described above, were extracted with MeOH and separated into basic, neutral, and acidic Et_2O soluble fractions by the procedure illustrated in Fig.5. Samples of each extract (equivalent to 1 g.f.w.) were separated by TLC in Et_2O : MeOH (6:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm) and the developed chromatograms bioassayed with C. herbarum. Wyerone acid was the only inhibitor present in the Et_2O soluble acid fraction from infected pod and cotyledon tissue. Wyerone and the other inhibitors were only detected in the basic and neutral fractions, traces of wyerone acid were present in these extracts.

These preliminary experiments showed that the broad bean produced at least two inhibitory substances in addition to wyerone acid and wyerone, after infection by B. cinerea. Extraction with MeOH followed by partitioning between Et_2O and water proved to be an efficient method for isolating all the inhibitors detected in infected broad bean tissues. This extraction procedure was employed throughout this project. All of these inhibitors may be considered phytoalexins since they were either absent from, or present at very low levels in uninfected tissue. Wyerone acid was the only inhibitor that behaved as an ether soluble acid.

3 Separation of phytoalexins from pod, leaf and cotyledon tissues bearing limited lesions caused by B. cinerea.

Pods, leaves and cotyledons were prepared and inoculated with suspensions of B. cinerea conidia. Two days later infected tissue was collected and extracted with MeOH. Et_2O extracts were subjected to TLC in hexane : acetone (3:1) and the developed chromatograms bioassayed with C. herbarum

Figure 5 Separation of MeOH extracts of infected tissue into basic and neutral, and acidic Et_2O soluble fractions.



(Plate 1). The four inhibitory bands previously detected were recognised as the major inhibitors in pod, leaf and cotyledon tissue. In addition less pronounced bands of inhibition were detected at RF 0.07 in extracts of pod and leaf tissue and at RFs 0.12 and 0.16 in extracts of leaf and cotyledon tissue respectively. The inhibitory band near the solvent front was subsequently shown to originate from the Et_2O used in the preparation of the extracts.

The separation of the inhibitors was improved by developing chromatograms in hexane : acetone (2:1, 15cm) followed by $CHCl_3$: petrol (2:1, 15cm). Using these solvents the inhibitory band previously termed $PA1^1$ was resolved into two components. The characteristic appearance of the inhibitors on TLC plates when examined under UV light and the nomenclature adopted for these inhibitors are shown in Table 2.

4 Characterization of the phytoalexins by UV spectrophotometry

Pod seed cavities were prepared and inoculated with suspensions of *B. cinerea* conidia. Three days later infected tissue was collected and Et_2O extracts made as previously described.

Wyerone acid was isolated from aliquots of the extracts (equivalent to 1 g.f.w.) separated by TLC in Et_2O : MeOH (6:1). The phytoalexin was detected as a blue fluorescent band at RF 0.4 - 0.6 under UV light (366nm). The area of silica gel corresponding to this band was eluted in MeOH and the UV absorption spectrum recorded (Fig. 6A). The eluate showed a peak of absorbance at 354nm. A similar λ max at 360nm has been reported for wyerone acid by Deverall (1967) and Mansfield (1972) in 50% EtOH. After solvent removal and resuspension in EtOH wyerone acid gave λ max at 350nm as reported by Letcher *et al.*(1970) and Mansfield (1972). Following further chromatography in $CHCl_3$: MeOH (10:1) wyerone acid ran as a single band at RF 0.23 which fluoresced pale blue under UV light (366nm) and was inhibitory towards

Plate 1

TLC plate bioassay of extracts from 0.25g pod, leaf, and cotyledon tissue collected 2 days after inoculation with conidial suspensions of B. cinerea. Solvent; hexane : acetone (3:1). WA, wyerone acid; W, wyerone; PA1[1] and PA2[1], unidentified phytoalexins.

Plate 1

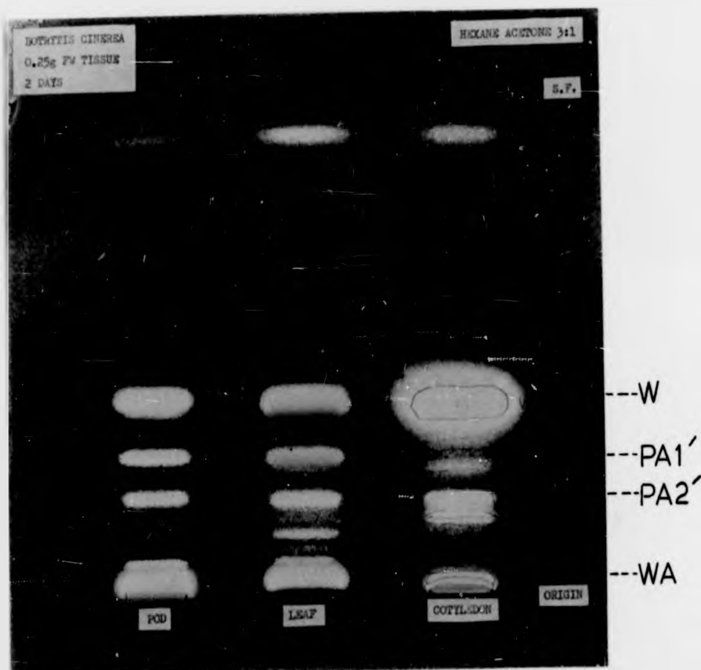


TABLE 2 Detection of inhibitory compounds, under UV light, in chromatograms of extracts from pod tissue 3 days after infection with *B. cinerea*. Solvents: Hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1).

INHIBITORY BAND	RF	UV LIGHT 366nm	UV LIGHT 254nm	NATURE
1	0.61	Bf†	Ab+	WYERONE
2	0.53	Bf	Ab	PA1
3	0.44	–	Ab	PA2
4	0.31	Bf	Ab	PA3
5*	0.12	–	Ab	PA4
6	0.06	Bf	Ab	WYERONE ACID

†-Bf, Blue fluorescent band.

+-Ab, Absorbing band

*-Inhibitor could not be detected as a single substance.

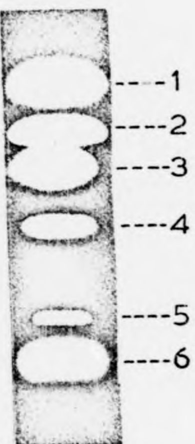
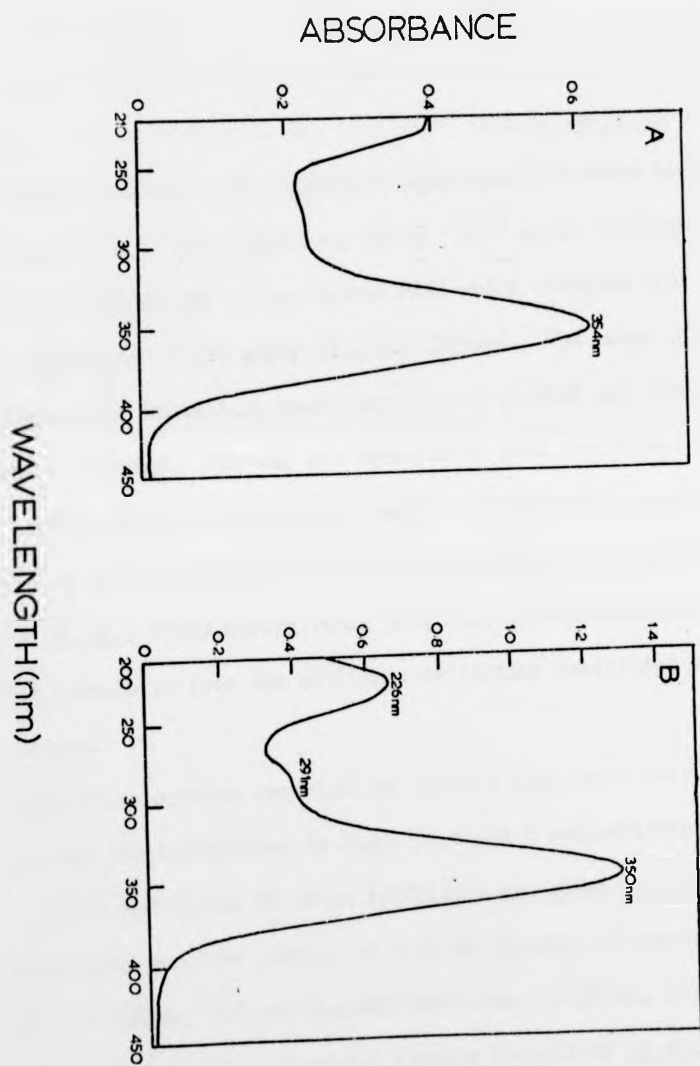


Figure 6

UV absorption spectra of wyerone acid (A) and wyerone (B) isolated from *B. cinerea* infected pod tissue.

Fig.6



C. herbarum in TLC plate bioassays. The yield of wyerone acid from infected tissue was calculated to be 58.3 µg/g.f.w. tissue.

Characterization of the other inhibitors was achieved by developing chromatograms in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). Duplicate chromatograms of extracts (equivalent to 1 g.f.w.) were developed in these solvents. One chromatogram was bioassayed with C. herbarum and the other examined under UV light. The inhibitory substances detected in the bioassay corresponded to 3 bands (wyerone, PA1 and PA3) which fluoresced blue under UV light (366nm) and to one (named PA2) which quenched the fluorescence of silica gel F 254 under UV light (254nm). The areas of silica gel corresponding to the inhibitory bands were eluted in MeOH and their UV absorption spectra recorded. PA4 was not detected in this experiment and appeared only intermittently in subsequent work. The band corresponding to wyerone (RF 0.65) gave identical spectrum to that published for synthetic wyerone (Fawcett et al., 1968) having λmax 350, 291, and 226nm (Fig.6B) The yield of wyerone calculated from the published extinction coefficent was 62.0µg/g.f.w. tissue.

TheUV absorption spectra recorded for eluates from areas corresponding to PA1, PA2, and PA3 are illustrated in Figs. 7A,B and C respectively and the absorbance maxima and yields of these inhibitors are given in Table 3. PA1 had a UV absorption spectrum similar to that of wyerone and wyerone acid with λmax at 347 and 230nm. PA2 had maximum absorbance at 312nm, identical to wyerol (Fawcett et al.,1968) and reduced wyerone (Mansfield et al.,1973). The band corresponding to PA3 appeared to be a mixture of at least two substances. The compound giving rise to the absorbance peaks at 287 and 282nm was thought to be of pterocarpanoid nature similar to other phytoalexins identified from leguminous plants.

Figure 7

UV absorption spectra of the unidentified inhibitors PA1(A), PA2(B), and PA3(C) isolated from *B. cinerea* infected pod tissue.

Fig.7

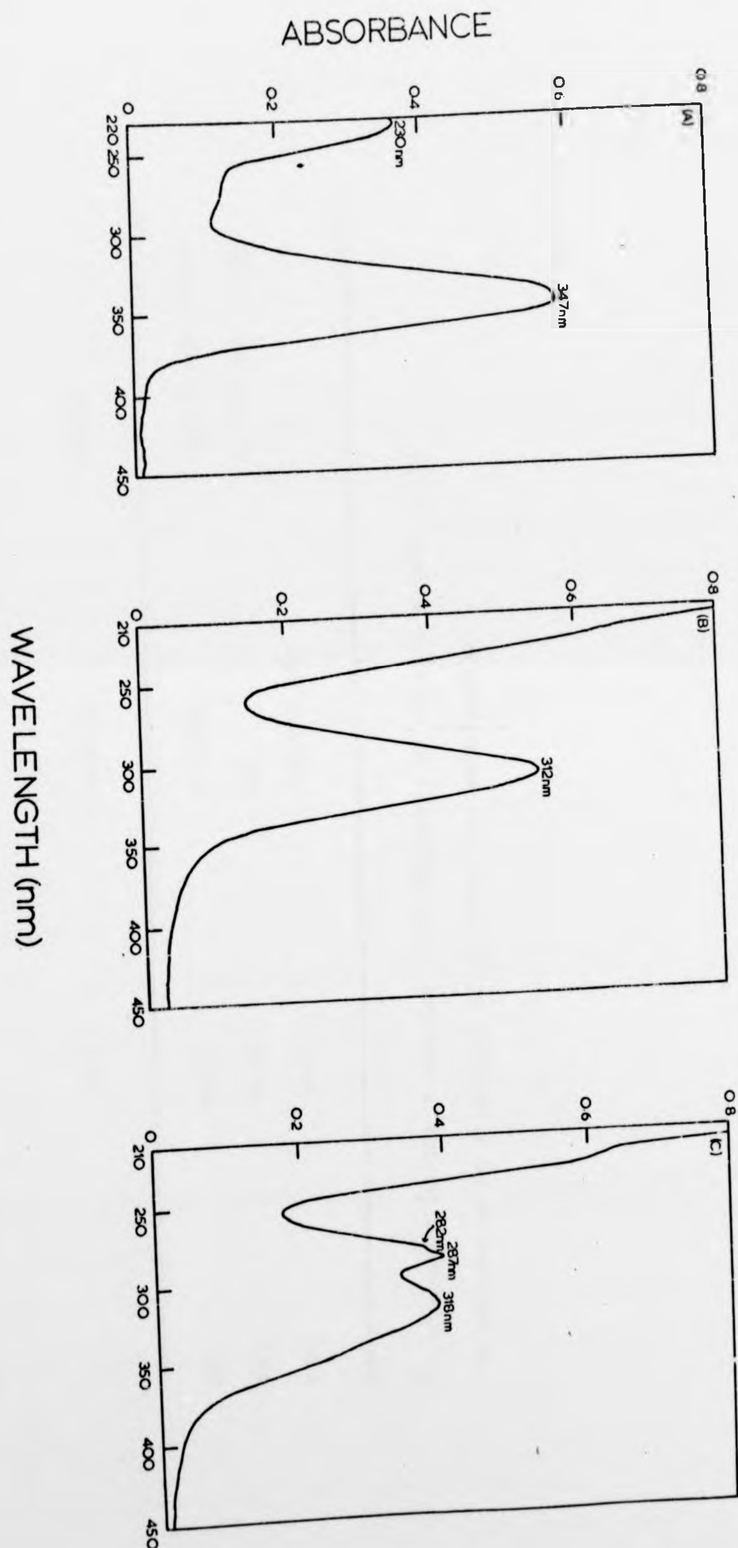


TABLE 3. Absorbance maxima and yields (AU/g.f.w.) of PA1, PA2 and PA3 isolated from B. cinerea infected pod tissue 3 days after inoculation.

	RF †	λmax(nm)	YIELD
PA1	0.55	347,230	2.8(λ max 347nm)
PA2	0.45	312	1.5(λ max 312nm)
PA3	0.33	318,287,282	-*

† - Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1)

*- Appears to be a mixture of at least two substances.

After rechromatography in pentane : Et_2O : acetic acid (75:25:1) wyerone (RF 0.9), PA1 (RF 0.85), PA2 (RF 0.8) and PA3 (RF 0.64) inhibited the growth of C. herbarum in TLC plate bioassays.

5 Characterization of the phytoalexins with spray reagents after two-way TLC separation of extracts.

Extracts were prepared from pod tissue 3 days after inoculation with B. cinerea and suspended in MeOH (1ml equivalent to 5 g.f.w.). Aliquots of the MeOH solutions (0.02ml) were applied as spots to TLC plates and developed two ways in hexane : acetone (2:1, 15cm) and CH_2Cl_2 (15cm). Seven replicate chromatograms were prepared. One was bioassayed with C. herbarum (Plate 2) and the remainder were each treated with different spray reagents after examination under UV light.

Wyerone, wyerone acid and the unidentified inhibitors PA1 and PA2 ran as single spots. However, the two-way development separated PA3 into three antifungal compounds (PA3 a, b, and c.)

All the inhibitors were visualized after treatment with the general spray reagents, $K_3Fe(CN)_6$: $FeCl_3$, $KMnO_4$ and iodine vapour as blue, yellow (on a pink background) and brown spots respectively. The colours which developed at spots corresponding to the inhibitors after spraying the chromatograms with isatin (acetylenes), 2,4 - DNP (keto groups) and p-DMA (Phenolics) are recorded in Table 4.

Visualization of the inhibitors with spray reagents suggested that PA1, PA2 and PA3b were acetylenic, possible related to wyerone where as PA3a appeared to be phenolic in character.

Plate 2

TLC plate bioassay of extracts from 0.1g pod tissue collected three days after inoculation with conidial suspensions of B. cinerea. The chromatogram was developed in two directions using dichloromethane (A) and hexane : acetone, 2:1 (B) as solvents. WA, wyerone acid; W, wyerone; PA1, PA2, PA3 (a,b and c), unidentified phytoalexins.

Plate 2

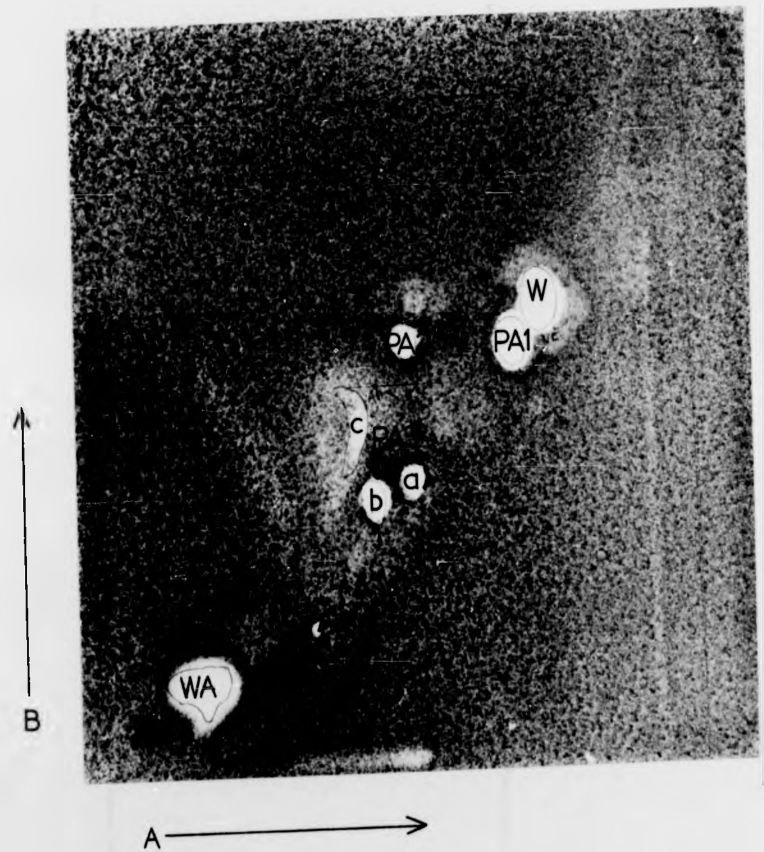


TABLE 4 Visualization of phytoalexins with spray reagents on TLC plates.

	ISATIN	2,4 - DNP	p- DNA
WYERONE	BROWN	BROWN/GREEN	-
PA1	GREEN	OLIVE GREEN	BROWN
PA2	BROWN	BROWN	-
PA3a	-	BROWN	YELLOW
PA3b	YELLOW	BROWN	-
PA3c	-	-	-
WYERONE ACID	BROWN	BROWN	-

6 Characterisation of PA3 by UV spectrophotometry.

The previous experiments suggested that PA3 was a mixture of three inhibitory compounds, one of which appeared to be a wyerone derivative and another a phenolic substance.

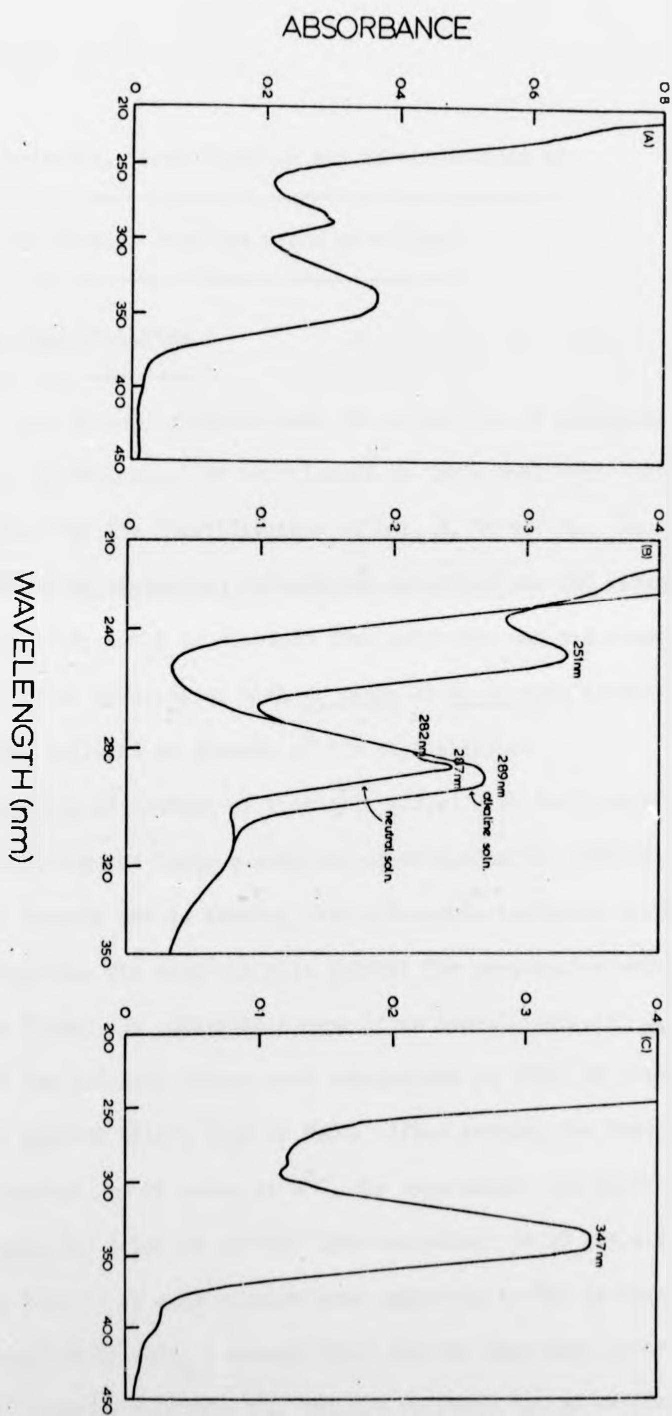
PA3 (Fig.8A) eluted from chromatograms of extracts of B. cinerea infected pod tissue developed in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) was resolved into two major inhibitory components by developing chromatograms twice in $CHCl_3$. A band at RF 0.34 corresponding to the p-DMA positive substance (PA3a) quenched the fluorescence of silica gel under UV light (254nm), where as the other inhibitor was detected by its blue fluorescence under UV light (366nm) at RF 0.29 (PA3b). The UV absorption spectra of the separated inhibitors were obtained after elution in MeOH. PA3a (Fig.8B) had a spectrum similar to the pterocarpanoid type phytoalexins (λmax 287, shoulder 282nm) and also exhibited a bathochromic shift in alkaline solution (λmax 251 and 289nm). The UV absorption spectrum of PA3b (Fig 8C) was identical to that recorded for PA1 (λ max 347nm). After rechromatography of these eluates in $CHCl_3$ the purified substances inhibited the growth of C. herbarum in TLC plate bioassays.

It was concluded that PA3 was composed of two major inhibitory components. One (PA3a) being pterocarpanoid in nature and the other (PA3b) being acetylenic and possible related to PA1. The third inhibitory component (PA3c) recognized in the previous experiments was not detected and may have been present in only trace amounts.

Figure 8

UV absorption spectra of the inhibitor PA3(A) isolated after TLC of extracts from *B. cinerea* infected pod tissue using hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) as solvents, and of its components, PA3a, in neutral and alkaline solution (B), and PA3b(C) after TLC as described in the text.

Fig8



CHAPTER 2

Isolation, identification and quantification of phytoalexins from the broad bean plant

1 Isolation and Identification

Methods were devised which allowed the collection of milligram quantities of the phytoalexins for experiments on their antifungal activity and metabolism and also for the identification of FA1, 2, 3a and 3b. In preliminary time course studies on phytoalexin accumulation, TLC bioassays showed that high yields could be obtained from cotyledon and pod tissue collected 6 days after inoculation with B. fabae or B. cinerea respectively. These tissues were utilized as sources of the phytoalexins.

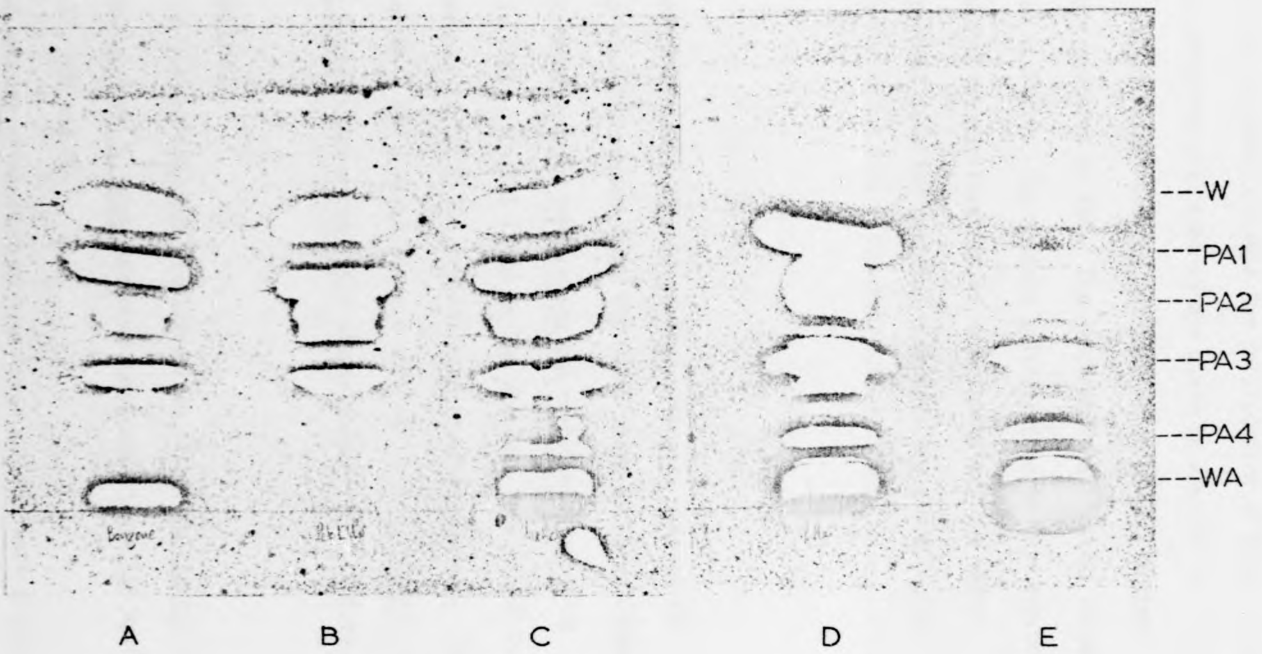
The extraction of samples of tissue $>$10 g.f.w. with MeOH proved ineffiœnt and also removed large quantities of contaminating material. The first experiment carried out to improve the phytoalexin isolation procedure was therefore, to determine the most suitable solvent for preparative extraction. Cotyledon tissue (100g) was collected 6 days after inoculation with B. fabae. Samples (20g) of the infected tissue were homogenised in 100ml of; benzene, petrol, hexane : acetone (2:1), Et_2O or MeOH. After soaking the homogenate in the respective solvent for 24 hours at $4^{o}C$, the supernatant was decanted off, evaporated *in vacuo* and taken up in MeOH (1ml equivalent to 10 g.f.w.).

Aliquots (0.1ml) of each extract were subjected to TLC in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) and the developed chromatograms bioassayed with C. herbarum (Plate 3). All the solvents tested extracted more of the unidentified inhibitors (based on the intensity of the inhibitory band)

Plate 3

TLC plate bioassays of extracts from 1g cotyledon tissue collected 6 days after inoculation with *B. fabae.* The tissueswere extracted with benzene (A), petrol (B), hexane : acetone, 2:1 (C), Et_2O (D), and MeOH (E). Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). WA, wyerone acid; W, wyerone; PA1, PA2, PA3 and PA4 unidentified phytoalexins.

Plate 3



than MeOH, Et_2O being the most efficient. It was decided that Et_2O would be used for large scale preparation of the inhibitors.

Attempts were made to separate the phytoalexins from Et_2O extracts by both PLC and gel filtration (LH20 sephadex). The use of LH20 sephadex gave mixed success but was most suitable for purification of PA3a. Details of the experiments performed with sephadex are given in Appendix 1. Milligram quantities of the phytoalexins used for identification and/or further experimentation were prepared by PLC. The preparative extraction procedure devised is outlined in Fig.9. The various modifications to this extraction procedure required for the purification of each inhibitor, and where appropriate the spectral data for purified compounds are given in the following sections.

A Wyerone.

(i) Isolation.

In chromatograms (PLC1, Fig.9) of Et_2O extracts, wyerone was detected as a deep blue fluorescent band (RF 0.86) under UV light (366nm). After elution, the crude wyerone preparation was rechromatographed by irrigation twice in $CHCl_3$: petrol (2:1) on PLC plates. Wyerone (RF 0.6) was eluted as described for PLC1 and crystallized from cyclohexane. Yields of 10.4 - 16.0 and 164 - 380 mg/Kg.f.w. were obtained from B. cinerea infected pod tissue and B. fabae infected cotyledon tissue respectively, 6 days after inoculation.

(ii) Spectral analysis.

Samples of wyerone (Fig.10A) isolated from either B. cinerea infected pod tissue (2.5 kg.f.w.) or B. fabae infected cotyledon tissue (15 kg.f.w.) were examined by IR, NMR and MS.

Spectra of wyerone obtained from both these sources were virtually

Figure 9 Preparative extraction procedure for the phytoalexins from infected tissue

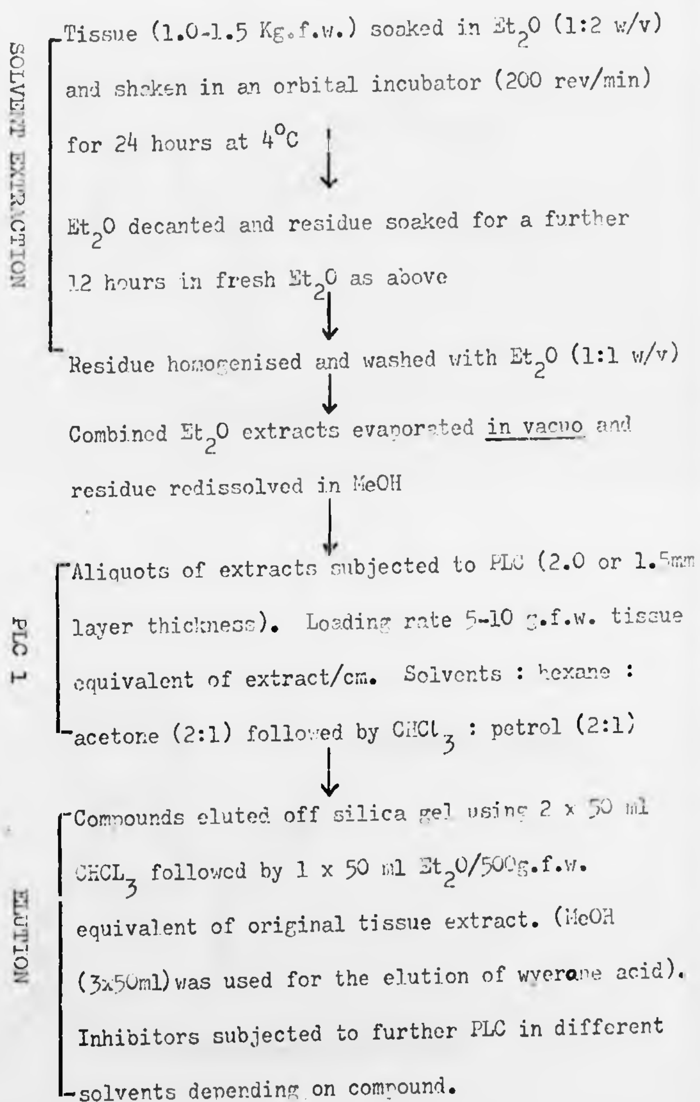
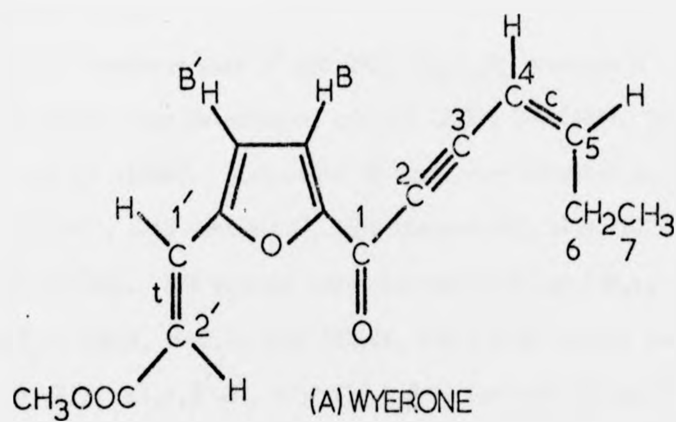
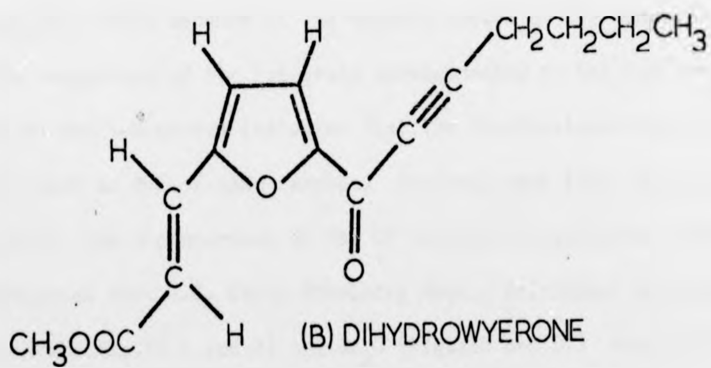


Fig.10





identical to that reported for synthetic wyerone (Fawcett et al., 1968) and wyerone isolated from broad bean shoots infected by Phytophora megasperma var sojae (Keen 1972).

The mass spectrum gave M^+ 258.0915 ($C_{15}H_{14}O_4$ requires M^+ 258.0892) prominent fragments were detected 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$\overset{t}{=}$CH). NMR signals were observed at δ 1.10 (3H,t, 7-H), 2.46 (2H,M,6-H), 3.79 (3H,s, $-OCH_3$), 5.67 (1H,dt, 4-H), 6.36 (1H,dt, 5-H), 6.59 (1H,d,2^1 -H), 6.73 (1H,d,β'-H), 7.32 (1H,d,β-H) and 7.45 (1H,d,1^1 -H) and the UV spectrum gave λ max (EtOH or MeOH) at 351, 291, and 226nm. The presence of a dihydrocontaminant (Fig.10B) m/e 260 (19%) was indicated in the mass spectrum as previously reported for natural wyerone (Fawcett et al., 1968). The percentage of dihydrowyerone could not be ascertained by comparison with peak intensities in mass spectra of synthetic material (Fawcett et al., 1968) because of the dynamic nature of the measurement. In NMR spectra comparison of the integrals corresponding to the two protons 1^1 and β H and to the 4-H proton indicated that the dihydro-impurity may have composed as much as 20% of some samples. However, much lower percentages were suggested from a comparison of the UV spectra of synthetic dihydrowyerone (obtained from R.O. Cain, Chemistry Dept., Stirling.) and natural wyerone in MeOH (Fig.11 A and B) and Et_2O (Fig.11C and D.) Ross (1970) used the differences in the UV spectra of these substances in Et_2O to calculate the ratio of wyerone and dihydrowyerone in mixtures, however, no dihydrowyerone could be detected in wyerone samples by UV spectrophotometry in either MeOH or Et_2O. Similarly no dihydro-contaminant could be detected by comparing the UV spectral changes obtained by the successive addition

WAVELENGTH (nm)

Figure 11

UV absorption spectra of natural wyerone and synthetic dihydrowyerone in MeOH and Et_2O.

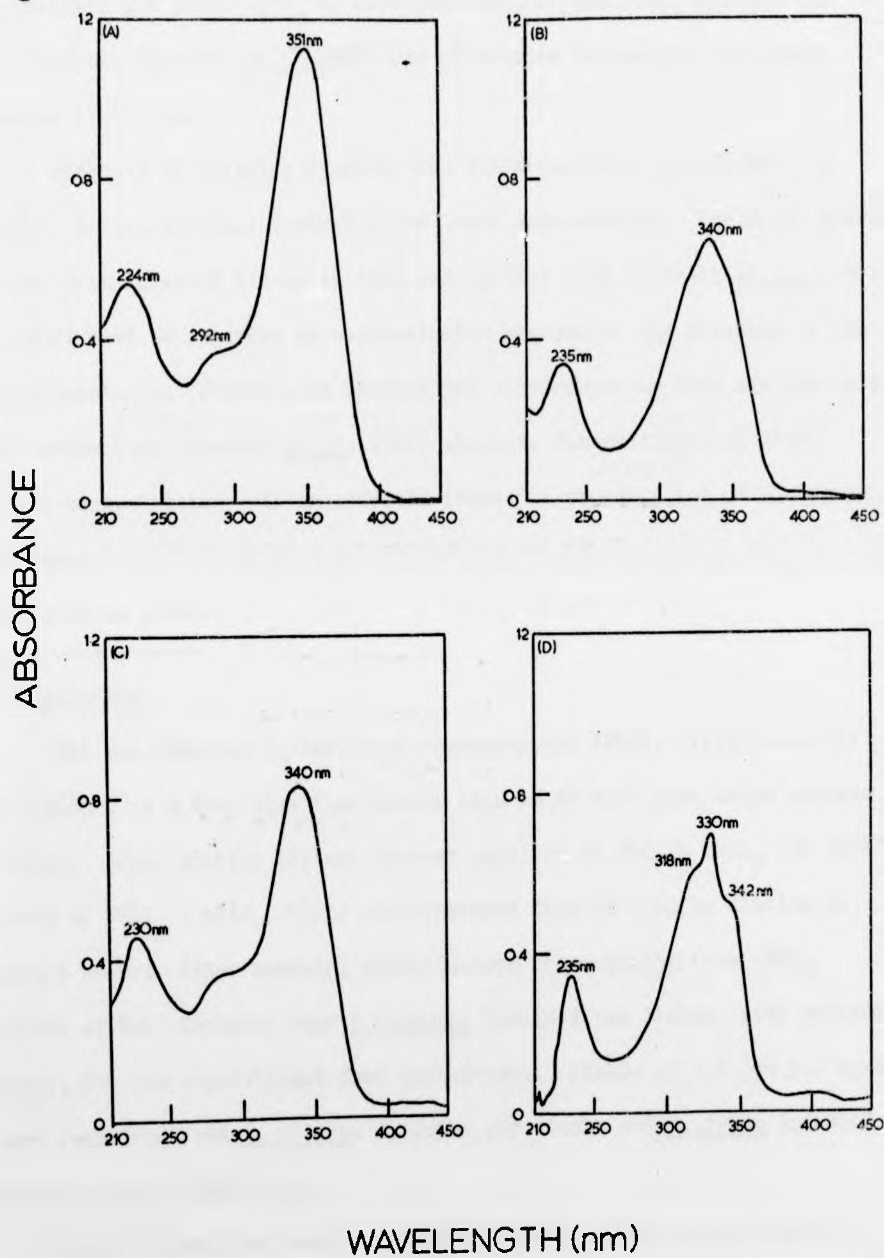
A. wyerone in MeOH

B. dihydrowyerone in MeOH

C. wyerone in Et_2O

D. dihydrowyerone in Et_2O

Fig.11



of piperidine and conc. H_2SO_4 to EtOH solutions of synthetic wyerone and dihydrowyerone (Fawcett et al.,1968) and of wyerone isolated by the above procedure (Table 5).

Attempts to separate wyerone from dihydrowyerone by TLC, HPLC or GLC (D.T. Coxon, personal communication) were unsuccessful. Yields of wyerone obtained from infected tissue in this and earlier work (Fawcett et al., 1971; Keen,1972) must be regarded as overestimates because of the presence of the dihydro-homologue. However, as wyerone and dihydrowyerone have similar anti-fungal activities (Fawcett et al.,1968) absolute determination of their relative concentrations within infected tissue is probably not of biological importance.

B Phytoalexin 1(PA1).

(i) Isolation.

PA1 was detected in developed chromatograms (PLC1, Fig.9) under UV light (366nm) as a deep blue fluorescent band at RF 0.78 just below wyerone (RF 0.86). After elution PA1 was further purified by PLC in $CHCl_3$ (2% EtOH) followed by $CHCl_3$: petrol (2:1) and recovered from RF 0.65 by elution as described above. After removing contaminating chlorophylls from $CHCl_3$ solutions of PA1 isolated from B. cinerea infected pod tissue, with activated charcoal, PA1 was crystallized from cyclohexane. Yields of 1.6 and 5.2 mg/Kg.f. w. were recovered from B. cinerea infected pod tissue and B. fabae infected cotyledon tissue respectively.

The UV absorption spectra of PA1 from both sources were identical (Fig.12) having maximum absorbance at 347 and 238nm. On TLC plates the samples of PA1 isolated, ran as single spots at identical RF values after development in $CHCl_3$: petrol (2:1, RF 0.12) and CH_2Cl_2 : MeOH (10:1, RF 0.92).

TABLE 5 UV light absorbance changes brought about by the successive addition of piperidine and conc. H_2SO_4 to EtOH solutions of synthetic wyerone*, dihydrowyerone*, and natural wyerone.

	λ_{max} (nm) (Rel.E)		
	EtOH	+PIPERIDINE	+PIPERIDINE +H_2SO_4
SYNTHETIC WYERONE	351.5(0.65)	397(0.6)	400(0.33)
	292(0.42)	307(0.54)	336(0.41)
	224(0.54)		324(0.45)
			294(0.54)
DIHYDROWYERONE	340(0.91)	389(0.89)	394(0.27)
	235(0.5)	305(0.85)	336(0.73)
			313(0.76)
			295.5infl(0.36)
NATURAL WYERONE	351(0.87)	398(0.75)	400(0.27)
	291(0.52)	307(0.63)	336(0.67)
	226(0.53)		324(0.68)
			294(0.53)

*- From Fawcett *et al.* (1968)

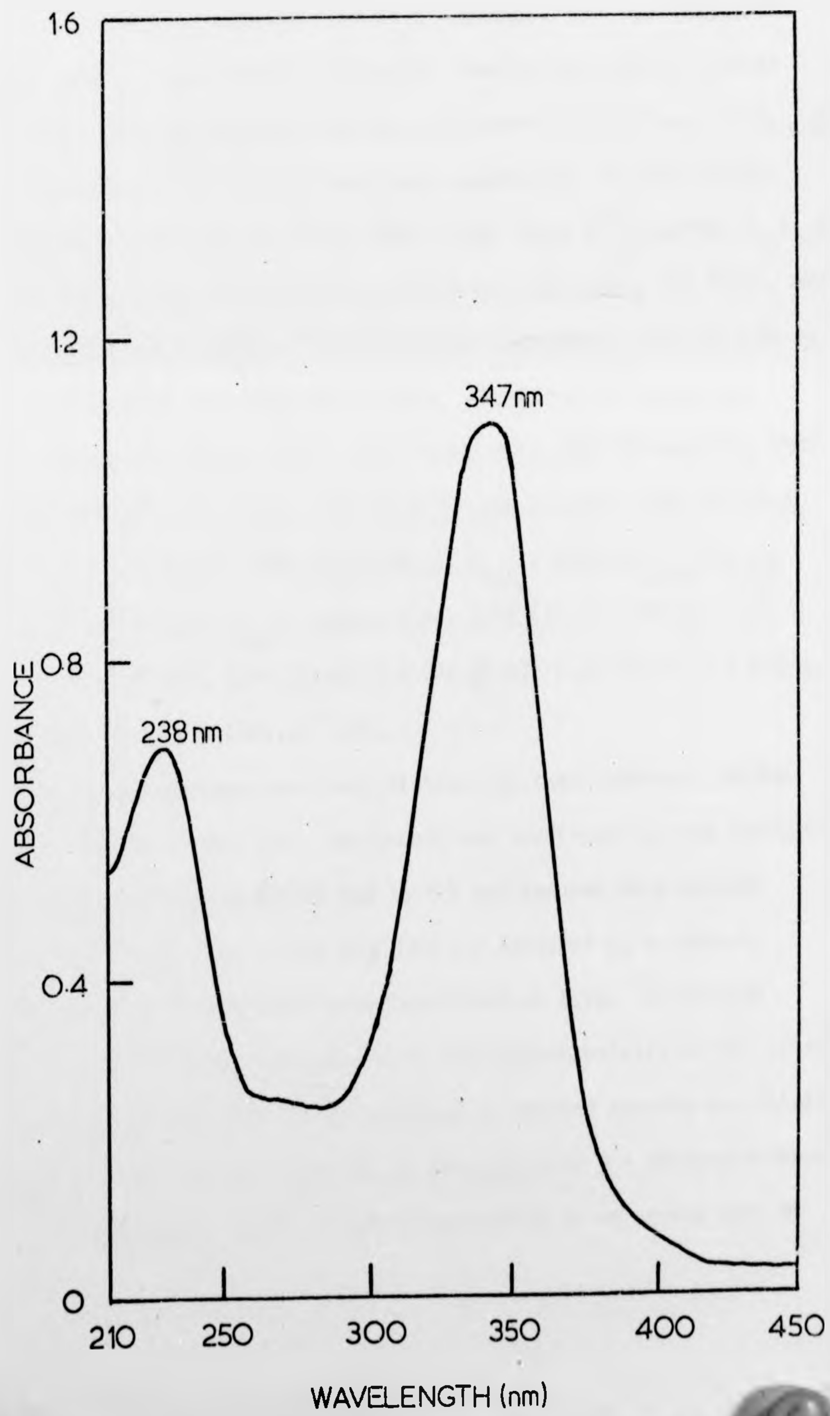
Figure 12

UV absorption spectrum of wyerone epoxide (PA1).

Figure 12

UV absorption spectrum of wyerone epoxide (PA1).

Fig. 12



TLC plate bioassays showed that the substance isolated was the active consituent of the PA1 inhibitory band detected in extracts of infected tissue.

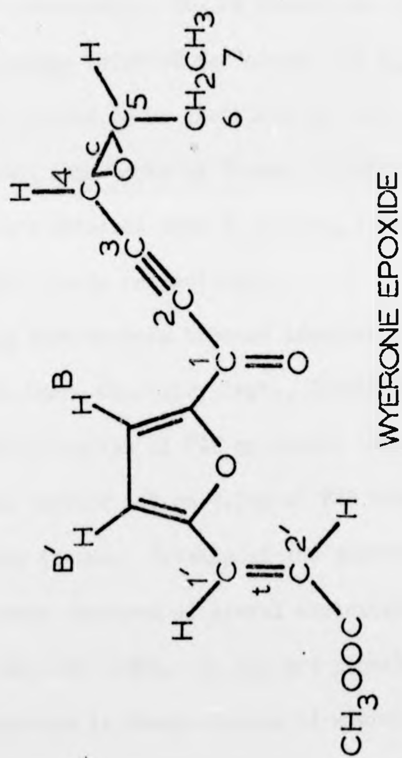
(ii) Spectral analysis.

After recrystallization from Et_2O - hexane. PA1 was identified as wyerone epoxide (m.p. 74-76°C) (Fig.13). Samples of wyerone epoxide isolated from either B. cinerea infected pod tissue (2.5 Kg.f.w.) or B. fabae infected cotyledon tissue (15 Kg f.w.) were examined by IR, NMR and MS.

The mass spectrum gave M^+ 274 (74%) (HRMS found M^+ 274.0856, $C_{15}H_{14}O_5$ requires 274.0842) and prominent fragments at m/e 245 (31%), 243 (34%), 242 (47%), 151 (53%) and 29 (100%). UV absorption occurred in EtOH or MeOH at λ max 347 (ϵ 28,100) and 238nm (ϵ 14,300). Diagnostic IR bands were detected at ν max (KBr) 2220 (C≡C), 1706 (Ester CO), 1640 (Ketone CO), 1500 (Furan) and 889 cm^{-1} (Epoxide). NMR signals were observed at δ 1.15 (3H,t, J=7.5Hz, 7-H), 1.76 (2H,m, 6-H), 3.21 (1H,dt, $J_{4,5}$ = 3.9Hz $J_{5,6\alpha}$ = $J_{5,6\beta}$ = 6.0Hz, 5-H), 3.66 (1H,d, $J_{4,5}$ = 3.9Hz, 4-H), 3.83 (3H,s, - OCH_3), 6.64 (1H,d, J = 16Hz, 2^1 -H), 6.75 (1H,d, J = 7Hz, β'-H), 7.37 (1H,d, J = 3.5Hz, β-H) and 7.48 (1H,d, J = 16Hz, 1^1 -H).

The epoxide protons were very distinct in their chemical shifts and coupling constants and their assignment was confirmed by spin decoupling experiments. The signal at δ 3.21 due to 5-H was reduced to a triplet (J = 6.0Hz) by irradiation of 4-H at δ 3.66 and appeared as a doublet (J = 3.9Hz) when the 6-H protons were irradiated at 1.76. The epoxide coupling constant of 3.9Hz indicated that the stereochemistry of the epoxide group was cis. Confirmation of the identity of wyerone epoxide was obtained by its partial synthesis from wyerone by reaction with p - chloroper-benzoic acid (Hargreaves et al., 1976) and by visualization as an orange spot on

Fig. 13



TLC plates after spraying with picric acid reagent, which is specific for epoxides.

C Phytoalexin 2 (PA2).

(i) Isolation and identification.

PA2 was detected on chromatograms (PLC1, Fig.9) as a dark quenching band under UV light (254nm) at RF 0.59. After elution PA2 was further purified by PLC twice using Et_2O : petrol (2:1) as solvents, and recovered from RF 0.51 as described previously. The UV absorption spectra of PA2 recorded in MeOH from B cinerea infected pod tissue and B.fabae infected cotyledon tissue (Fig.14A) proved to be identical to that of wyerol (Fawcett et al., 1968) having maximum absorbance at 312nm. Yields of 1.2 and 8.3 mg/Kg.f.w. of purified PA2 were obtained from B. cinerea infected pod tissue and B. fabae infected cotyledon tissue respectively.

The inhibitor from both sources behaved identically to synthetic wyerol (obtained from R.O. Cain, Chemistry dept., Stirling) in various TLC solvent systems. The identification of PA2 as wyerol (Fig.15A) was confirmed by NMR, IR and MS analysis carried out on 4.5mg of PA2 isolated from B. fabae infected cotyledon tissue. Details of the spectra of wyerol are given in Chapter 5. The mass spectrum of wyerol indicated the presence of dihydrowyerol (Fig.15B), m/e 262 (63%). It was not possible to determine the amount of dihydro-contamination in these samples of wyerol by UV spectrophotometry since dihydrowyerol has an identical UV absorption spectrum to wyerol (Fig.14B). Neither substance showed any changes in UV absorption on the successive addition of piperidine and H_2SO_4. Comparison of the NMR integrals as described for wyerone (p33) indicated that the dihydro-contamination did not compose more than 10% of the samples.

Figure 14

UV absorption spectra of natural wyerol, PA2(A) and of synthetic dihydrowyerol (B).

Fig.14

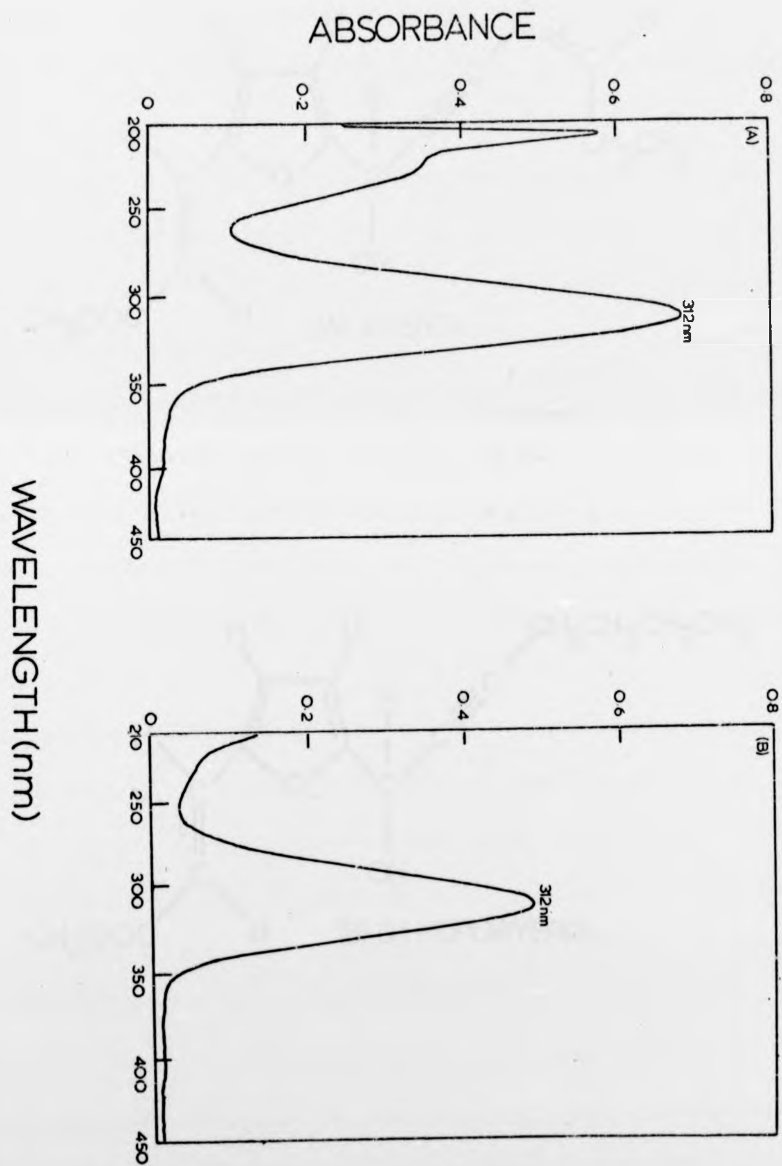
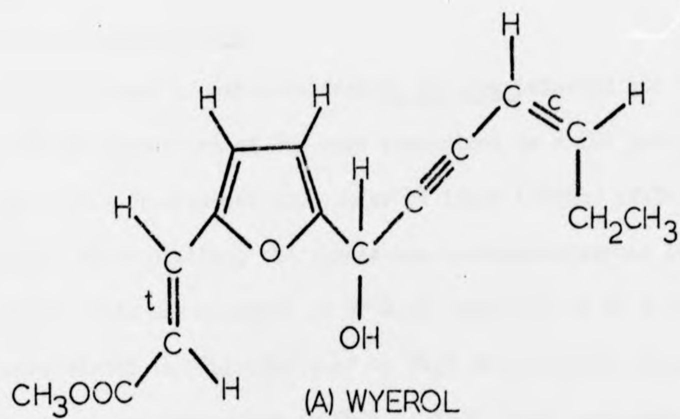
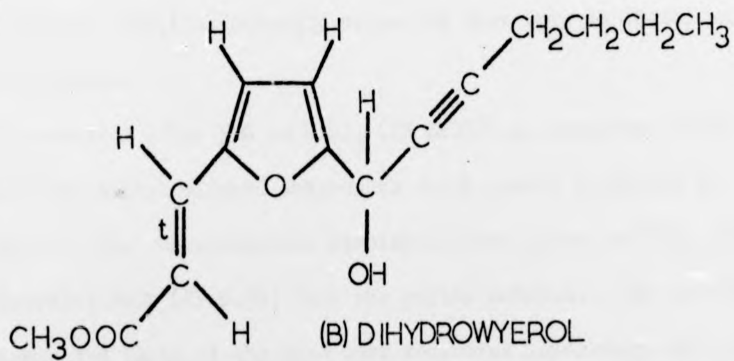


Fig. 15



(A) WYEROL



(B) DIHYDROWYEROL

D Phytoalexin 3 (PA3)

(i) Isolation of PA3a and PA3b

In chromatograms of extracts from B. cinerea infected pod tissue (PLC1, Fig. 9) the components of PA3 were recognised as a DMA positive band (PA3a) and as a blue fluorescent band under UV light (366nm) (PA3b) at RF 0.31 - 0.38. After elution, the eluate was rechromatographed in $CHCl_3$ (2% EtOH) twice. PA3a was detected at RF 0.35, and PA3b at RF 0.28, both substances were eluted in $CHCl_3$ followed by Et_2O as previously described.

PA3a was rechromatographed in $CHCl_3$: MeOH (10:1) and detected as a single quenching band under UV light (254nm) at RF 0.6. After elution and removal of chlorophyll contamination from $CHCl_3$ solutions with activated charcoal, PA3a was obtained as a white solid (7.6 mg/Kg.f.w.). The UV absorption spectrum of purified PA3a and its bathochromic shift on addition of 0.1ml of 1M NaOH (Fig.16A) strongly suggested that this inhibitor was of a pterocarpanoid nature.

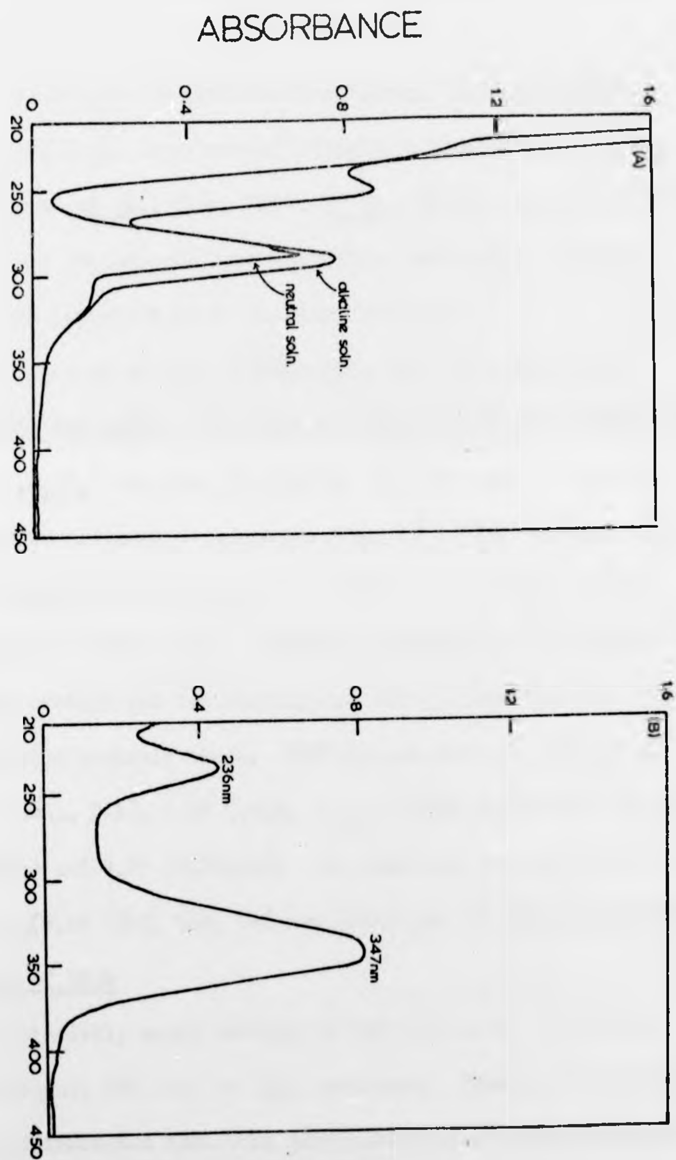
PA3b recovered after PLC in $CHCl_3$ (2% EtOH), as described above, was contaminated with yellow coloured substances which proved difficult to remove by further PLC. Chromatograms developed three times in $CHCl_3$ (2% EtOH) partially separated PA3b (RF 0.34) from the yellow material. The uncontaminated and the contaminated parts of the band were recovered separately and eluted as above. The contaminated eluate was subjected to further PLC in $CHCl_3$ (2% EtOH) three times and the isolation procedure repeated. Samples of purified PA3b were combined and the UV absorption spectrum of the phytoalexin recorded (Fig.16B). Approximately 1mg (81 AU at λmax 347nm) of PA3b was recovered. Both substances inhibited the growth of C. herbarum in TLC plate bioassays.

Figure 16

UV absorption spectrum of medicarpin, PA3a, in neutral and akaline solution (A) and of PA3b(B).

Fig.16

ABSORBANCE



WAVELENGTH (nm)

tral

(ii) Identification of PA3a

After crystallization from Et_2O - hexane, the inhibitor was identified as medicarpin ((-) 3-hydroxy-9-methoxypterocarpan) (Fig.17A) on the basis of the following data:-

Physical constants for the phytoalexin; melting point (129-130°C); $[\alpha]_D^{22}$ - 214° (C,0.19 in $CHCl_3$), corresponded closely to the values reported in the literature (Harper et al., 1965; Smith et al., 1971). UV, MS, and NMR spectra obtained for the phytoalexin were virtually identical to spectra obtained for medicarpin (supplied by V. Higgins, Toronto).

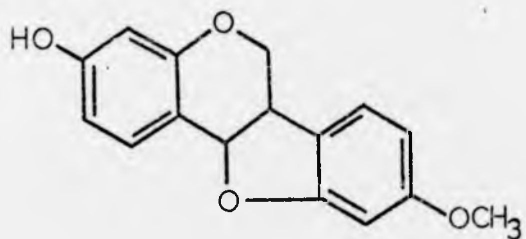
UV absorption occured in MeOH at λmax 210, 276, 282, and 286nm (Log ε 4.53, 4.14, 3.38 and 3.94). The mass spectrum gave M^+ 270 (100%), HRMS found M^+ 270.0897 ($C_{16}H_{14}O_4$ requires 270.0892). The NMR spectrum contained a singlet which could be assigned to a methyl group (δ 3.77). Signals from the remaining 10 protons could be divided into three sub-spectral systems which cannot be analysed by first order analysis (Fig.18); an ABMX system from the four heterocyclic protons and two overlapping AKM systems from the protons on the two trisubstituted aromatic rings. NMR signals were observed at δ 7.40 (1H,d, $J_{1,2}$ = 8.0Hz, H-1), 7.14 (1H,d, $J_{7,8}$ = 8.7Hz, H-7), 5.50 (1H,d, $J_{6a,11a}$ = 6.3Hz, H-11a) and 4.25 (m,H-6eq). The remaining protons are in the envelopes centered at δ6.65 (H-2, H-4, H-8 and H-10) and 3.6 (H-6ax and H-6a).

(iii) Identification of PA3b

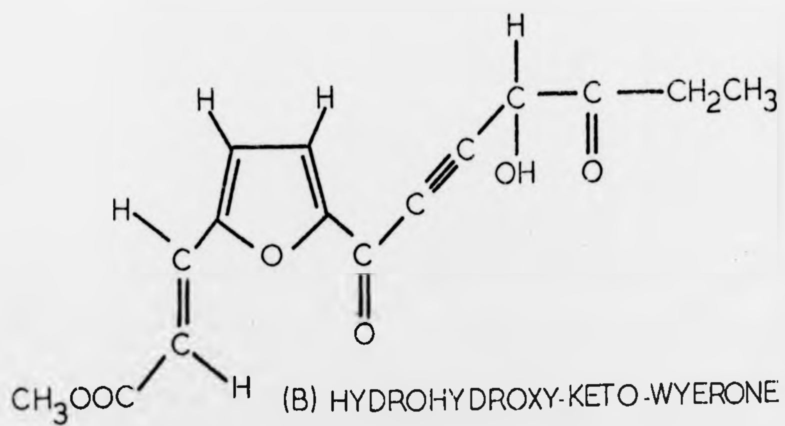
Due to the relatively small amounts of PA3b recovered it was not possible to obtain adequate NMR data on this substance. However, IR and mass spectral data obtained indicated that this phytoalexin may be 4-hydrohydroxy - 5 Keto - wyerone (Fig.17B).

The hypothetical MS fragmentation pathways for hydrohydroxyketo wyerone illustrated in Fig.19 closely fit the observed fragmentation patterns of

Fig.17



(A) MEDICARPIN



(B) HYDROHYDROXY-KETO-WYERONE

Figure 18

NMR spectrum of medicarpin

Fig.18

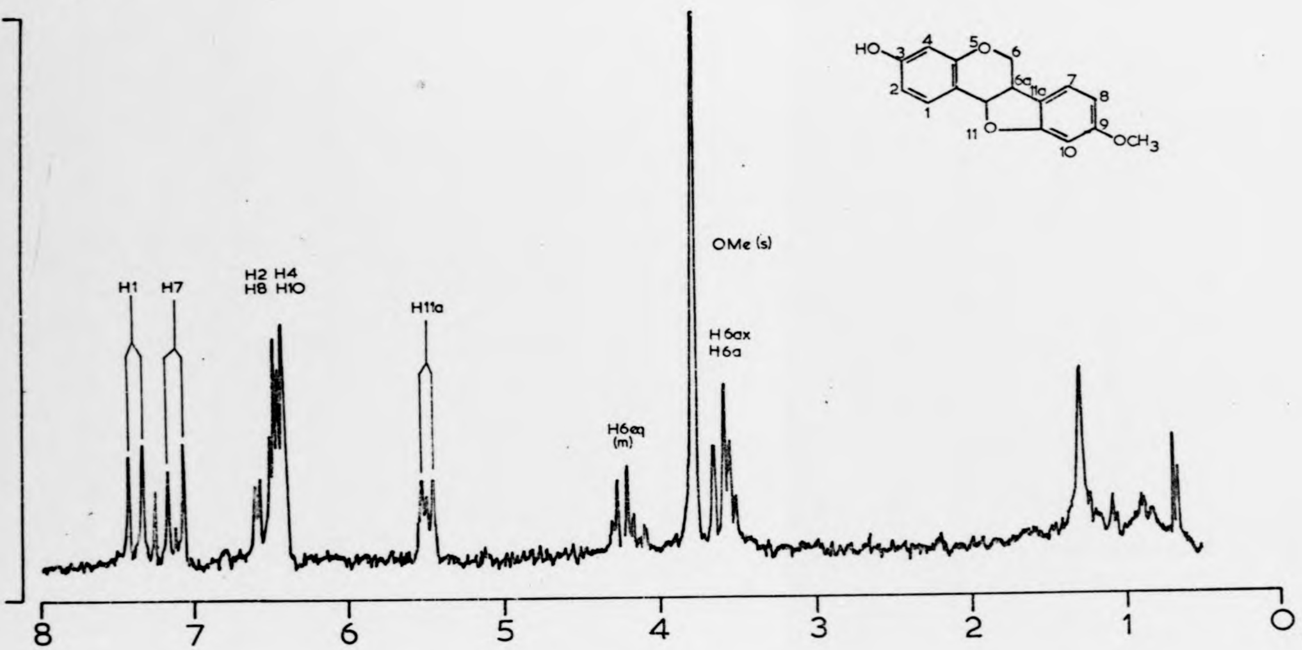
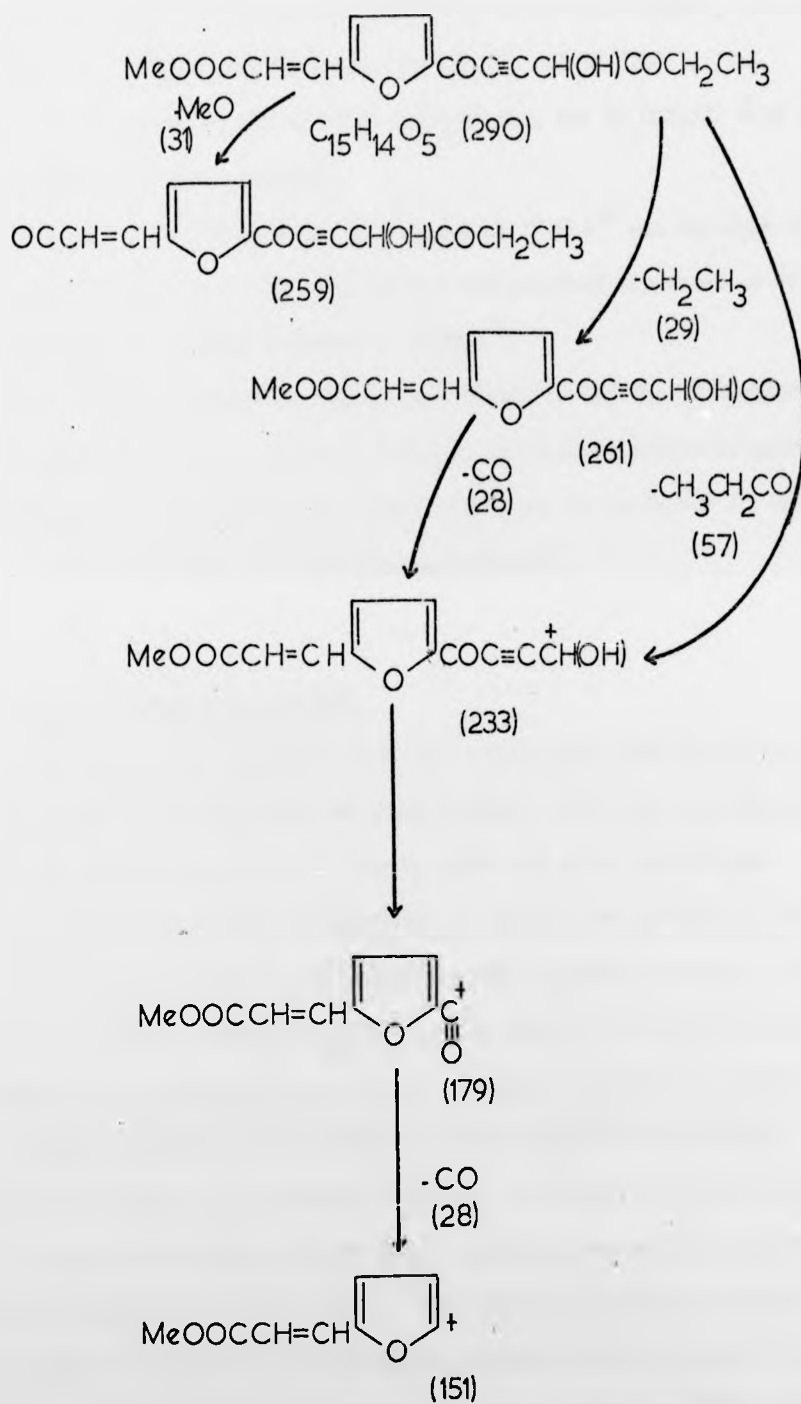


Figure 19

A hypothetical MS fragmentation pathway for hydrohydroxy-keto - wyerone.

Fig. 19



PA3b, M^+290 ($C_{15}H_{14}O_6$) with prominent fragments at m/e 261 (52.9%), 259 (4.7%), 233 (13.3%), 179 (38.9%), 163 (12.3%), 151 (38.2%) and 43 (100%). The appearance of fragments at 179 and 163 suggests that the $CH_3OOC.CH \overset{t}{=} CH$ side chain is retained in this compound.

In the IR spectrum carbonyl absorption at 1716cm^{-1} was stronger than that at 1642cm^{-1} which would be expected for the proposed structure of PA3b. Hydroxyl absorption was also detected at 3509cm^{-1}.

The observed polarity on TLC systems (Table 6) and the UV spectrum, which is identical to that of wyerone epoxide, are also in agreement with this structure. However before any firm conclusions can be formed on the structure of PA3b adequate NMR data must be obtained.

E Wyerone acid

(i) Isolation and spectral analysis

Wyerone acid was detected at RF 0.1 - 0.15 after PLC (Fig 9) as a light blue fluorescent band under UV light (366nm). Care was taken to remove the least contaminated area of the wyerone acid band since preliminary experiments had revealed that the sacrifice of quality for quantity at this stage lead to difficulties of purification in the subsequent stages. The inhibitor was eluted in MeOH and subject to PLC in Et_2OH : MeOH (8:1). The loading rates were calculated so that crude extracts equivalent to 5 g.f.w. of the original extract/ cm were applied to 18cm origins on 1.5mm thick layers; higher loading rates inevitably lead to 'streaking' of wyerone acid over the length of the plate. In developed chromatograms the main wyerone acid band was detected at RF 0.3 - 0.4. Two areas of silica gel corresponding to the middle of the band and the peripheral upper and lower areas of the band, which were contaminated with other substances, were eluted separately. The

peripheral regions from each plate were combined and rechromatographed as described above. In all these chromatograms silica gel was only removed from the middle of the wyerone acid band, since the advancing edge of the band was contaminated with yellow impurities. The 'tail' of the band was also disregarded.

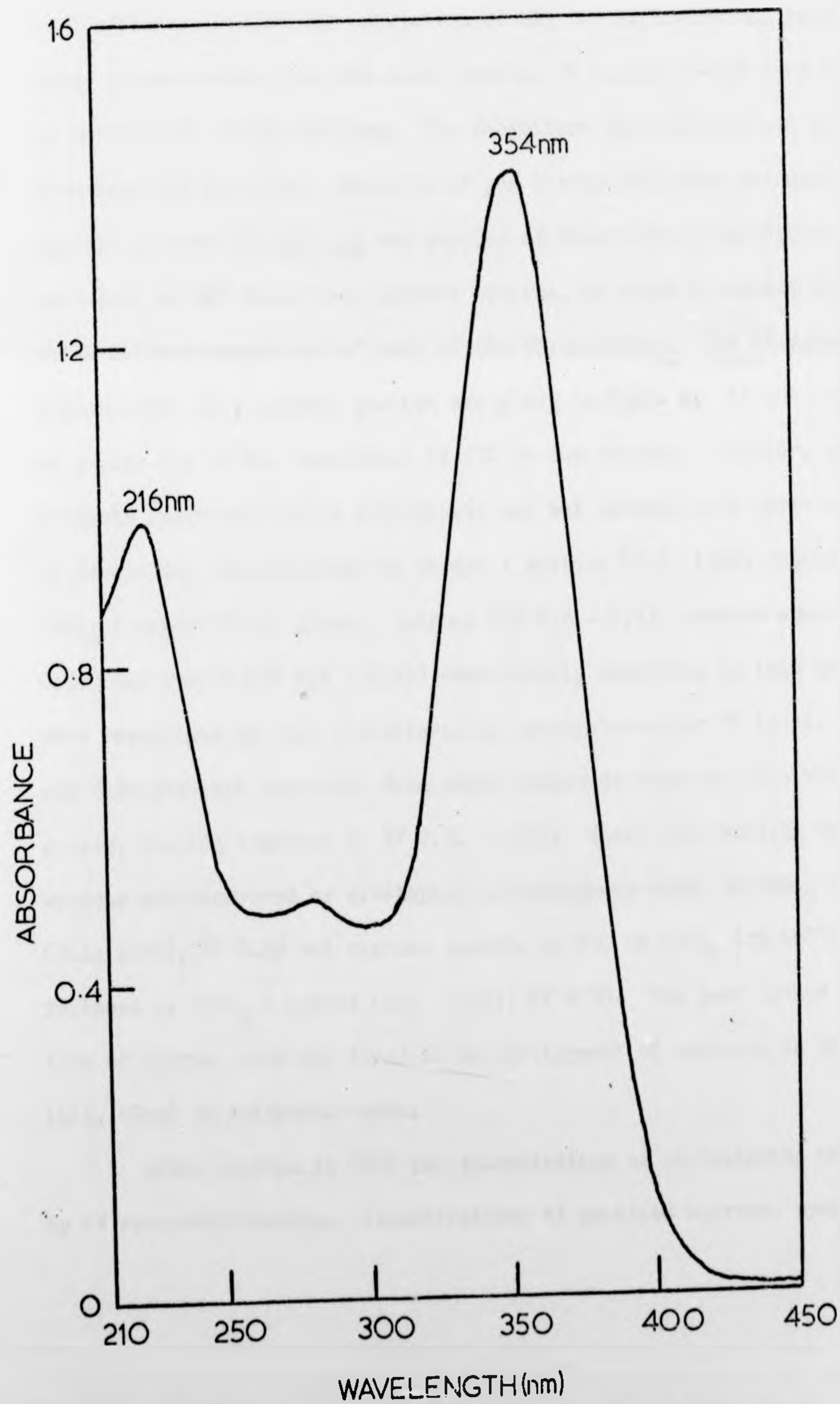
The combined eluates containing wyerone acid were filtered through a cotton wool plug in a pasteur pipette and the solvent evaporated in vacuo. The residue was taken up in a minimum amount of warm MeOH and Et_2O added dropwise and in excess as the vial was cooled in ice. Wyerone acid precipitated out as a pale yellow solid and was collected by centrifugation at 850g for 5 minutes and washed twice with Et_2O. Very little wyerone acid was detected in the supernatant fraction. After drying in vacuo wyerone acid was recovered as a pale yellow solid, a typical absorption spectrum obtained by this procedure is shown in Fig. 20. Yields of 55 and 17 mg/Kgf.w. were obtained from B. cinerea infected pod tissue and B. fabae infected cotyledons respectively, 6 days after inoculation. Before each experiment samples of wyerone acid were rechromatographed in either $CHCl_3$: MeOH (10:1, RF 0.35 - 0.43) or hexane : acetone (2:1, RF 0.21 - 0.28).

NMR and IR analysis of wyerone acid proved difficult because of its insolu bility in less polar solvents such as $CHCl_3$ and no useful MS data was obtained because of its low volatility. NMR data, however was obtained in deuteriomethanol and diagnostic NMR signals were obtained at δ 1.12 (3H,t, 7-H), 2.5 (2H, m, 6-H), 5.74 (1H,dt, 4-H), 6.44 (1H,dt, 5-H), 6.65 (1H,d, 2-H), 6.78 (1H,d,β'-H) 7.28 (1H,d,β-H) and 7.42 (1H,d,1^1 -H). The NMR spectrum was virtually identical to that of wyerone with the exception that no signal due to the methyl ester protons (δ 3.79) was detected. This evidence strongly suggests that this inhibitor is wyerone acid. Attempts to methylate wyerone acid with diazomethane proved unsuccessful. Details of the methylation procedure are

Figure 20

UV absorption spectrum of wyerone acid.

Fig.20



given in Appendix II.

2 Measurement of phytoalexin concentrations in infected tissue and inoculum droplets.

Maceration of infected tissue in Et_2O (50 ml/g) was found to be the most efficient method for extraction of all the phytoalexins, including the most polar wyerone acid, from the small samples of tissue (1-6g) used for estimation of phytoalexin concentrations. The inhibitors also partitioned into Et_2O from inoculum droplets. Extracts of pod tissue collected six days after inoculation with B. cinerca and samples of the purified inhibitors were subjected to TLC in several solvent systems, in order to select the system which allowed separation of most of the phytoalexins. The RF values of the phytoalexins in 9 solvent systems are given in Table 6. It was not possible to purify all of the inhibitors by TLC in one solvent. However, providing extracts contained little chlorophyll all but wyerone acid could be separated by developing chromatograms in hexane : acetone (2:1, 15cm) followed by $CHCl_3$: petrol (2:1, 15cm). Wyerone (RF 0.6 - 0.7), wyerone epoxide (RF 0.5 - 0.55) and wyerol (RF 0.4 - 0.45) were clearly separated in this solvent and were recognised by their characteristic appearance under UV light. Medicarpin and PA3b although separated from other compounds were not resolved in this system, running together at RF 0.31 - 0.38. Where chlorophylls were present wyerone was recovered by developing chromatograms twice in $CHCl_3$: petrol (2:1, 15cm), RF 0.66 and wyerone epoxide by TLC in $CHCl_3$ (2% EtOH) (15cm) followed by $CHCl_3$: petrol (2:1. 15cm), RF 0.50. The best method for purification of wyerone acid was found to be development of extracts in Et_2O : MeOH (6:1, 10cm) in saturated tanks.

After elution in MeOH the concentration of phytoalexins were determined by UV spectrophotometry. Concentrations of purified wyerone, wyerone

TABLE 6 RF values for phytoalexins on nine TLC systems. *

SOLVENT SYSTEM	WYERONE	WYERONE EPOXIDE	WYEROL	MEDICARPIN	PA3b	WYERONE ACID
$CHCl_3$(2%EtOH)	0.6	0.53	0.32	0.19	0.15	0.00
$CHCl_3$:CCl_4(3:1)	0.56	0.45	0.21	0.10	0.10	0.00
$CHCl_3$:MeOH(10:1)	0.9	0.9	0.75	0.69	0.62	0.11
$CHCl_3$:petrol(2:1)	0.42	0.34	0.17	0.07	0.05	0.00
Cyclohexane:EtAc(1:1)	0.5	0.41	0.48	0.46	0.46	0.00
Et_2O:MeOH(6:1)	SF	SF	SF	SF	SF	0.65
Hexane:acetone(1:1)	0.74	0.68	0.68	0.61	0.6	0.03
Hexane:acetone(3:1)	0.35	0.3	0.28	0.2	0.18	0.00
Hexane:EtAc:acetic acid(50:40:1)	0.49	0.39	0.44	0.41	0.4	0.00

SF = Solvent Front

*- Plates were silica gel (0.25mm thick) with fluorescent indicator from Merck, Germany

epoxide and wyerone acid were estimated directly using published (Fawcett *et al.*, 1968) or determined extinction coefficents. Because of their different λ max, the concentrations of medicarpin (λ max 287nm) and PA3b (λ max 347nm) in the mixture eluted from chromatograms developed in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) could also be determined by UV spectrophotometry. Medicarpin has negligible absorbance at 347nm, therefore absorbance at this wavelength was considered to be entirely due to PA3b. Absorbance of the mixture at 287nm due to PA3b was then estimated from the absorbance ratio of 347nm : 287nm for pure PA3b (Fig.16B) which was calculated to be 5.9. Subtraction of the estimated absorbance by PA3b at 287nm from the absorbance of mixture gave the absorbance due to medicarpin. The yield of PA3b was expressed in absorbance units and of medicarpin was determined from the published extinction coefficent (Smith *et al.*, 1971).

The yield of each identified phytoalexin in chromatographic eluates was calculated from the following formula:-

$$\text{Yield } (\mu g) = \text{Absorbance at } \lambda \text{ max} \times \text{Conversion factor} \times \text{Volume of MeOH (ml)}$$

Conversion factors were calculated from extinction coefficents to be for wyerone, 9.55; wyerone epoxide, 9.75; wyerol, 9.29; wyerone acid, 9.04; and medicarpin, 34.78.

3 Measurement of reduced wyerone acid concentrations in infected tissue and inoculum droplets.

Reduced wyerone acid, the metabolite of wyerone acid produced by *B. fabae* (Mansfield and Widdowson, 1973; Mansfield *et al.*, 1973) was detected in extracts of plant tissues as a dark quenching band under UV light (254nm) at Rf 0.64 after chromatography in Et_2O : MeOH (6:1). On elution in MeOH

reduced wyerone acid gave an identical UV absorption spectrum to that published (Mansfield and Widdowson, 1973) with λmax at 300nm (Fig.21). Assuming that reduced wyerone acid has a similar extinction coefficent to that of reduced wyerone (Mansfield et al., 1973) concentrations of reduced wyerone acid were calculated from the following formula:-

$$\text{reduced wyerone acid } (\mu g) = \text{Absorbance at 300nm} \times 9.8 \times \text{Volume of MeOH (ml)}$$

4 Extraction of known quantities of wyerone, wyerone epoxide, wyerol and wyerone acid from healthy pod and cotyledon tissue.

In order to determine the efficiency of extraction of the phytoalexins from pod and cotyledon tissue, the recovery of known amounts of the phytoalexins from healthy tissue, when extracted with either MeOH followed by partitioning between Et_2O and water, or with Et_2O directly was investigated.

The amounts of each phytoalexin added to and recovered from MeOH or Et_2O extracts of pod or cotyledon tissue (5g) are shown in Table 7. Between 59 and 79% recoveries of the phytoalexins were obtained. MeOH was slightly less efficient than Et_2O extraction. This may have been due to the additional extraction step required following MeOH extraction.

The recorded yields of these phytoalexins in the following chapter must therefore be considered an underestimate of the actual level within the tissues.

Figure 21

UV absorption spectrum of reduced wyerone acid isolated from *B. fabae* infected pod tissue.

Fig. 21

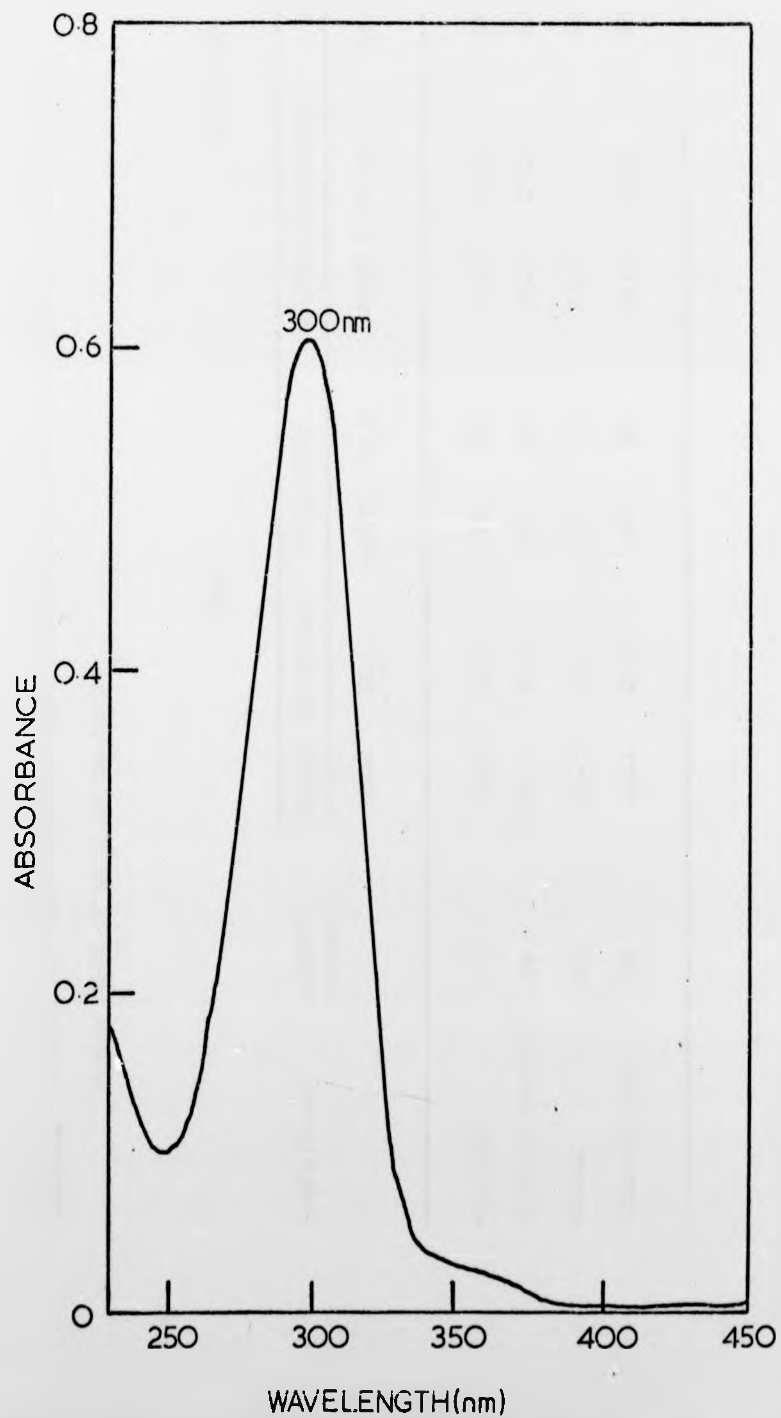


TABLE 7 Extraction efficencies of phytoalexins from pod and cotyledon tissue using either MeOH or Et_2O as solvents

		POD				COTYLEDON			
(μg/g.f.w.)	AMOUNT ADDED	AMOUNT RECOVERED		%RECOVERY		AMOUNT RECOVERED		%RECOVERY	
		MeOH	Et_2O	MeOH	Et_2O	MeOH	Et_2O	MeOH	Et_2O
WYERONE	37	21.8	23.3	59	63	23.7	24.8	64	67
WYERONE EPOXIDE	24	15.6	18.9	65	79	14.4	15.6	60	65
WYEROL	35	23.5	25.2	67	72	22.0	22.7	63	65
WYERONE ACID	33	21.1	23.8	64	72	21.5	20.1	65	61

CHAPTER 3

Accumulation of phytoalexins in V. faba infected by species of Botrytis.

1 Accumulation of phytoalexins in different tissues.

Different tissues of the broad bean plant (cotyledons, pods and leaves) were prepared and inoculated with conidial suspensions of B. cinerea, B. fabae or sterile distilled water as previously described. After 4 days incubation the symptoms that developed were recorded (Table 8) and the infected tissue collected.

Both fungi had caused restricted orange/brick red lesions in cotyledon tissues beneath inoculum droplets. B. cinerea caused similar brown limited lesions in pod seed cavities, however B. fabae blackened and rotted the tissue beneath the inoculum droplets and had spread into adjacent pod endocarp by 4 days after inoculation. Leaf tissue was similarly susceptible to B. fabae, but sympton development at sites inoculated with B. cinerea varied from no visible symptoms to spreading lesions. Inoculation sites collected from leaves were therefore divided into two groups, those which bore restricted lesions and those from which spreading lesions had developed.

The appearance of slight flecking in leaves at sites inoculated with sterile distilled water alone was thought to be due to infection by epiphytic micro organisms as the leaves used in this experiment were collected from field grown plants late in the growing season (August, 1975). However, the leaves showed no sign of senescence throughout the experiment. Inoculum droplets which had persisted on the tissues were collected and combined

TABLE 8 Symptoms and classification of disease reaction (resistant/susceptible) in different tissues of *V. faba** 4 days after inoculation with *B. cinerea*, *B. fabae* or sterile distilled water

	TISSUE	SYMPTOMS	DISEASE REACTION+
B. CINEREA	COTYLEDON	ORANGE/BRICK RED RESTRICTED LESIONS	R
	POD	LIGHT BROWN/BLACK RESTRICTED LESIONS	R
	LEAF	(i) BROWN/BLACK LESIONS GRADE 6.5-100† (41.2%)▲	R
		(ii) BLACK SPREADING LESIONS (58.8%)▲	S
B. FABAE	COTYLEDON	ORANGE/BRICK RED RESTRICTED LESIONS	R
	POD	BLACK SPREADING LESIONS MYCELIUM VISIBLE	S
	LEAF	BLACK SPREADING LESIONS MYCELIUM VISIBLE	S
WATER	COTYLEDON	NO VISIBLE SYMPTOMS	-
	POD	VERY SLIGHT FLECKING	-
	LEAF	LESION GRADES (0-6.5)	-

* - Leaves and pods were collected from field grown plants late in the growing season (August 1975)

† - As described by Mansfield and Deverall (1974a).

\+ - R = Resistant S = Susceptible

▲ - % of total inoculation sites collected.

with the tissue collected from the respective treatment for extraction.

Et_2O extracts were prepared from the tissues and aliquots (equivalent to 0.25 g.f.w.) were subjected to TLC in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). Developed chromatograms were bioassayed with C. herbarum. The plate bioassays showed (Plate 4) that phytoalexins accumulate in all tissues undergoing a resistant response to fungal invasion. Very little inhibitory activity could be detected in extracts of pod tissue infected by B. fabae or inoculation sites collected from leaves bearing spreading lesions of B. cinerea. No phytoalexins could be detected in B. fabae lesions on leaves. The appearance of inhibitors in tissues inoculated with sterile distilled water alone was considered to be due solely to infection by contaminating micro-organisms since previous experiments had indicated that none of the inhibitors are preformed.

The concentrations of the phytoalexins; wyerone, wyerone epoxide, wyerol, medicarpin, PA3b and wyerone acid in infected tissues were determined as previously described (Chapter 2) and are shown in Table 9. Quantitative data reflects the results of TLC plate bioassays. All the phytoalexins were recovered at much higher levels from resistant than susceptible tissue. In general, proportionately similar levels of the phytoalexins were detected in pod and leaf tissue bearing limited lesions. Wyerone acid, wyerone and wyerone epoxide were the dominant inhibitors in these tissues, the acid being present in greatest concentration in leaves. The accumulation of wyerone in cotyledon tissue infected by either species of Botrytis was most striking. Yields from cotyledons inoculated with B. fabae were three times those from tissues inoculated with B. cinerea. Despite the high yields of wyerone derivatives from cotyledons they contained only traces of medicarpin. Low levels of the phytoalexins were detected in tissue inoculated with water,

Plate 4

TLC plate bioassays of extracts from 0.25g cotyledon (C), pod endocarp (P), and leaf (L) tissue collected 4 days after inoculation with either conidial suspensions of *B. cinerea* or *B. fabae* or with sterile distilled water. Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). W, wyerone; WE, wyerone epoxide; WO, wyerol; PA3 medicarpin and PA3b; WA, wyerone acid.

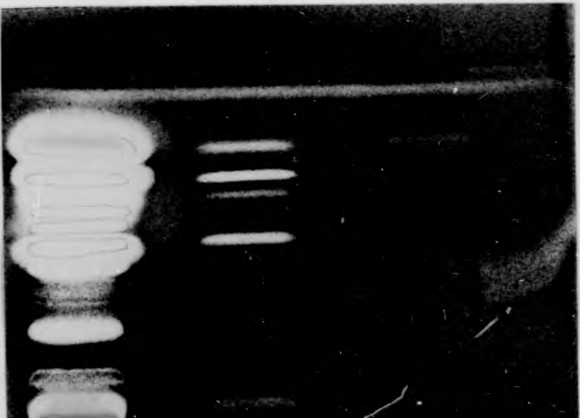
L_R - leaf resistant

L_S - leaf susceptible

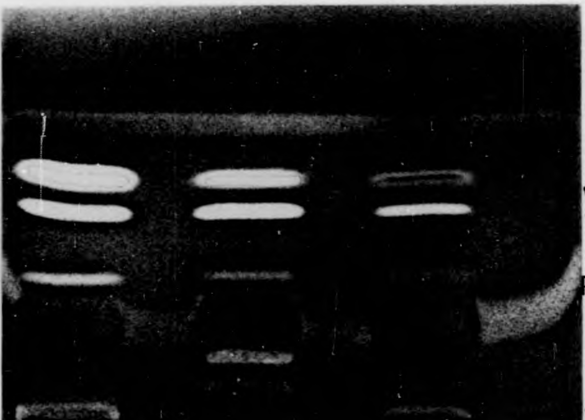
Plate 4

B.CINEREA

B.FABAE



WATER



W
WE
WO
PA3
WA

<u>TABLE 9</u> Yields (μg/g.f.w.) of phytoalexins from cotyledon, pod, and leaf tissue 4 days after inoculation with <u>B. cinerea</u> (BC), <u>B. fabae</u> (BF), or sterile distilled water (W).

<table>
<tr><th></th><th colspan="3">COTYLEDON</th><th colspan="3">POD</th><th colspan="4">LEAF</th></tr>
<tr><th></th><th>BC</th><th>BF</th><th>W</th><th>BC</th><th>BF</th><th>W</th><th>BC_r*</th><th>BC_s*</th><th>BF</th><th>W</th></tr>
<tr><td>WYERONE</td><td>538</td><td>1789</td><td>45</td><td>217</td><td>2.6</td><td>14.6</td><td>66.4</td><td>-</td><td>-</td><td>D</td></tr>
<tr><td>WYERONE EPOXIDE</td><td>54.5</td><td>106.7</td><td>D</td><td>30.7</td><td>D</td><td>D</td><td>10.8</td><td>-</td><td>-</td><td>D</td></tr>
<tr><td>WYEROL</td><td>77.9</td><td>180</td><td>-</td><td>8.9</td><td>-</td><td>-</td><td>D</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>MEDICARPIN</td><td>D</td><td>D</td><td rowspan="2">D</td><td>19.5</td><td rowspan="2">D</td><td>-</td><td rowspan="2">D</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>PA3b†</td><td>5.2</td><td>5.8</td><td>0.85</td><td>-</td><td>-</td><td>-</td><td>-</td></tr>
<tr><td>WYERONE ACID</td><td>28.8</td><td>122</td><td>-</td><td>219</td><td>-</td><td>-</td><td>104</td><td>D</td><td>-</td><td>-</td></tr>
</table>

* - r = Resistant reaction s = Susceptible reaction.
† - Yield AU λmax 347nm/g.f.w.
\- - Not detected.
D - Detected under UV light and/or in bioassays but not identified by UV spectrophotometry.

in comparison with levels recovered from infected tissue exhibiting a resistant response.

In conclusion it appears that phytoalexins accumulate in tissue of V. faba undergoing a resistant reaction to fungal invasion, however the pattern of accumulation in cotyledons differed from that in pod and leaf tissue.

2 Changes in concentrations of phytoalexins and reduced wyerone acid in cotyledons and pod endocarp following inoculation with B. cinerea and B. fabae

In order to determine the relative importance of the different phytoalexins in the resistance of tissues of the broad bean plant to fungal infection the time course of accumulation of phytoalexins and reduced wyerone acid in pod and cotyledon tissue was examined. Leaves were unsuitable for this quantitative work because of the great variability in symptom development at sites inoculated with B. cinerea and difficulty experienced in separating wyerone epoxide, wyerol, medicarpin and PA3b from contaminating chlorophylls in certain extracts. Further details of experiments carried out with leaves are given in Appendix III.

Inoculated tissue (and inoculum droplets from pods) was collected for extraction and isolation of fungi at intervals after inoculation. MeOH extracts were partitioned between Et_2O and water (droplets were partitioned directly with Et_2O). Samples of the Et_2O extracts were subjected to TLC in hexane : acetone (2:1) followed by $CHCl_3$; petrol (2:1) and developed chromatograms bioassayed with C. herbarum. The levels of phytoalexins and reduced wyerone acid in tissues and droplets were determined using samples of extracts equivalent to 0.5 g.f.w. tissue.

A In cotyledons

TLC plates bioassays showed that all the phytoalexins accumulated after

fungal infection (Plate 5). Wyerone was the first phytoalexin detected after inoculation with either B. cinerea or B. fabae, and the only inhibitor present in cotyledon tissue after prolonged incubation with sterile distilled water. Accumulation of wyerone, wyerone epoxide, wyerol, PA3b and wyerone acid in tissues infected with B. cinerea and B. fabae are illustrated in Figs 22 and 23 respectively. Medicarpin, although detected with DNA, was only present in trace amounts. No reduced wyerone acid was detected at any time after inoculation with B. fabae or B. cinerea.

The pattern of phytoalexin accumulation in lesions caused by either species was qualitatively similar, however, B. fabae consistently induced higher levels of all phytoalexins than B. cinerea. In both cases accumulation of inhibitors was associated with the appearance of macroscopic flecking at inoculation sites. Wyerone increased at greater rates and to much higher levels than the other phytoalexins. After prolonged incubation with both fungi the level of wyerone reached a sustained maximum 6 days after inoculation (Fig.24). Only wyerone was detected in tissues incubated with sterile distilled water alone and accumulated to 14.8 - 46.5 μg/g.f.w. 6 days after inoculation. Both fungi could not be isolated from infected tissue 4 days after inoculation.

B In pod endocarp.

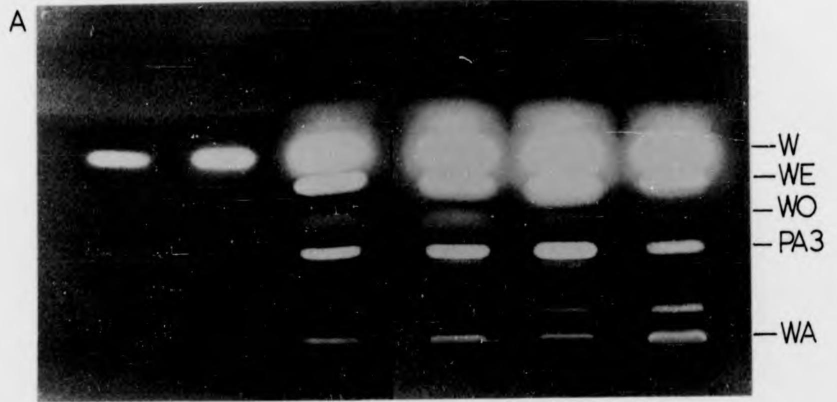
Each phytoalexin accumulated with time after inoculation with B. cinerea (Plate 6A and Fig.25). Wyerone epoxide, wyerol, medicarpin and PA3b however, did not reach concentrations $>$25μg/g.f.w., even after prolonged incubation periods and were mainly confined to the tissue. Although wyerone acid and wyerone were the predominant phytoalexins present, their rates of accumulation and distribution between inoculum droplets and tissue differed

Plate 5

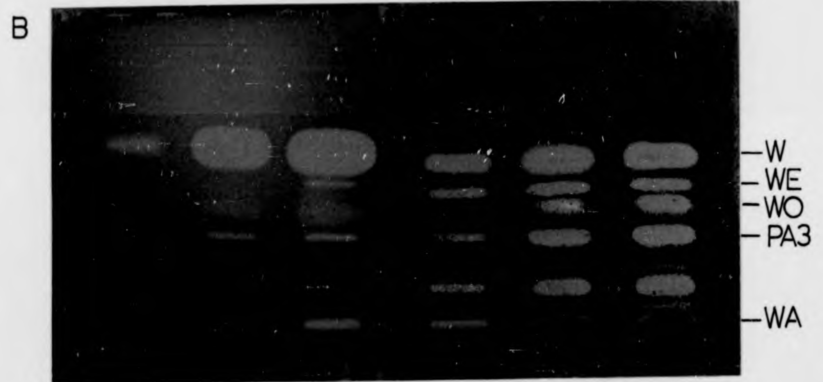
TLC plate bioassays of extracts from cotyledon tissue (0.1g) collected 1,2,3,4,6 and 10 days after inoculation with either B. cinerea (A) B. fabae (B) or sterile distilled water (C). Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) W, wyerone; WE, wyerone epoxide; WO wyerol; PA3, medicarpin and PA3b; WA, wyerone acid.

Plate 5

A



B



C

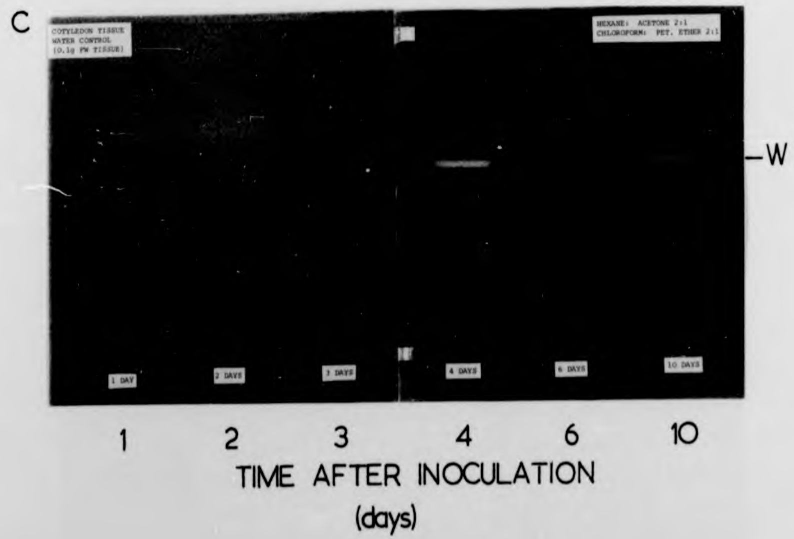


Figure 22

Accumulation of phytoalexins in cotyledon tissue following infection with *B. cinerea.* Only traces of medicarpin were detected even after prolonged incubation.

Fig. 22

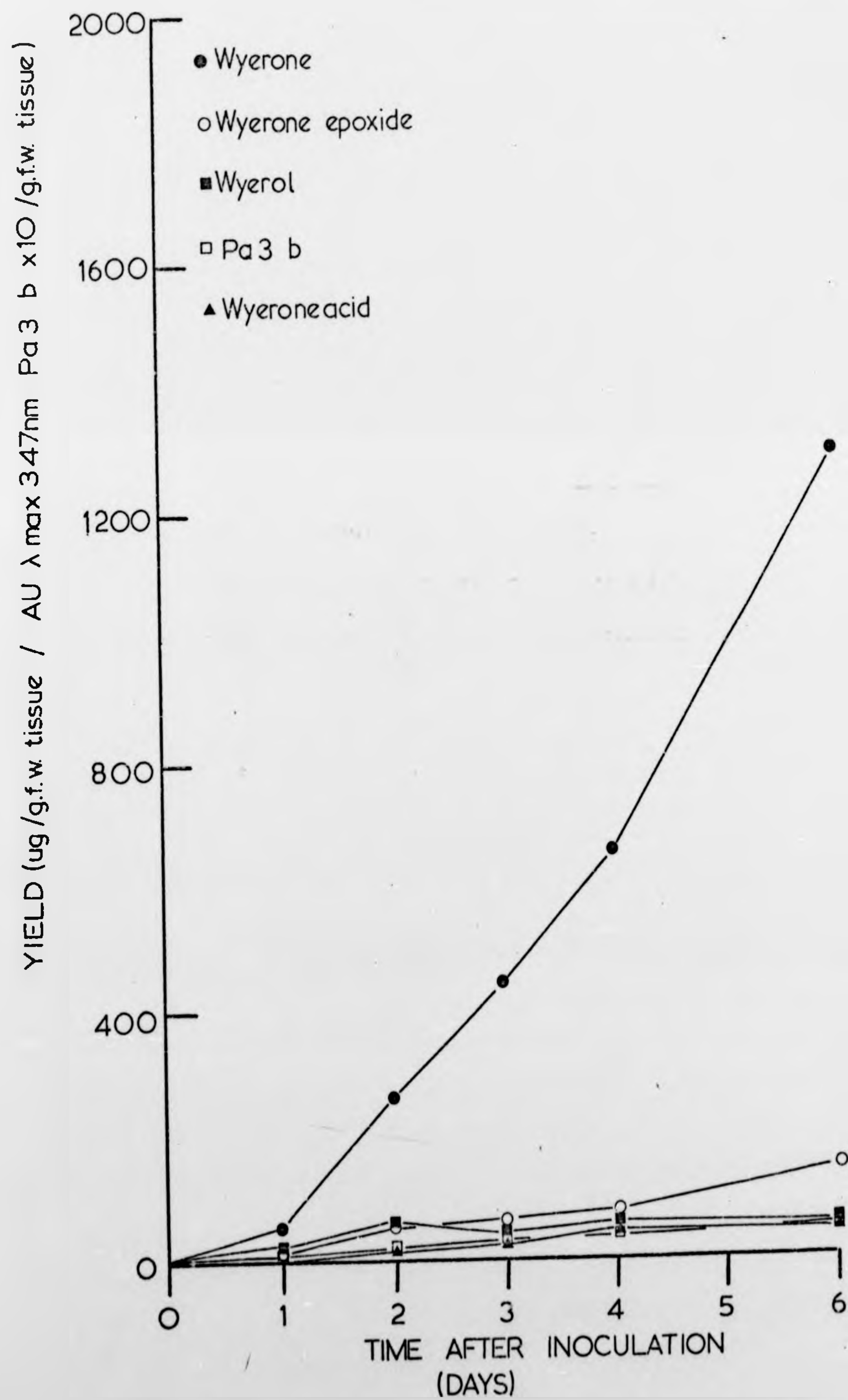
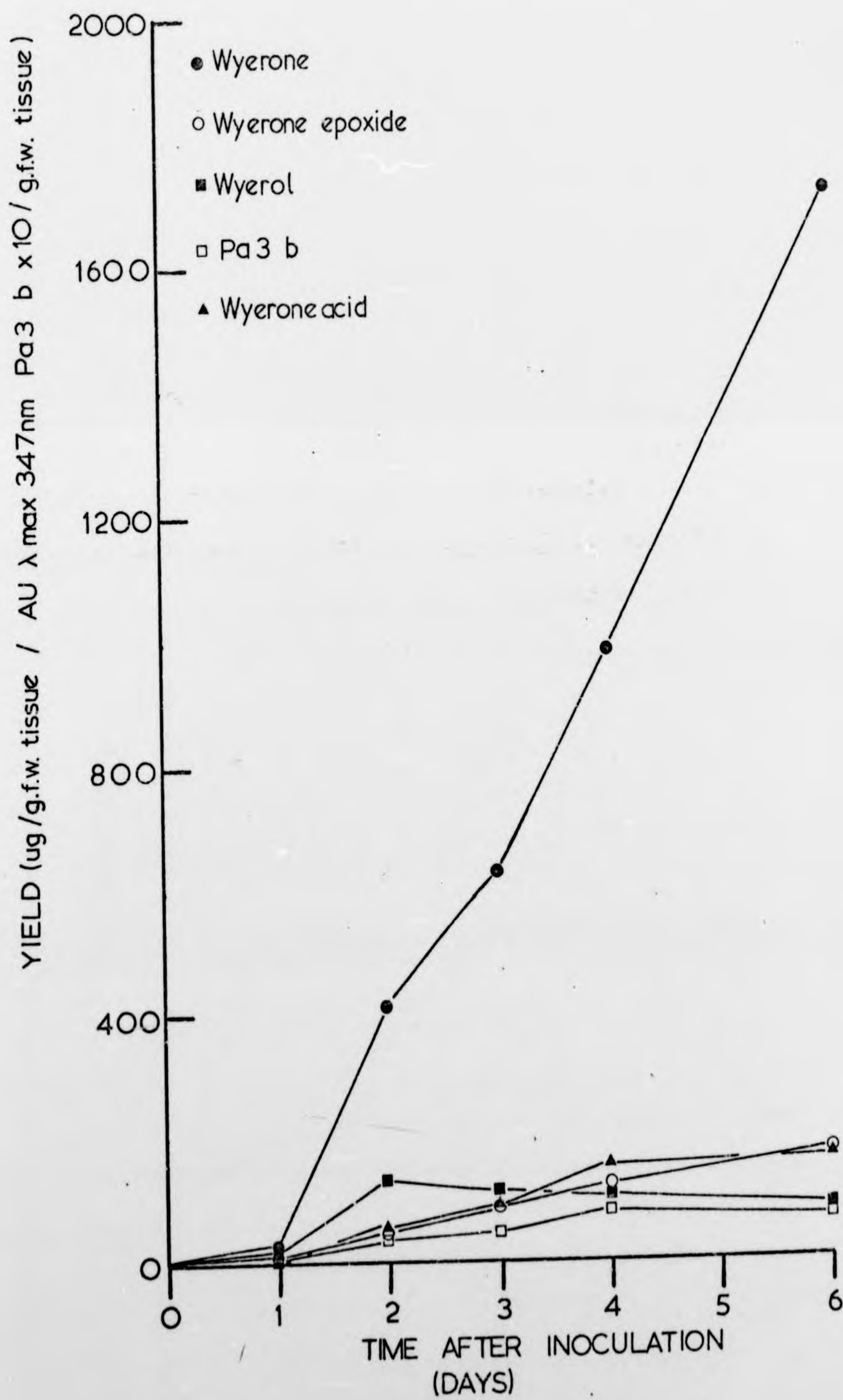


Figure 23

Accumulation of phytoalexins in cotyledon tissue following infection with *B. fabae*. Only traces of medicarpin were detected even after prolonged incubation.

Fig. 23



owing

etected

Figure 24

Accumulation of wyerone in cotyledon tissue following prolonged incubation with either *B. cinerea*, (O) or *B. fabae* (●). Each point represents the mean of two replicate experiments.

Figure 24

Accumulation of wyerone in cotyledon tissue following prolonged incubation with either _B. cinerea_, (O) or _B. fabae_ (●). Each point represents the mean of two replicate experiments.

Fig.24

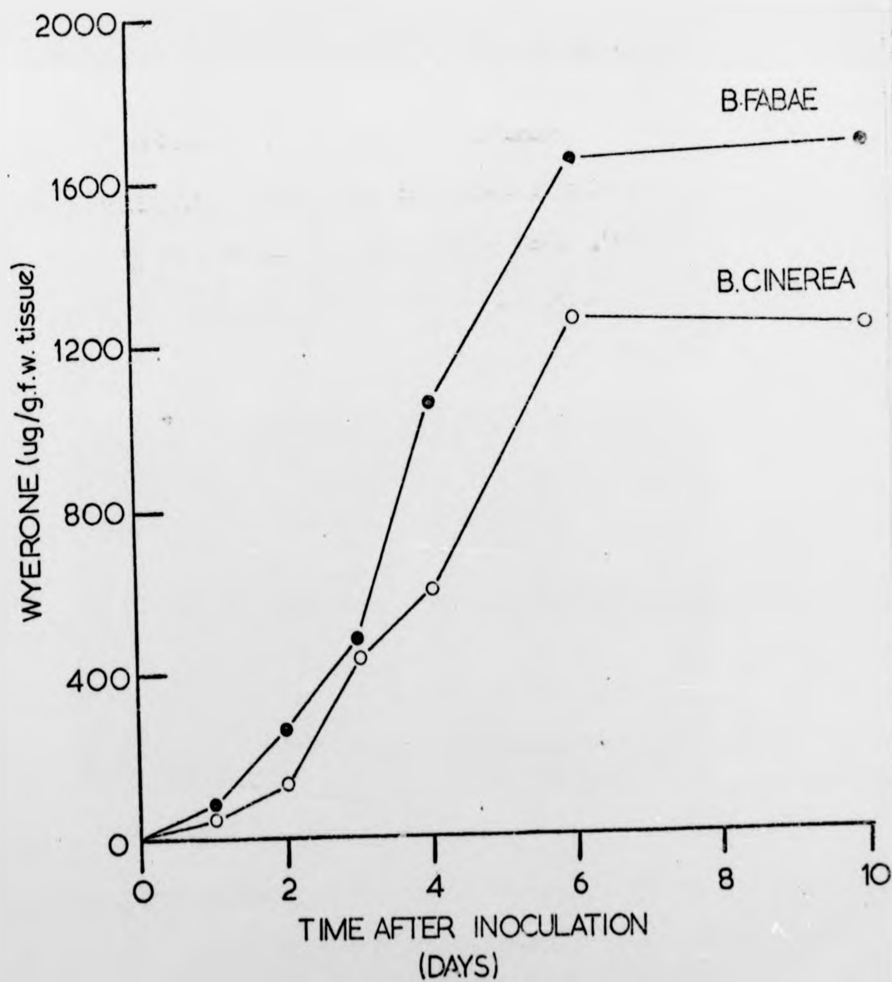


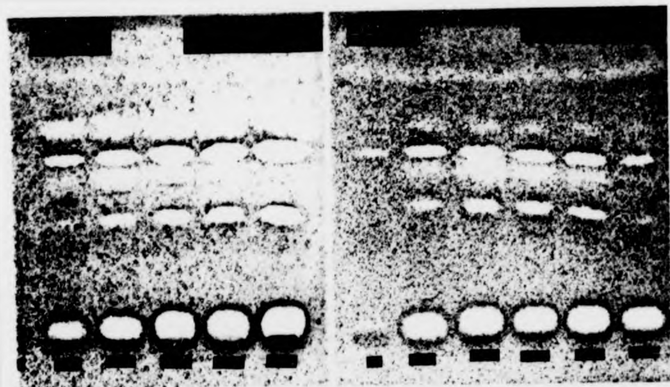
Plate 6

TLC plate bioassays of extracts from pod tissue (0.25g) and overlying inoculum droplets (equivalent to 0.25g tissue) collected 12h, 1,2,3,4 and 6 days after inoculation with either _B. cinerea_ (A), _B. fabae_ (B) or sterile distilled water (C). Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). W, wyerone; WE wyerone epoxide; WO, wyerol; PA3, medicarpin and PA3b; WA, wyerone acid.

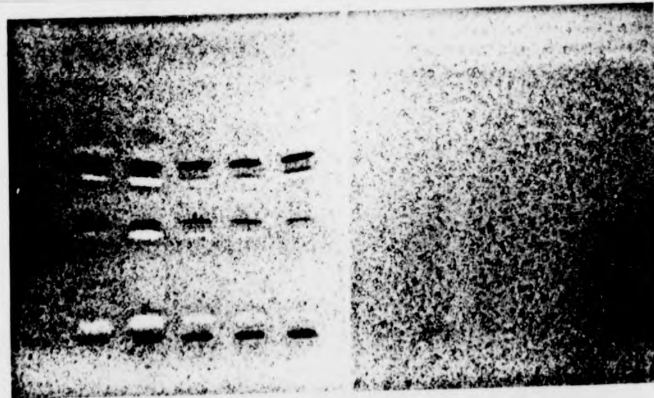
Plate 6

TISSUE INOCULUM DROPLET

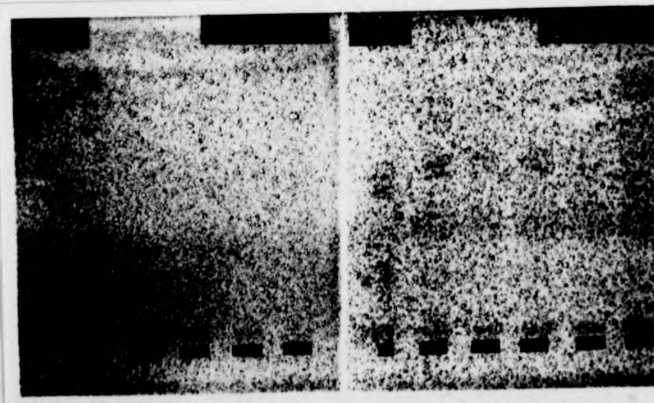
A



B



C

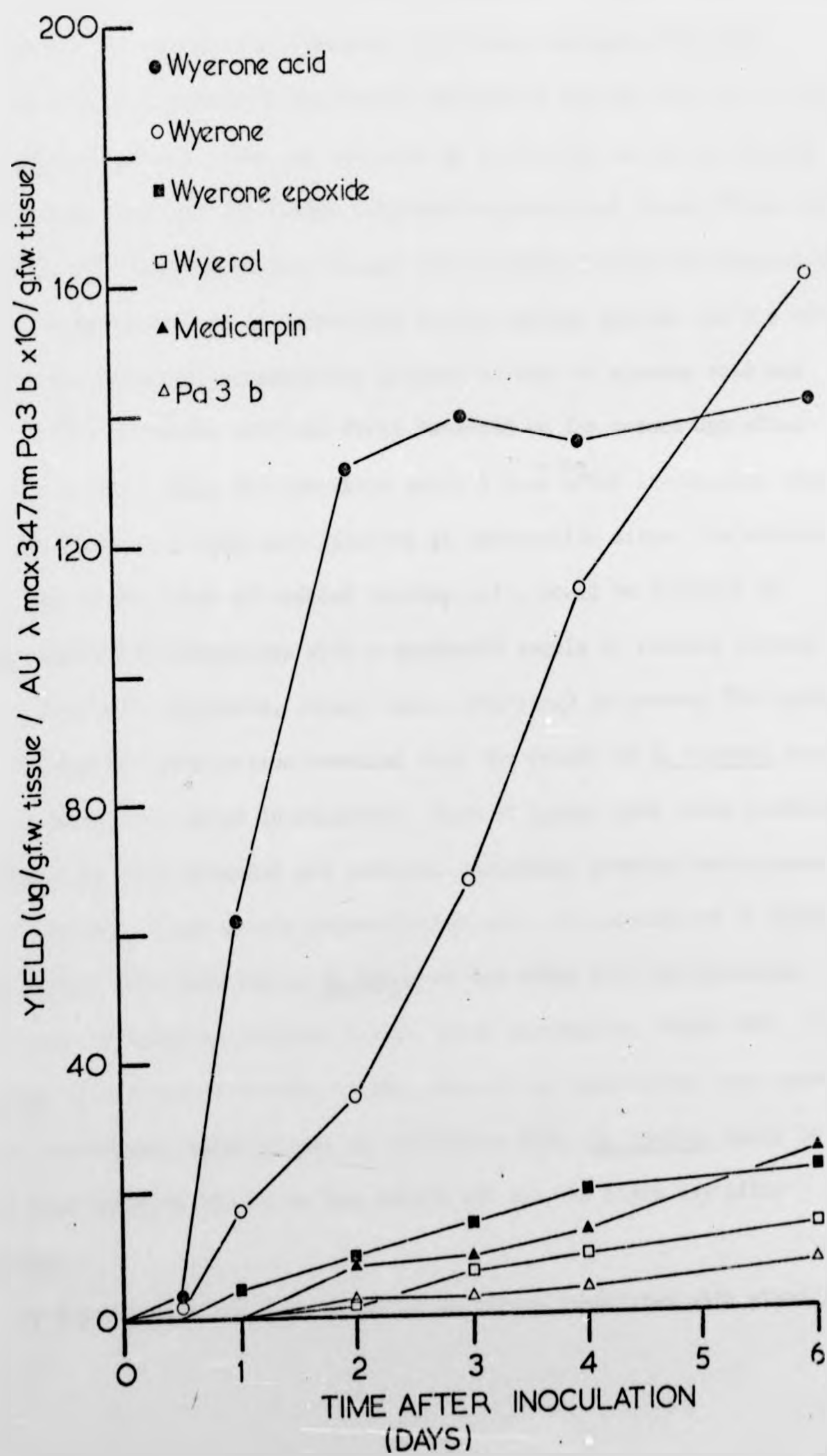


TIME AFTER INOCULATION

Figure 25

Accumulation of phytoalexins in pods following infection with B. cinerea.

Fig. 25



markedly. Wyerone acid accumulated rapidly in tissues and inoculum droplets during the first and second days after inoculation with B. cinerea, reaching maximum concentration 2 days after inoculation. In contrast, wyerone increased more slowly reaching maximum concentrations 6 days after inoculation and almost all the wyerone was recovered from tissue extracts (Fig.26).

An initial increase in the concentrations of wyerone acid and wyerone at B. fabae inoculation sites was followed by a decrease as tissues became completely blackened and the fungus colonized uninoculated tissue (Plate 6B and Fig.27). TLC plate bioassays showed that no phytoalexins accumulated in inoculum droplets and that in underlying tissue wyerone epoxide and the mixture PA3 followed a pattern of accumulation similar to that of wyerone acid and wyerone. Reduced wyerone acid was first detected on the second day after inoculation with B. fabae and increased until 4 days after inoculation when only traces of wyerone acid were detected at inoculation sites. No reduced wyerone, the methyl ester of reduced wyerone acid, could be detected in B. fabae extracts by comparison with a synthetic sample of reduced wyerone (obtained from J.W. Mansfield, Biology Dept., Stirling) in several TLC systems.

Microscopic examination revealed that the growth of B. cinerea ceased within the second day after inoculation. Tips of hyphal germ tubes produced by conidia were often branched and swollen, containing granular cytoplasm. (Plate 7A and B). These growth abnormalities were most pronounced at hyphal tips in contact with host cells. B. fabae on the other hand had produced a hyphal mesh in inoculum droplets 2 days after inoculation (Plate 7C). Hyphae of B. fabae also appeared healthy at the edge of the inoculation site where they were penetrating uninoculated tissue (Plate 7D). B. cinerea could be isolated from infected tissue on the fourth but not the sixth day after inoculation.

No phytoalexins could be detected in tissue inoculated with sterile

Figure 26

The accumulation of wyerone, wyerone epoxide and wyerone acid in inoculum droplets and pod tissue following infection with *B. cinerea*. Only traces of wyerol, medicarpin and PA3(b) were detected in inoculum droplets.

Fig.26

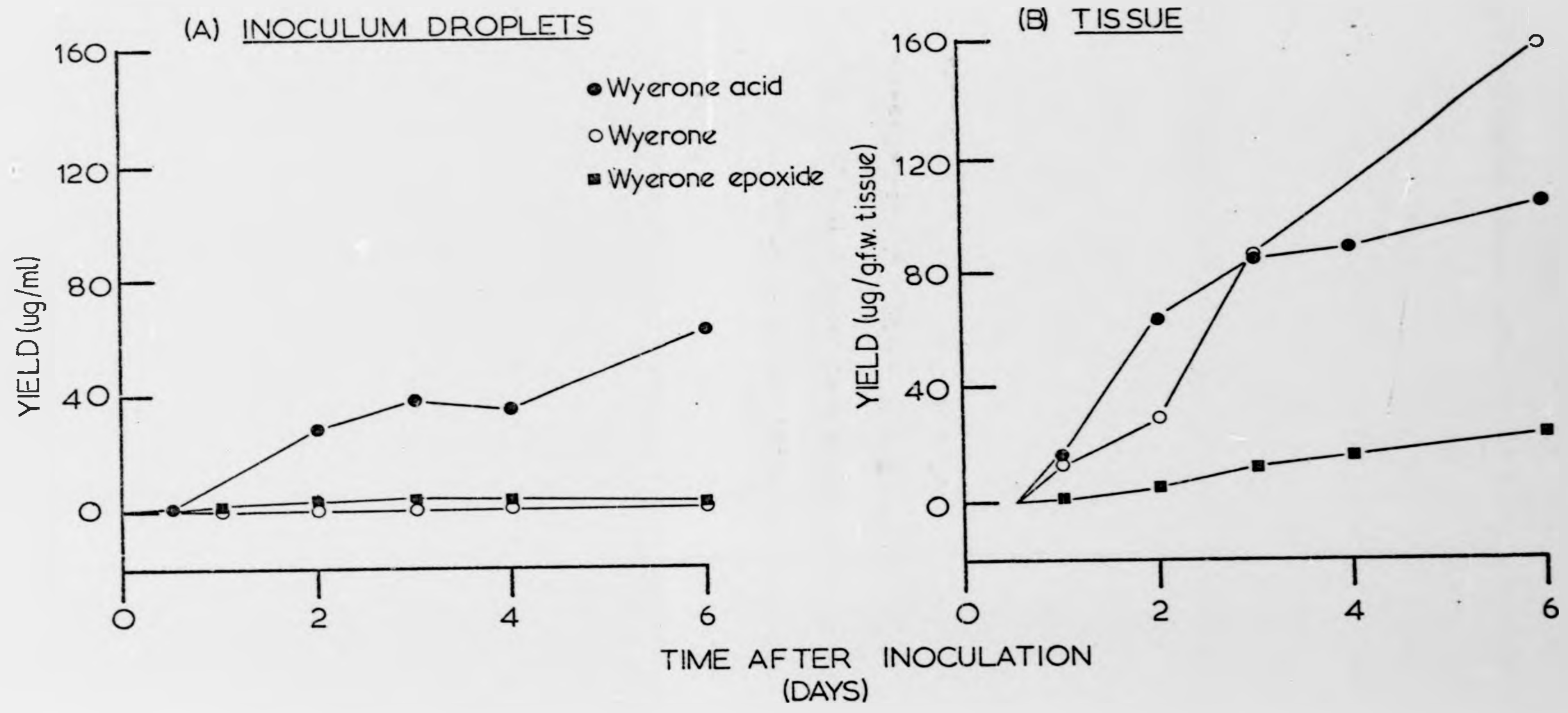


Figure 27

Changes in the concentrations of wyerone, wyerone acid and reduced wyerone acid in pod tissue following infection with *B. fabae*.

Fig. 27

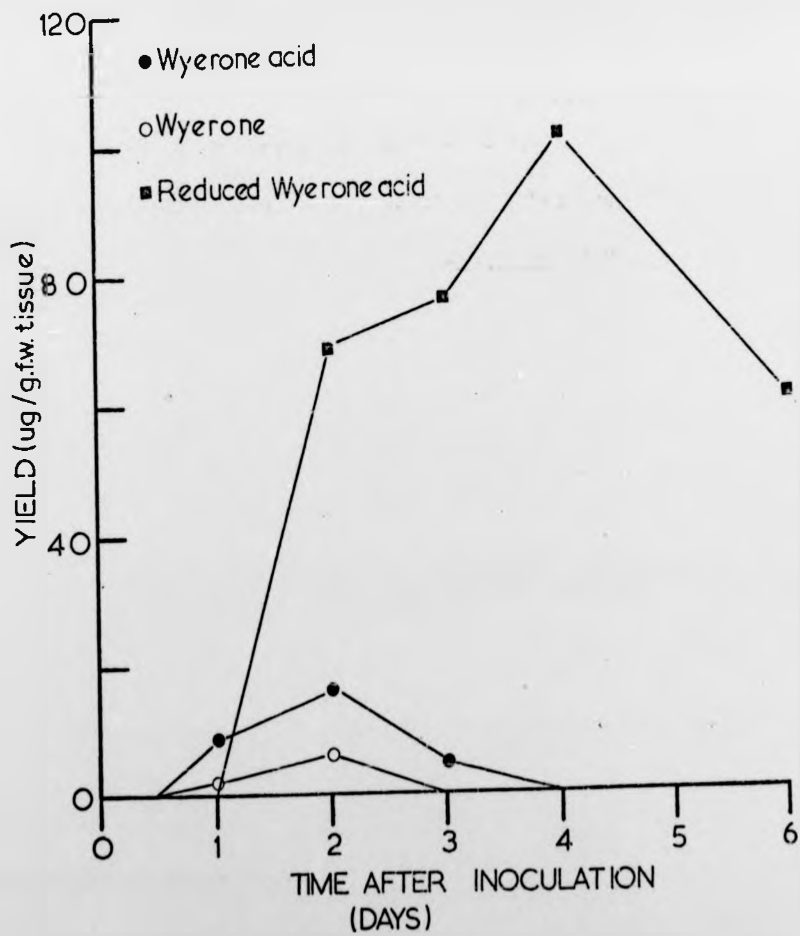
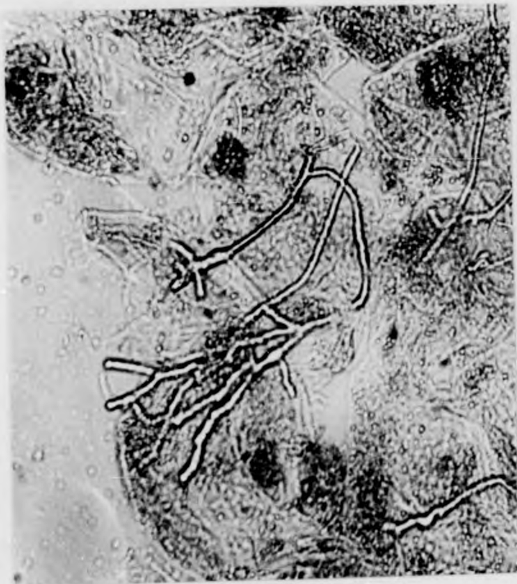


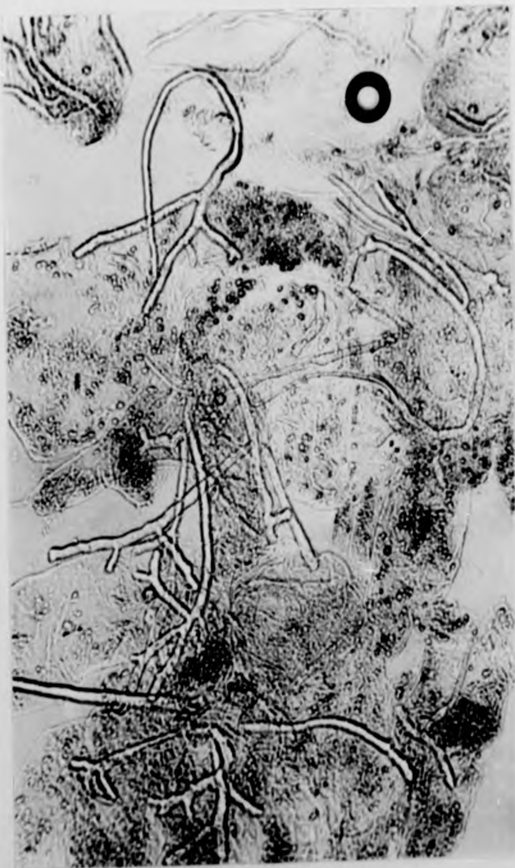
Plate 7.

The growth of B. cinerea and B. fabae in pod endocarp two days after inoculation.

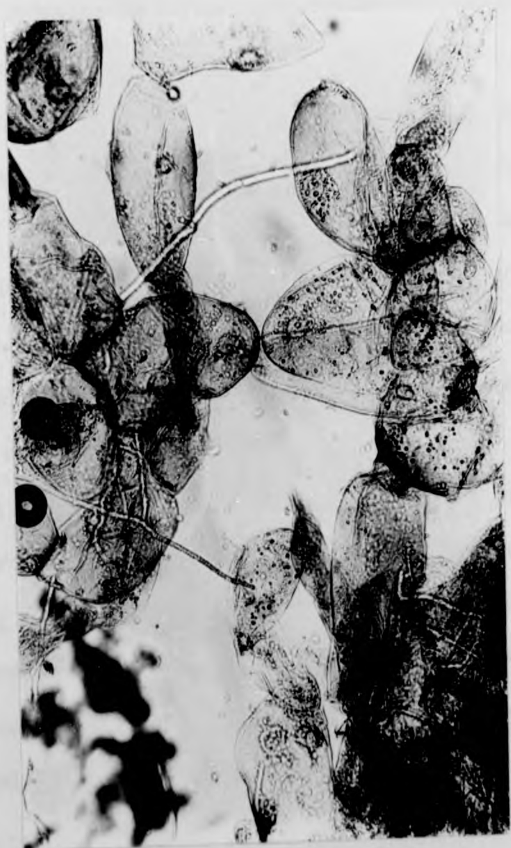
A and B The growth of B. cinerea. Hyphae are swollen and branched and also contain granular cytoplasm. These growth abnormalities appear more pronounced in hyphae in contact with the hosts cell wall.

C and D The growth of B. fabae. A hyphal mesh is present in the inoculum droplet (C) and hyphae on the periphery of the lesion appear unaffected (D).


A


B

C


D

distilled water alone at any time after inoculation in this experiment (Plate 6C).

The pH of inoculum droplets containing either fungus decreased from 5.5 to between 4.0 and 4.5 during the first two days after inoculation and remained at this level throughout the experiment. The pH of water incubated alone in seed cavities did not drop below 5.0.

3 Accumulation of phytoalexins in pod tissue following inoculation with species of *Botrytis* and *C. lindemuthianium*.

This experiment was carried out to determine if the phytoalexin response of *V. faba* is a general reaction to fungal infection, and also to examine the ability of different isolates of *B. cinerea* and *B. fabae* and of different species of *Botrytis* to induce phytoalexins and metabolize wyerone acid to reduced wyerone acid *in vivo*. *C. lindemuthianium* was included as an example of an unrelated fungus which is not a pathogen of *V. faba*.

Pods were prepared and inoculated with spore suspensions of the fungi or sterile distilled water. All fungi germinated and had caused browning of the tissue beneath the inoculum droplets 3 days after inoculation, when the tissue was collected. Only isolates of *B. fabae* had spread from the inoculation site into adjacent uninoculated tissue. Inoculum droplets and tissue from each treatment were combined for extraction.

Where as all the phytoalexins accumulated in tissue bearing restricted lesions, very low levels were detected at infection sites inoculated with isolates of *B. fabae* (Plate 8). Proportionately similar levels of each phytoalexin were recovered from tissues bearing limited lesions caused by the *Botrytis* spp. However, the concentrations of inhibitors were much greater in tissues inoculated with isolates of *B. cinerea* than with the other species.

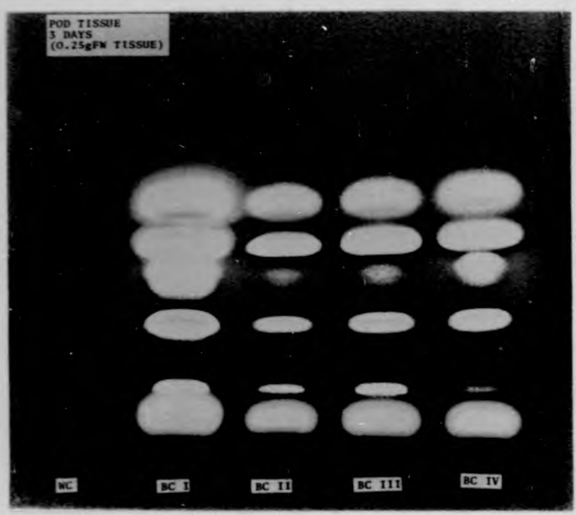
Plate 8

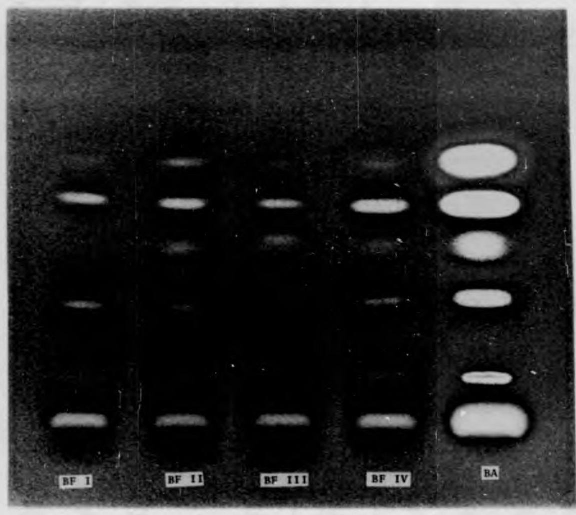
TLC plate bioassays of extracts from pod endocarp (0.25g) collected 3 days after inoculation with different species of *Botrytis* and *C. lindemuthianium*.

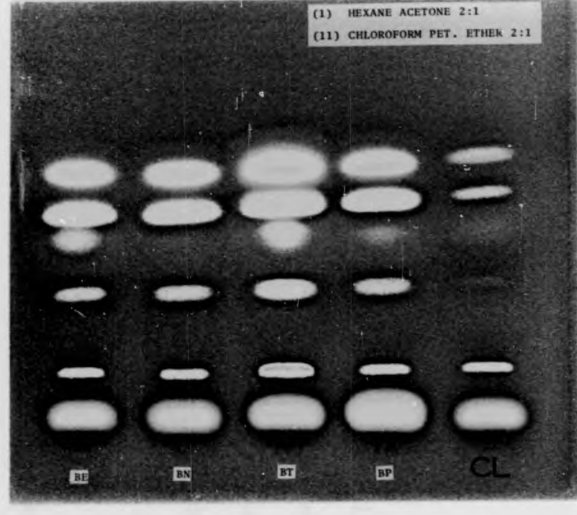
WC	-	water control		
BCI	-	*B. cinerea* isolate	1	(origin : broad beans)
BCII	-	" "	2	(" " ")
BCIII	-	" "	3	(" vine)
BCIV	-	" "	4	(" rose)
BFI	-	*B. fabae* "	1	(" broad beans)
BFII	-	" "	2	(" " ")
BFIII	-	" "	3	(" " ")
BFIV	-	" "	4	(" " ")
BA	-	*B. allii*		
BE	-	*B. elliptica*		
BN	-	*B. narcissicola*		
BT	-	*B.tulipae*		
BP	-	*B. paeoniae*		
CL	-	*C. lindemuthianium*		

Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). W, wyerone; WE, wyerone epoxide; WO, wyerol; PA3, medicarpin and PA3b; WA, wyerone acid.

Plate 8







(Table 10). In general phytoalexin concentration appeared to be correlated with tissue browning in limited lesions; symptoms were most marked at sites inoculated with B. cinerea and least evident following inoculation with C. lindemuthianium. Neither medicarpin nor PA3b were detected in tissues infected with C. lindemuthianium and reduced wyerone acid was detected only in lesions caused by B. fabae.

In conclusion it appears that multiple phytoalexin production by V. faba is a general response to fungal invasion. B. fabae was the only fungus able to spread from the inoculation site into adjacent uninoculated tissue and to metabolize wyerone acid to reduced wyerone acid in vivo.

4 Accumulation of phytoalexins in pod tissue and inoculum droplets following infection with B. cinerea, B. allii and B. fabae.

In the previous experiment species of Botrytis which are not pathogens of V. faba caused less browning of cells at the inoculation site, and induce lower levels of the phytoalexins than B. cinerea, which is essentially a weak pathogen. Since little cellular damage occurs on infection by these non-pathogenic fungi the possibility that the phytoalexins responsible for fungal inhibition are those which are present in the inoculum droplet rather than the tissue was investigated. B. allii was selected as an example of a non-pathogenic member of Botrytis. B. fabae was included as an example of a pathogen, and B. cinerea as an example of a weak pathogen, of V. faba.

Inoculum droplets and underlying tissue were collected and analysed for inhibitors 3 days after inoculating pod seed cavities with conidial suspensions of the fungi. In this experiment the degree of browning caused

TABLE 10 Yields (μg/g.f.w.)* of phytoalexins from pod tissue 3 days after inoculation with different *Botrytis* sp. and *C. lindemuthianium*.

<table>
<tr><th></th><th>WYERONE</th><th>WYERONE EPOXIDE</th><th>WYEROL</th><th>MEDICARPIN</th><th>PA3b†</th><th>WYERONE ACID</th><th>REDUCED WYERONE ACID</th></tr>
<tr><td>B. CINEREA isolates 1</td><td>114</td><td>68</td><td>46</td><td>13</td><td>0.67</td><td>144</td><td>-</td></tr>
<tr><td>" " 2</td><td>42</td><td>18</td><td>15</td><td>D</td><td>D</td><td>82</td><td>-</td></tr>
<tr><td>" " 3</td><td>108</td><td>59</td><td>19</td><td>11</td><td>0.58</td><td>98</td><td>-</td></tr>
<tr><td>" " 4</td><td>72</td><td>44</td><td>22</td><td>9</td><td>0.45</td><td>97</td><td>-</td></tr>
<tr><td>B. FABAE " 1</td><td>2</td><td>D</td><td>D</td><td colspan="2">D</td><td>D</td><td>49</td></tr>
<tr><td>" " 2</td><td>5</td><td>D</td><td>D</td><td colspan="2">D</td><td>D</td><td>66</td></tr>
<tr><td>" " 3</td><td>2</td><td>D</td><td>D</td><td colspan="2">D</td><td>D</td><td>56</td></tr>
<tr><td>" " 4</td><td>1</td><td>D</td><td>D</td><td colspan="2">D</td><td>D</td><td>62</td></tr>
<tr><td>B. TULIPAE</td><td>65</td><td>26</td><td>39</td><td>7</td><td>0.35</td><td>50</td><td>-</td></tr>
<tr><td>B. ELLIPTICA</td><td>42</td><td>15</td><td>31</td><td>D</td><td>D</td><td>40</td><td>-</td></tr>
<tr><td>B. ALLII</td><td>36</td><td>10</td><td>9</td><td>D</td><td>D</td><td>48</td><td>-</td></tr>
<tr><td>B. PAEONIA</td><td>39</td><td>15</td><td>31</td><td>D</td><td>D</td><td>63</td><td></td></tr>
<tr><td>B. NARCISSICOLA</td><td>47</td><td>14</td><td>20</td><td>D</td><td>D</td><td>37</td><td>-</td></tr>
<tr><td>C. LINDEMUTHIANIUM</td><td>12</td><td>D</td><td>4</td><td>-</td><td>-</td><td>33</td><td>-</td></tr>
</table>

* - mean of two experiments (other symbols as for Table 9)

by *B. allii* was much less than that recorded in the previous experiment. Microscopic observations showed that although *B. allii* conidia had germinated, little germ tube growth had been attained by the third day after inoculation.

Wyerone acid and wyerone epoxide were the major inhibitory bands detected in TLC plate bioassays of extracts of inoculum droplets from limited lesions caused by *B. allii* and *B. cinerea* (Plate 9B). As shown previously no inhibitors were present in inoculum droplets from spreading *B. fabae* lesions. Where as all the phytoalexins were detected in *B. cinerea* infected tissue, only traces of wyerone and wyerone acid were found in tissue infected with *B. allii*, (Plate 9A and Table 11).

Although wyerone epoxide produced an inhibitory band of similar intensity as wyerone acid on TLC plate bioassays the level of this inhibitor was much lower than that of wyerone acid. This anomaly was subsequently shown to be due to the greater activity of the epoxide against *C. herbarum* on TLC plates (Appendix IV).

This experiment provides evidence which suggests that restriction of *B. allii* and also perhaps *B. cinerea* to the inoculation site in pods may directly relate to the phytoalexins present in the inoculum droplet, of which wyerone acid is predominant.

5 Localization of wyerone in pod tissue after inoculation with *B. cinerea*.

In previous experiments very low levels of wyerone were detected in inoculum droplets despite the accumulation of the phytoalexin to concentrations $>100\mu g/g$.f.w. within certain infected pod tissues. This suggests that wyerone may be bound in some way within the tissue. This possibility was investigated

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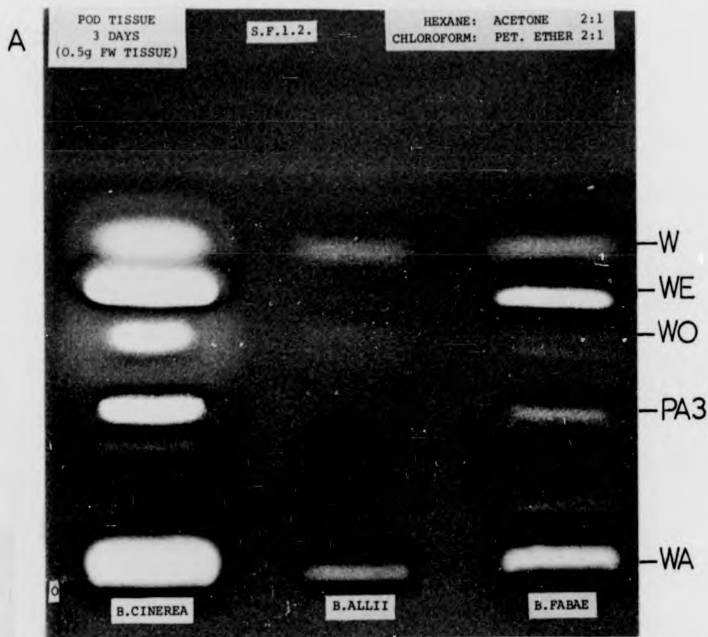
In previous experiments very low levels of wyerone were detected in inoculum droplets despite the accumulation of the phytoalexin to concentrations $>100\mu g/g$ f.w. within certain infected pod tissues. This suggests that wyerone may be bound in some way within the tissue. This possibility was investigated

Plate 9

TLC plate bioassays of extracts from pod tissue (0.5g) (A) and from inoculum droplets (equivalent to 0.5g)(B) collected three days after inoculation with B. cinerea, B. allii, or B. fabae. Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). W, wyerone; WE, wyerone epoxide; WO, wyerol; PA3, medicarpin and PA3b; WA, wyerone acid.

Plate 9

A



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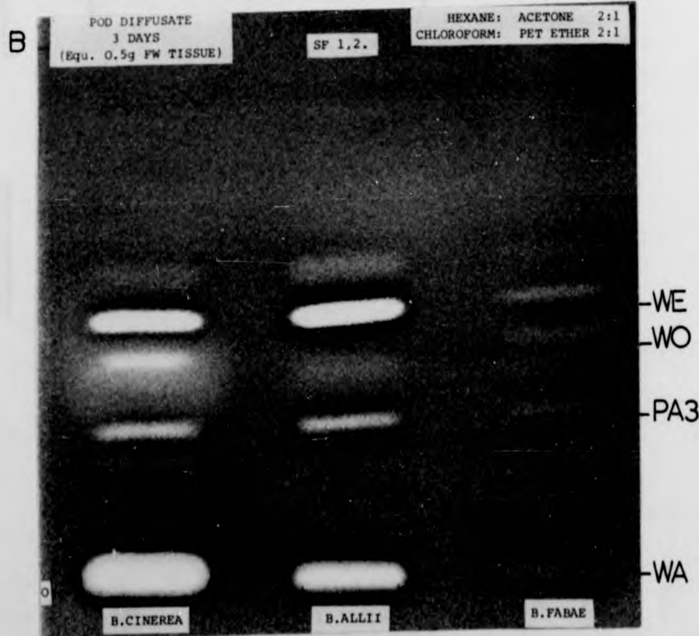

<u>TABLE 11</u> Yield (μg/g.f.w.) of phytoalexins from pod tissue (PT) and overlying inoculum droplets (ID) 3 days after inoculation with *B. cinerea*, *B. allii* and *B. fabae*.

	B. CINEREA		B. ALLII		B. FABAE	
	PT	ID	PT	ID	PT	ID
WYERONE	35	-	5	-	7	-
WYERONE EPOXIDE	29	4	-	3	2	-
WYEROL	15	4	-	-	-	-
MEDICARPIN	15	-	-	-	-	-
PA3b *	0.66	-	-	-	-	-
WYERONE ACID	93	40	2	10	5	-

* - yield AU λmax 347nm/g.f.w.

by examining the recovery of inhibitors at different stages during the fractionation of infected tissue. After removal of inoculum droplets, infected tissue was collected 6 days after inoculation with B. cinerea and divided into two batches (c 5g f.w. tissue each). One sample was extracted with MeOH and partitioned between Et_2O and water in the usual way, the other sample was fractionated as described on p.18 Fractions collected were: water washings, phosphate buffer fractions, and a crude cell wall fraction. Each fraction collected was compared with the whole tissue on TLC plate bioassays (Plate 10) and the yields of wyerone and wyerone acid from each fraction determined (Table 12).

Wyerone was detected mainly in the cell wall preparation. Analysis of the yields of wyerone and wyerone acid showed that the combined yields of wyerone recovered from fractionated tissue was considerable less than that from comparable MeOH extracts, whereas the combined yields of wyerone acid in each fraction were much higher than that from MeOH extracts. The highest yields of wyerone acid were detected in water washings of intact tissue. The loss of 98 μg/g.f.w. of wyerone was associated with the appearance of 114 μg/g.f.w. of wyerone acid, suggesting that wyerone was converted to wyerone acid during this extraction procedure. Experiments carried out to investigate the conversion of wyerone to wyerone acid are described in Appendix V.

In TLC plate bioassays wyerone acid was detected in all fractions, whereas the components of PA3 were only present in the distilled water washing and phosphate buffer fractions. The detection of the unknown phytoalexin, PA4, in the phosphate buffer and cell wall fractions suggests that this inhibitor, like wyerone may be closely associated with cell walls.

Plate 10

TLC plate bioassay of fractions of 1g 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 residual cell walls were compared with an extract of the original tissue (whole tissue) Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). W, wyerone; WE, wyerone epoxide; PA3, medicarpin and PA3b; PA4 unidentified phytoalexin; WA, wyerone acid.

Plate 10

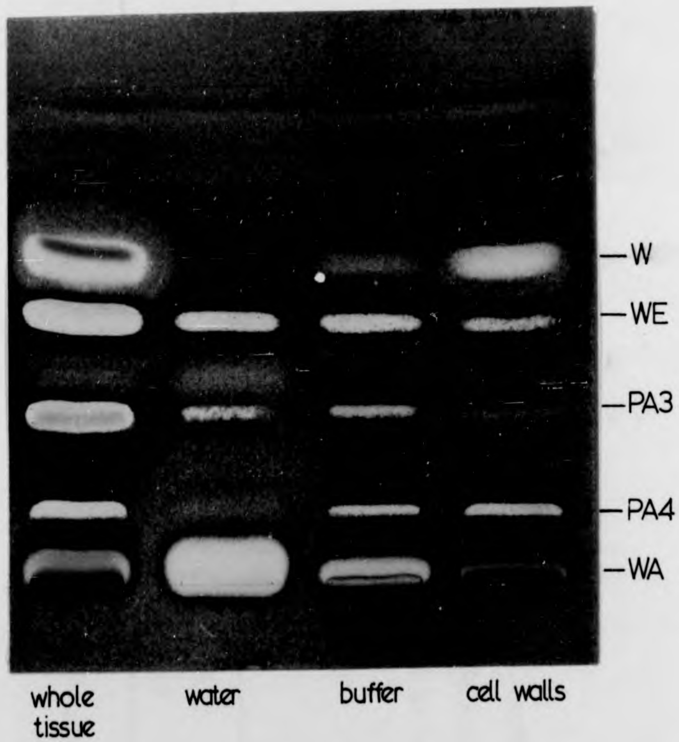

TABLE 12 Yields (μg/g.f.w.) of wyerone and wyerone acid recovered from whole pod and fractionated pod tissue 6 days after inoculation with B. cinerea.

	WHOLE TISSUE	WATER[a]	BUFFER[b]	CELL WALLS[c]	COMBINED FRACTIONS
WYERONE	159	2	8	51	61
WYERONE ACID	85	143	48	8	199

a - water washings of whole tissue.

b - phosphate buffer (pH7.0) extract of washed tissue.

c - cell wall residue.

TABLE 12 Yields (μg/g.f.w.) of wyerone and wyerone acid recovered from whole pod and fractionated pod tissue 6 days after inoculation with *B. cinerea*.

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a - water washings of whole tissue.

b - phosphate buffer (pH7.0) extract of washed tissue.

c - cell wall residue.

CHAPTER 4

The antifungal activity of phytoalexins from V. faba towards Botrytis.

Mansfield (1972) reported that wyerone acid was inactive against B. cinerea in distilled water and Deverall and Rodgers (1972) showed that the antifungal activity of wyerone acid was affected by pH and also unknown components of natural media. In previous studies pod nutrients, comprised of the residual phase recovered after Et_2O extraction of inoculum droplets collected 24 hours after inoculation of pod seed cavities with B. cinerea has been used as a medium for bioassays (Deverall, 1967; Mansfield and Deverall, 1974 b). Preliminary experiments showed that different batches of pod nutrient solutions varied in their ability to support the growth of Botrytis. In order to standardize tests on antifungal activity of phytoalexins a synthetic medium was therefore devised which was considered to approach the nutrient conditions prevailing within infected tissue.

The medium developed for bioassays, synthetic pod nutrients (SPN), was based on the analysis of inoculum droplets carried out by Deverall(1967). SPN solutions adjusted to pH 3.5, 4.0, 4.5, and 5.0 with either galacturonic acid or H_3PO_4 promoted both germination and germ tube growth by B. cinerea and B. fabae (Table 13). Phytoalexins were added to SPN solutions in MeOH (final concentration 0.33% MeOH).

1 Antifungal activity of wyerone acid.

A series of bioassays were carried out with different concentrations of wyerone acid (0 to 40 μg/ml) in SPN at pH values ranging from 3.5 - 5.0.

TABLE 13 Germ tube growth (GTG, µm) and germination (G,%) of B. cinerea and B. fabae in SPN solutions at different pH values, adjusted with either galacturonic acid or H_3PO_4.

	GALACTURONIC ACID				H_3PO_4			
	B. CINEREA		B. FABAE		B. CINEREA		B. FABAE	
pH	GTG	G	GTG	G	GTG	G	GTG	G
5.0	213	98-100	228	98-100	182	98-100	234	98-100
4.5	217	98-100	239	98-100	226	98-100	251	98-100
4.0	185	98-100	222	98-100	221	98-100	249	98-100
3.5	200	98-100	229	98-100	210	98-100	248	98-100

The percentage reduction in germination of B. cinerea and B. fabae conidia, as compared to controls, at the highest wyerone acid concentration examined (40μg/ml) is recorded in Table 14. The activity of wyerone acid against germ tube growth by B. cinerea and B. fabae in media of different pH values are illustrated in Figs. 28 and 29 respectively, where the percentage reduction in germ tube growth is plotted against Log. phytoalexin concentration. ED_{50}s for the activity of wyerone acid against germ tube growth and by extrapolation the minimum concentration of acid which prevented germ tube growth by B. cinerea and B. fabae were determined from these graphs. The effect of pH (adjusted with either galacturonic acid or H_3PO_4) on these parameters is shown in Fig.30.

At all pH values wyerone acid was more active against germination and germ tube growth of B. cinerea than B. fabae. With decreasing pH the activity of wyerone acid increased against both fungi. However, whereas the relationship between pH and activity against B. cinerea appeared to be Log. linear, in that against B. fabae the difference in activity between pH 3.5 and 4.0 was much less than the difference between pH 4.5 and 5.0. This suggests that the inactivity of wyerone acid at higher pH values against B. fabae may not be solely due to a direct effect of pH on the dissociation of wyerone acid.

The results obtained after adjusting the pH of solutions with either galacturonic acid or H_3PO_4 were virtually identical. Therefore, it was decided that in subsequent experiments pH would be adjusted with galacturonic acid, since this substance occurs at relatively high levels in diffusates from B. cinerea infected pod seed cavities (Deverall, 1967).

2 Antifungal activity of wyerone and wyerone epoxide.

A series of bioassays were carried out with a range of concentrations

The percentage reduction in germination of B. cinerea and B. fabae conidia, as compared to controls, at the highest wyerone acid concentration examined (40μg/ml) is recorded in Table 14. The activity of wyerone acid against germ tube growth by B. cinerea and B. fabae in media of different pH values are illustrated in Figs. 28 and 29 respectively, where the percentage reduction in germ tube growth is plotted against Log. phytoalexin concentration. ED_{50}S for the activity of wyerone acid against germ tube growth and by extrapolation the minimum concentration of acid which prevented germ tube growth by B. cinerea and B. fabae were determined from these graphs. The effect of pH (adjusted with either galacturonic acid or H_3PO_4) on these parameters is shown in Fig.30.

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2 Antifungal activity of wyerone and wyerone epoxide.

A series of bioassays were carried out with a range of concentrations

TABLE 14 Percentage reduction in germination of *B. cinerea* and *B. fabae* conidia in SPN solutions containing 40 µg/ml wyerone acid at different pH values, adjusted with either galacturonic acid or H_3PO_4.

	GALACTURONIC ACID		H_3PO_4	
	B. CINEREA	B. FABAE	B. CINEREA	B. FABAE
5.0	0	0	0	0
4.5	4	5	0	0
4.0	96	74	85	27
3.5	100	81	100	79

Figure 28

The effect of pH on the antifungal activity of wyerone acid in SPN solutions against germ tube growth of *B. cinerea*. The pH of the solutions was adjusted with either galacturonic acid, (A) or H_3PO_4,(B).

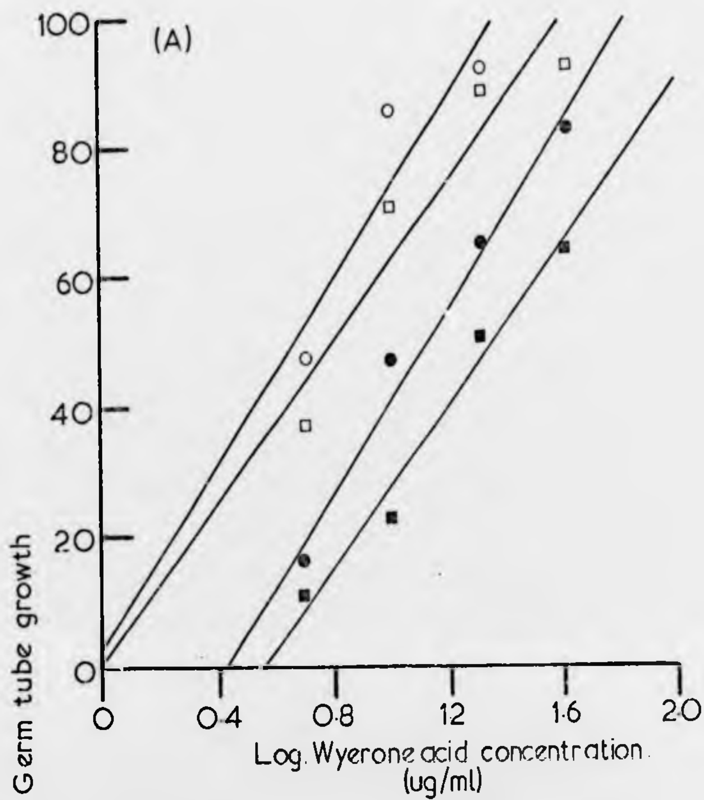
○ pH3.5

□ pH4.0

● pH4.5

■ pH5.0

Fig.28



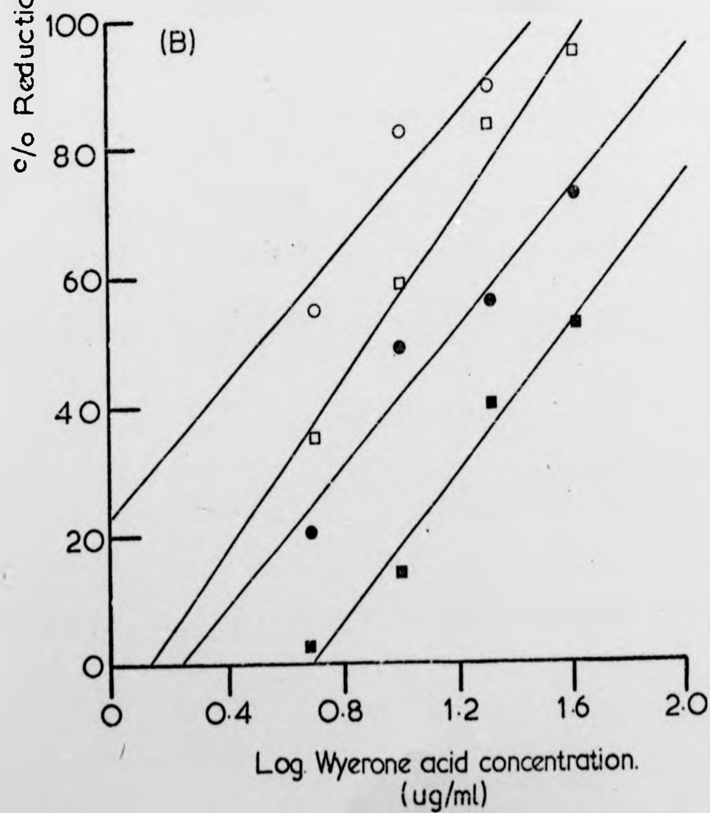


Figure 29

The effect of pH on the antifungal activity of wyerone acid in SPN solutions against germ tube growth of B. fabae. The pH of the solutions was adjusted with either galacturonic acid (A) or H_3PO_4 (B).

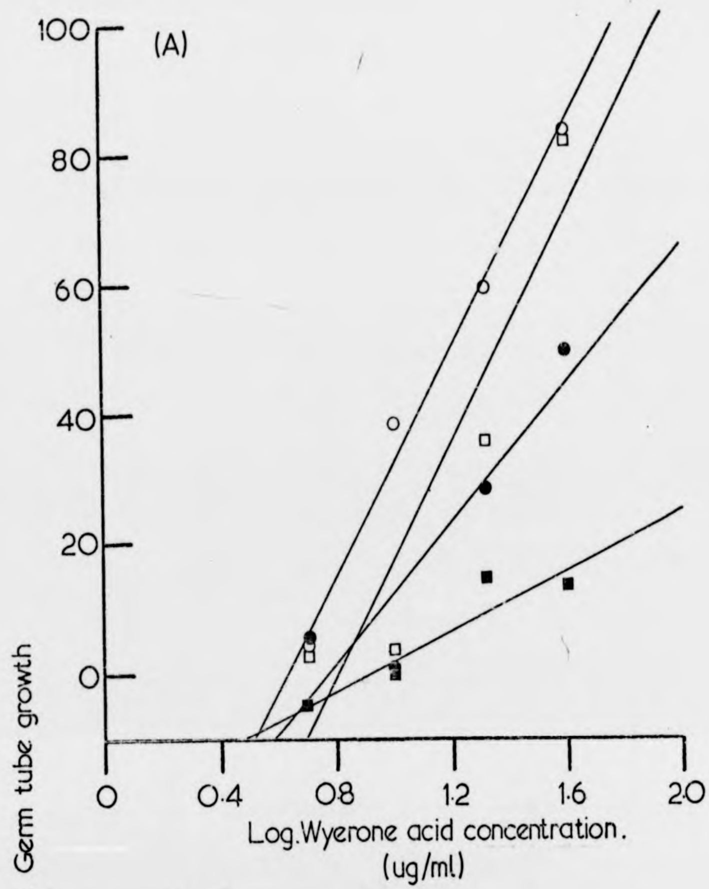
O pH3.5

□ pH4.0

● pH4.5

■ pH5.0

Fig.29



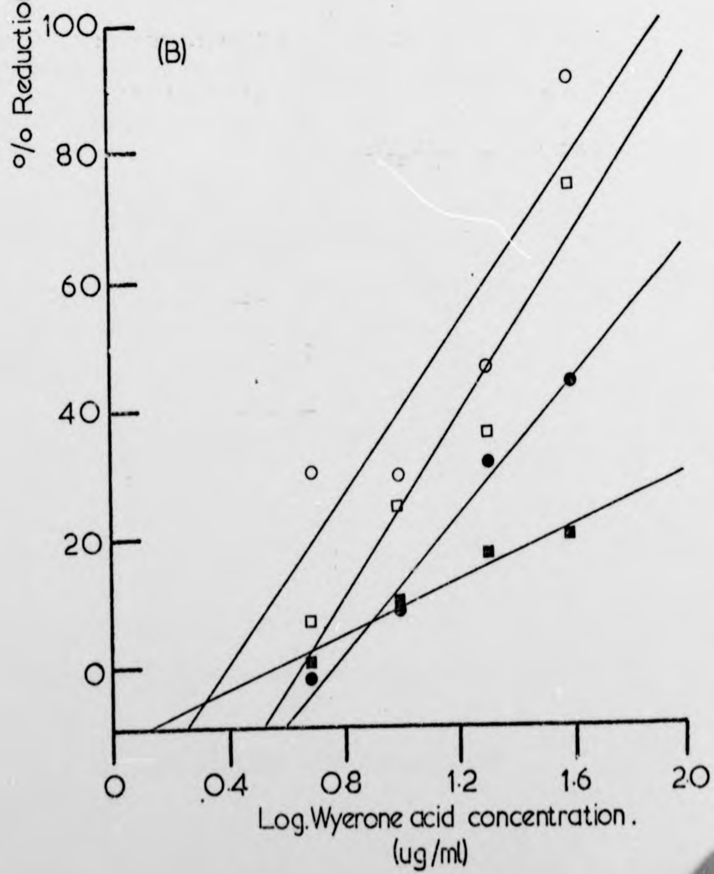


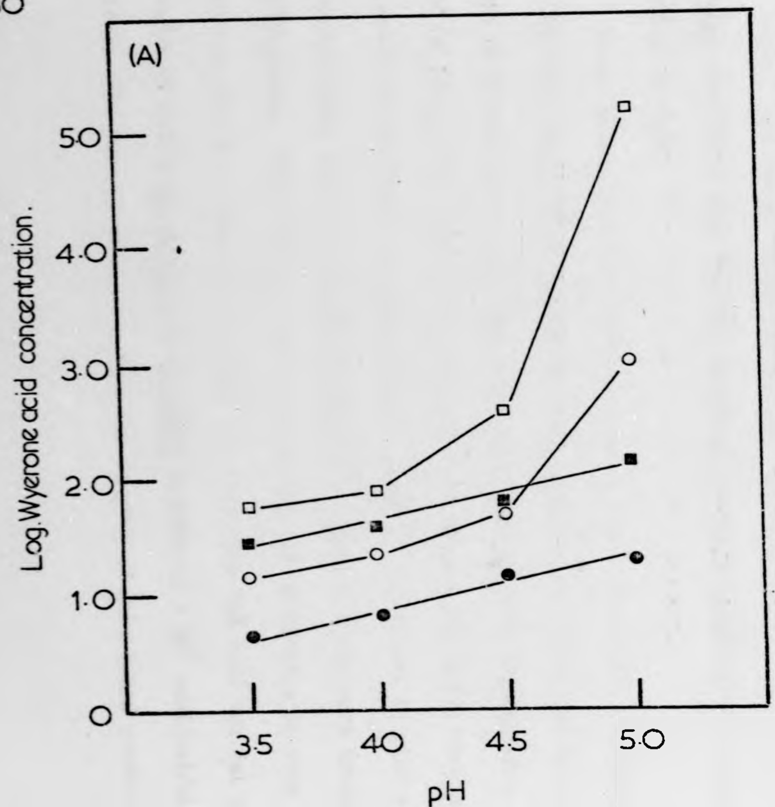
Figure 30

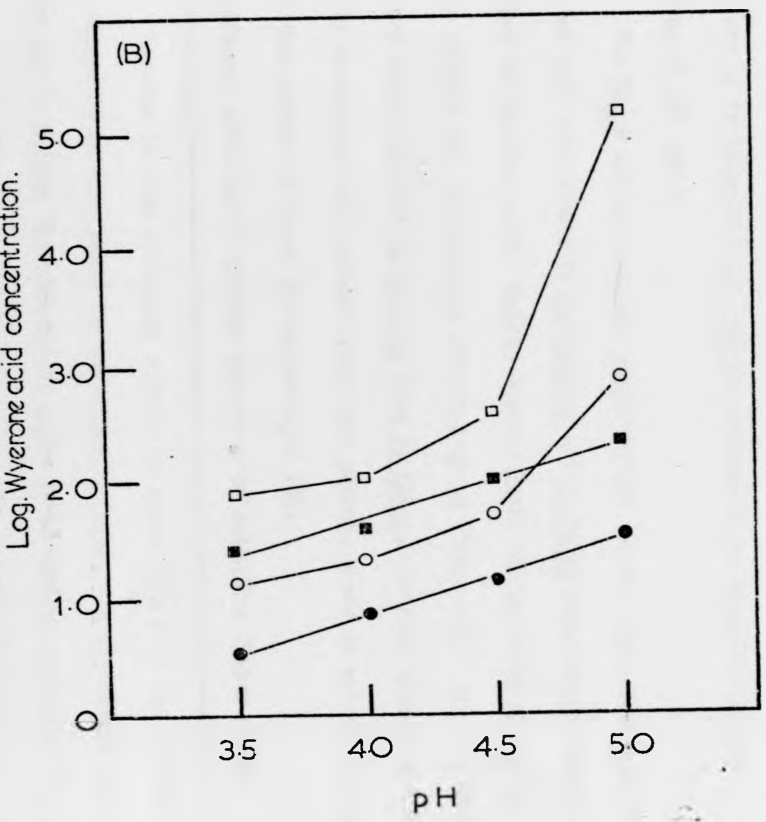
The effect of pH on the ED50 and minimum concentration of wyerone acid which prevents germ tube growth of *B. cinerea* and *B. fabae*. The pH of the solution was adjusted with either galacturonic acid (A) or H_3PO_4 (B).

● ○ ED50 against [*B. cinerea* / *B. fabae*] germ tube growth

■ □ minimum concentration which prevents [*B. cinerea* / *B. fabae*] germ tube growth.

Fig.30





of wyerone (0 to 100μg/ml) and wyerone epoxide (0 to 40 μg/ml) in SPN solutions at pH4 and 5.

The ED_{50}S and the minimum concentrations of the phytoalexins which prevented germ tube growth by B. cinerea and B. fabae were determined as described for wyerone acid. Unlike wyerone acid, the activity of wyerone and wyerone epoxide was not markedly affected by pH (Table 15). Both phytoalexins were more active against B. cinerea than B. fabae. Wyerone epoxide had similar activity as wyerone acid against germ tube growth, where as wyerone was less active than either of these phytoalexins, at pH4.

3 Antifungal activity of wyerone deposited on cellulose filter paper.

Wyerone is only sparingly soluble in water (<5μg/ml) and appears to be deposited on cell walls in the infected plant. An experiment was carried out to examine the antifungal activity of wyerone deposited on cellulose filter paper. The use of deposited wyerone was considered to be more analogous to in vivo conditions than the use of wyerone held in solution by low concentrations of MeOH.

Discs (0.4mm diameter) were cut with a paper punch from chromatography paper (Whatman No.1) after papers had been washed with dilute ammonia solution followed by dilute acetic acid and dried overnight at room temperature. Wyerone in $CHCl_3$ at concentrations ranging from 100 to 1000 μg/ml were added in 5μl aliquots to the discs on glass slides, under an airstream, to give a range of concentrations from 3 to 30 μg wyerone/cm^2. Control discs were treated with 5 μl $CHCl_3$ alone. After leaving the discs to dry for 30 minutes at room temperature they were covered with 10μl SPN solutions (pH 4.0) and 5μl spore suspension of either B. cinerea or B. fabae conidia (1×10^5 conidial/ml SPN solution). The percentage germination and lengths of germ tubes produced by

TABLE 15 Antifungal activities of wyerone acid, wyerone and wyerone epoxide in SPN solutions against germ tube growth of B. cinerea and B. fabae conidia at pH4 and pH5.

(ug/ml)	pH4		pH5	
	B. CINEREA	B. FABAE	B. CINEREA	B. FABAE
ED_{50} against germ tube growth				
WYERONE ACID	6.1	23.3	33.8	1230
WYERONE	18.1	28.8	20.6	34.6
WYERONE EPOXIDE	6.4	20.0	5.6	16.0
Minimum concentration which prevents all germination				
WYERONE ACID	41.3	84.1	144.5	18400
WYERONE	91.6	450	120.8	602
WYERONE EPOXIDE	46.6	102	28.7	75.2

conidia were recorded after incubation for 18 hours at 18°C in the dark.

Germination and germ tube growth were both affected by deposited wyerone (Fig.31). However, the effect against germ tube growth was more pronounced than against germination. For example, on discs containing 3µg/cm^2 mean germ tube length was reduced 58 and 86%, whereas, germination was reduced only 2 and 29% for B. fabae and B. cinerea respectively. These results suggest that germ tube elongation was selectively inhibited. This conclusion was supported by microscopical examination of the bioassays. On germinating, germ tubes produced by conidia of both fungi appeared to be inhibited when they came into contact with cellulose fibrils coated with wyerone (Plate 11). As in the previous experiment B. fabae was less sensitive than B. cinerea to wyerone.

4 The effect of combinations of wyerone acid and wyerone epoxide on the growth of B. cinerea and B. fabae germ tubes.

Previous experiments had shown that wyerone acid and wyerone epoxide were the major inhibitors present in inoculum droplets collected from B. cinerea infected pod seed cavities. This experiment was carried out to determine if the acid and the epoxide interacted additively or synergistically in preventing the growth of Botrytis.

Bioassays involving a de-Wit combination (Donald, 1963) of these two phytoalexins were carried out. The total concentrations of the phytoalexins were calculated to give less than 50% inhibition of germ tube growth and no inhibition of germination, thus two levels of phytoalexin concentration were employed, a high level (10µg/ml) for B. fabae and a low level (5µg/ml) for B. cinerea. Figure 32 illustrates the % reduction in the growth of

Figure 31

The effect of wyerone, deposited on filter paper on the germination and germ tube growth of B. cinerea and B. fabae in SPN solutions.

Symbol	Species	Measure
●	B. cinerea	germination
■	B. fabae	germination
○	B. cinerea	germ tube growth
□	B. fabae	germ tube growth

At 30µg/cm^2 wyerone completely inhibited the germination of B. cinerea conidia.

Fig.31

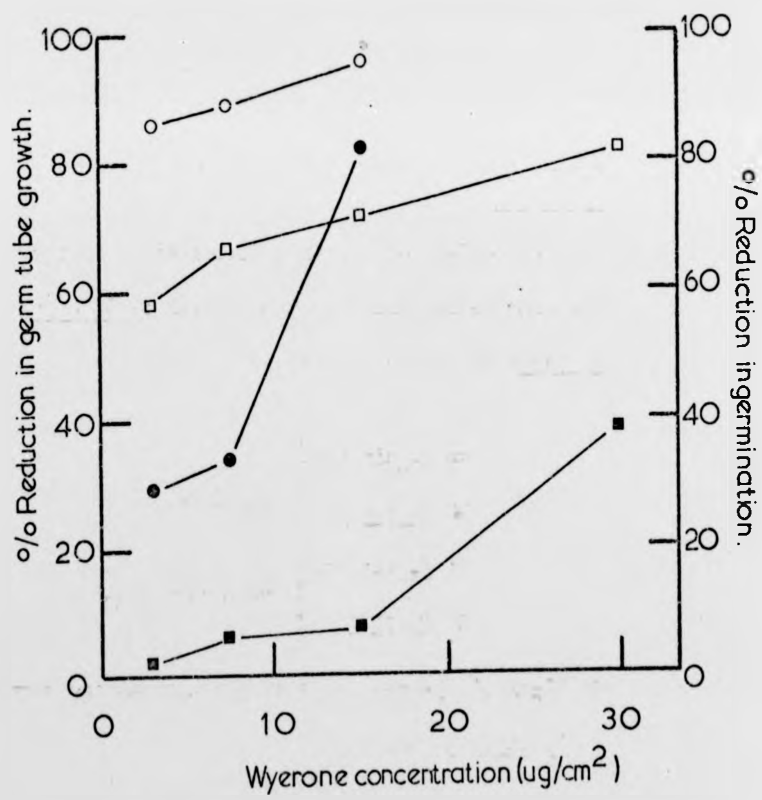


Plate 11

The growth of B. cinerea and B. fabae conidia on filter paper in the presence and absence of wyerone

A. The growth of B. cinerea on filter paper in the presence of 7.5µg wyerone/cm^2, germ tubes appeared to be inhibited when they came into contact with the cellulose fibrils.

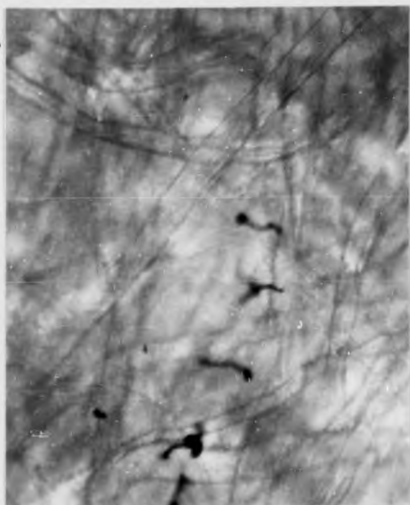
B. The growth of B. cinerea on filter paper in the absence of wyerone.

C. The growth of B. fabae on filter paper in the presence of 30µg wyerone/cm^2, germ tubes appeared to be inhibited when they came into contact with the cellulose fibrils.

D. The growth of B. fabae on filter paper in the absence of wyerone.

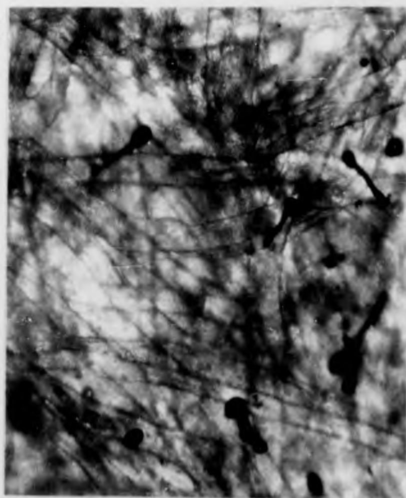
Plate 11

A



B

C



D

Figure 32

The effect of combinations of wyerone acid (WA) and wyerone epoxide (WE) in SPH solutions, on the growth of *B. cinerea* and *B. fabae* germ tubes.

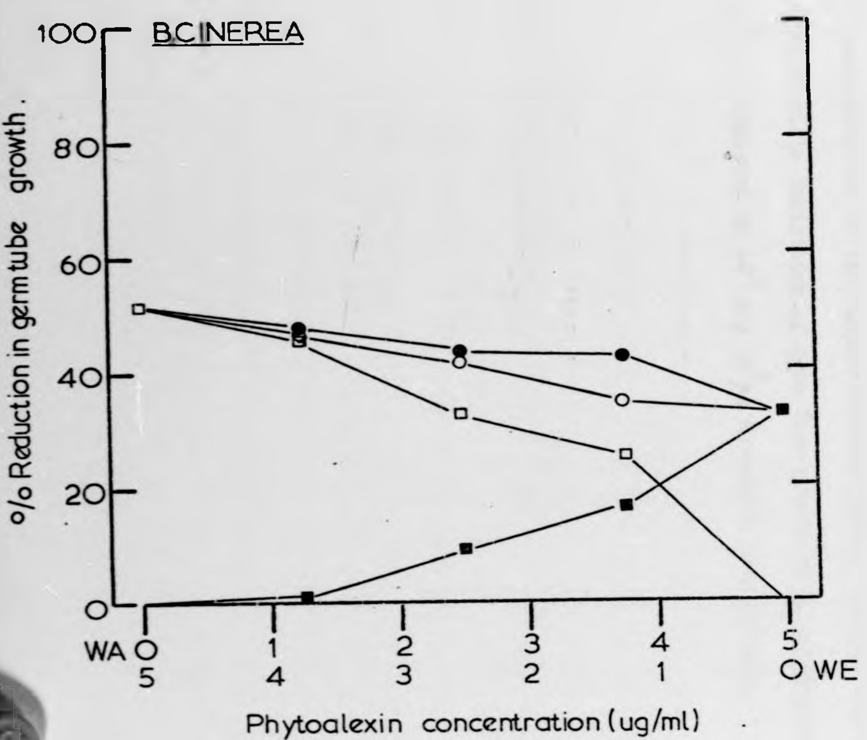
■ wyerone acid alone.

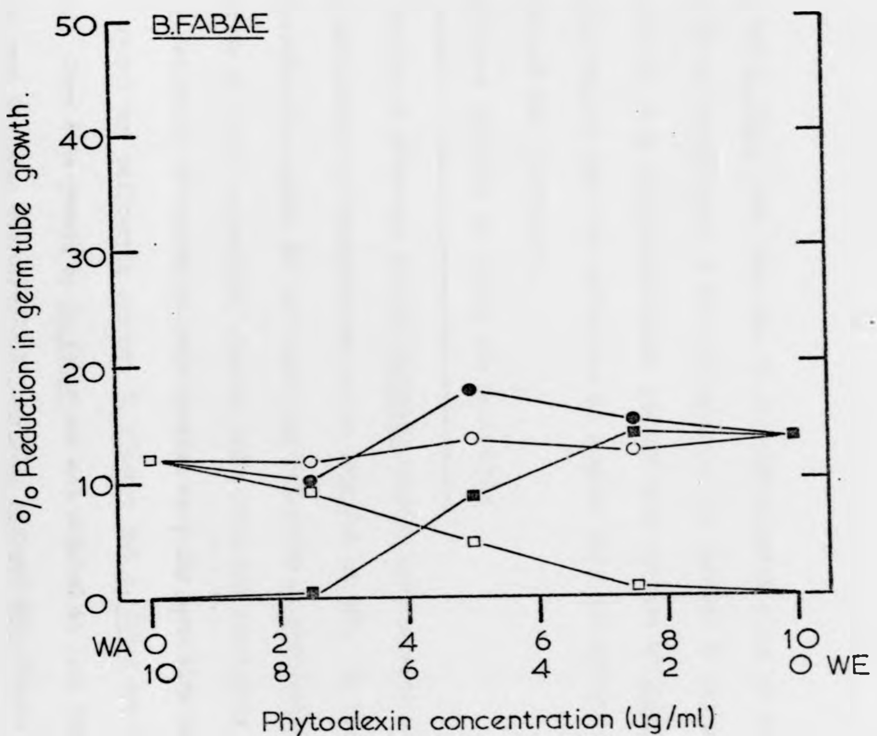
□ wyerone epoxide alone.

○ combination of wyerone acid and wyerone epoxide.

● expected reduction in germ tube growth if the effect of the phytoalexins additive.

Fig.32





B. cinerea and B. fabae germ tubes due to the phytoalexins alone or incombination. The activities of combination of the phytoalexins was similar to the sum of the activities of each phytoalexin alone against both species of Botrytis. These results suggest that the activities of wyerone acid and wyerone epoxide are additive and not synergistic.

5 The antifungal activity of wyerol and medicarpin

A series of bioassays against Botrytis conidia were carried out with wyerol and medicarpin at concentrations up to 100μg/ml at pH4. At the highest concentration wyerol did not cause any reduction in germination recorded after 18 hours incubation, whereas medicarpin had completely prevented germination of conidia of both species over the same time period. The ED_{50} for wyerol and medicarpin against B. cinerea and B. fabae are given in Table 16. Germ tube growth by B. fabae was not reduced to less than 50% of controls even in 100μg wyerol/ml S N, the ED_{50} against this fungus was estimated by extrapolation of the dosage/response curve. Further extrapolation indicated that complete inhibition of germ tube growth by B. cinerea and B. fabae would be achieved by 10^3 and 10^5μg wyerol/ml respectively.

TABLE 16 Antifungal activities of wyerol and medicarpin in SPN solutions against germ tube growth of *B.cinerea* and *B.fabae* conidia at pH4.

(ug/ml)	B.CINEREA	B.FABAE
ED_{50} against germ tube growth.		
WYEROL	75.9	1905
MEDICARPIN	8.4	10.4
Minimum concentration which prevents all germination.		
WYEROL	1620	–
MEDICARPIN	79.3	175.4

CHAPTER 5

Investigation of the metabolism of phytoalexins by *B. cinerea* and *B. fabae*

It was shown in Chapter 3 that very little phytoalexin accumulation occurs in pod tissues invaded by *B. fabae*, whereas high concentrations of the inhibitors are reached in endocarp undergoing a resistant response to infection by *B. cinerea*. The low levels of phytoalexins recovered after inoculation with *B. fabae* could result from 1) the failure of *B. fabae* to induce their biosynthesis, 2) suppression of phytoalexin synthesis, or 3) metabolism of the phytoalexins as they are produced.

An initial increase in phytoalexin concentrations within pod tissue after inoculation with *B. fabae* was followed by a decrease as inoculation sites became completely blackened and colonized by the fungus. A similar pattern has been shown for changes in wyerone acid concentrations in leaves after infection by *B. fabae* (Mansfield and Deverall, 1974 b). This evidence suggests that metabolism of the phytoalexins may play an important role in the pathogenicity of *B. fabae*.

A previous report showed that growing germ tubes of *B. fabae* can detoxify wyerone acid at a greater rate than those of *B. cinerea* *in vitro* (Mansfield and Widdowson, 1973). The metabolite of wyerone acid was identified as reduced wyerone acid (Fig. 3) and shown to accumulate in *B. fabae* inoculum droplets incubated in pod seed cavities (Mansfield and Widdowson, 1973; Mansfield *et al*., 1973)

In the following series of experiments the hypothesis that differences in rates of and/or products of phytoalexin metabolism could afford some basis for explaining the differential pathogenicity of *B. cinerea* and *B. fabae* has

been investigated. The metabolism of wyerone and wyerone epoxide has been examined and detoxification of wyerone acid by B. cinerea and B. fabae re-investigated. In these studies it was believed that a knowledge of the chemical structure, antifungal activity and rates of production and accumulation of the metabolites in vitro was required prior to investigating the detoxification process in vivo.

1 Metabolism of wyerone.

A Metabolism of wyerone by germinating conidia.

Aliquots of sterile SPN solutions containing conidia of B. cinerea and B. fabae or SPN solutions alone were added to solutions of wyerone (final concentration 14 μg/ml SPN) or SPN alone in 100ml conical flasks and incubated in the dark at 18°c. After incubation for 24 hours, triplicate solutions were extracted with Et_2O. Examples of the UV absorption spectra of Et_2O extracts in MeOH are shown in Fig. 33.

Wyerone (λmax 350nm) had completely disappeared from solutions containing germinating conidia of both B. cinerea and B. fabae. Loss of wyerone was associated with the appearance of 310 nm absorbing substances in cultures of both fungi. Neither fungus produced substantial amounts of UV absorbing Et_2O soluble substances in SPN solutions alone. Examination of the incubation flasks after extraction of the cultures showed that both B. cinerea and B. fabae conidia had produced a mesh of hyphae after incubation in the presence or absence of the phytoalexin. On the basis of UV absorbance of Et_2O extracts 84% of the wyerone added was recovered from solutions incubated without conidia.

Et_2O extracts from three replicate flasks of wyerone solutions incubated with and without conidia were combined and examined by TLC. Extracts (equivalent to 3ml culture solution) were applied to 2.5cm origins in MeOH and chromatograms developed in hexane : acetone (2:1, 15cm). Examination

been investigated. The metabolism of wyerone and wyerone epoxide has been examined and detoxification of wyerone acid by *B. cinerea* and *B. fabae* re-investigated. In these studies it was believed that a knowledge of the chemical structure, antifungal activity and rates of production and accumulation of the metabolites *in vitro* was required prior to investigating the detoxification process *in vivo*.

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Et_2O extracts from three replicate flasks of wyerone solutions incubated with and without conidia were combined and examined by TLC. Extracts (equivalent to 5ml culture solution) were applied to 2.5cm origins in MeOH and chromatograms developed in hexane : acetone (2:1, 15cm). Examination

Figure 33

UV absorption spectra of Et_2O extracts, in 5ml MeOH, from SPN solutions incubated for 24 hours in the presence or absence of wyerone (14μg/ml) and conidia of either _B. cinerea_ or _B. fabae_.

(1) Wyerone solution incubated without conidia.

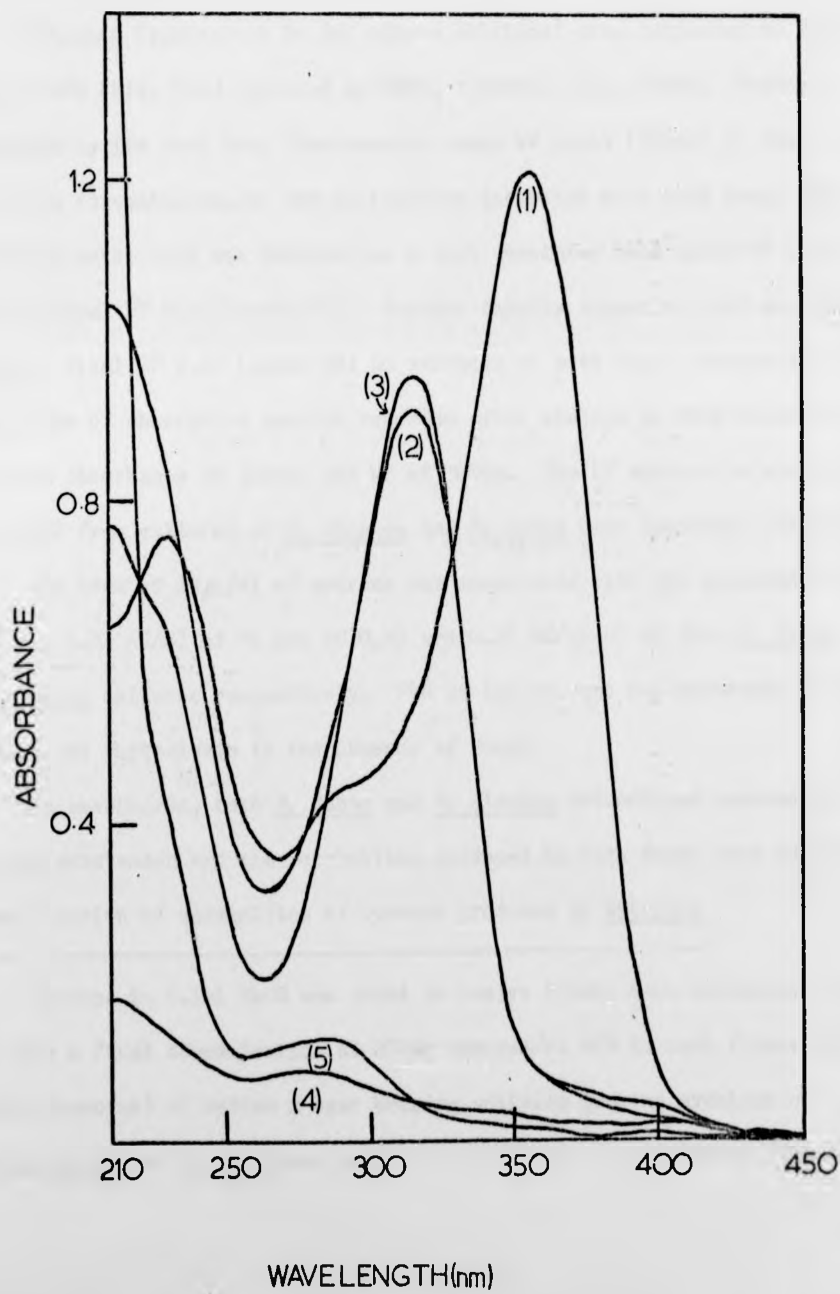
(2) " " " with _B. fabae_ conidia.

(3) " " " " _B. cinerea_ conidia.

(4) SPN solutions incubated with _B. fabae_ conidia.

(5) " " " " _B. cinerea_ conidia.

Fig.33



under UV light (254nm) revealed that both B. cinerea and B. fabae cultures the disappearance of wyerone (RF 0.45) was associated with the appearance of a dark quenching band (RF 0.35). Developed chromatograms bioassayed with C. herbarum showed wyerone, present in extracts of solutions incubated without conidia, to be the only inhibitor detected.

Extracts (equivalent to 6ml culture solution) were subjected to TLC in Et_2O : MeOH (6:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm). Wyerone was detected by its dark blue fluorescence under UV light (366nm) at final RF 0.71. In chromatograms of the phytoalexin incubated with both fungi the major fungal metabolite was detected as a dark quenching band under UV light (254nm) at final RF 0.55 (named W1). Another faintly quenching band was also detected at final RF 0.42 (named W2) in extracts of both fungi incubated with wyerone. The UV absorption spectra recorded after elution in MeOH showed W1 had maximum absorbance at 312nm, and W2 at 300nm. The UV absorption spectra of W1 and W2 from cultures of B. cinerea and B. fabae were identical (Fig.34)

The loss of 14µg/ml of wyerone was associated with the appearance of 0.78 and 1.22 AU/ml of W1 and of 0.42 and 0.18 AU/ml of W2 from B. fabae and B. cinerea cultures respectively. 74% of the wyerone was recovered after incubating the phytoalexin in the absence of fungi.

In conclusion, both B. fabae and B. cinerea metabolized wyerone to less inhibiting substances and the metabolites produced by both fungi were identical.

B Identification of metabolites of wyerone produced by Botrytis.

Wyerone in 0.1ml MeOH was added to twelve flasks each containing 20ml SPN to give a final concentration of 200µg wyerone/ml SPN in each flask. One disc (5mm diameter) of medium x agar bearing actively growing mycelium of either B. cinerea or B. fabae was added to each flask. Six cultures of each

Figure 34

UV absorption spectra of the metabolies, W1 (1), and W2 (2), of wyerone produced by _B. fabae_,(A) and _B. cinerea_, (B).

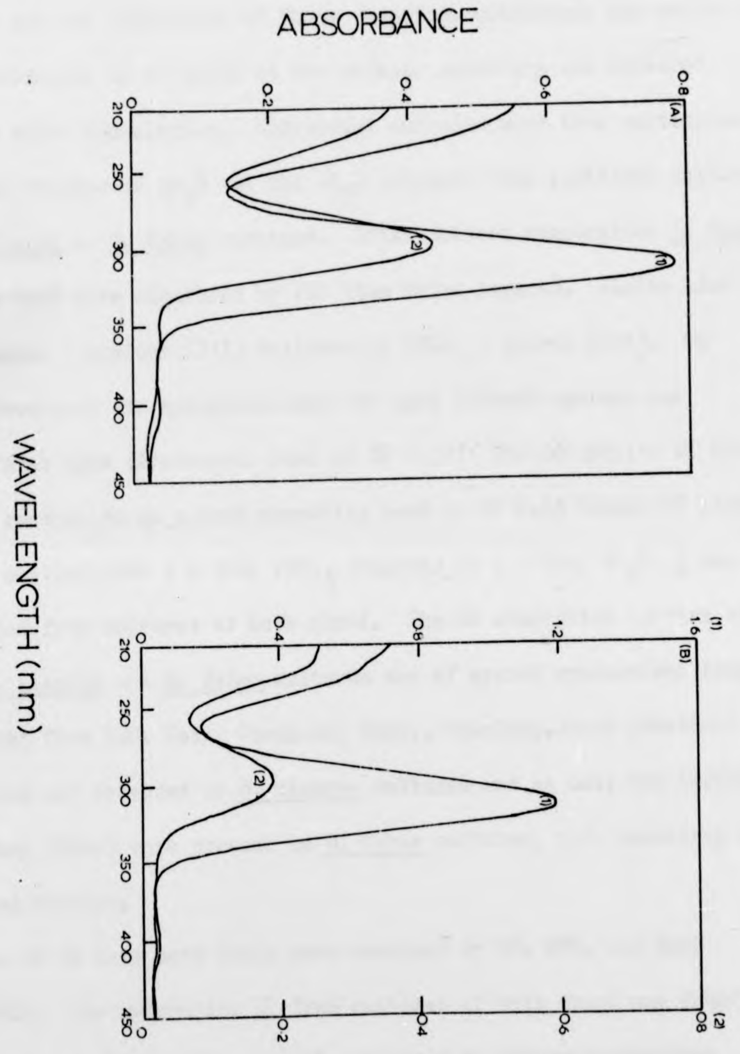


Fig.34

fungus were incubated at $18^{o}c$ in the dark on an orbital incubator (200 rev/min). Loss of wyerone and the appearance of 310nm absorbing substances was monitored by UV spectrophotometry of aliquots of the bathing solutions and appeared complete 4 days after inoculation. Individual cultures were then partitioned twice with equal volumes of Et_2O and the Et_2O extracts from replicate cultures of either B. cinerea or B. fabae combined. After solvent evaporation in vacuo the extracts in MeOH were separated by PLC (1mm thick layers). Plates were developed in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). On examining the developed chromatograms under UV light (366nm) wyerone was detected as a faint blue fluorescent band at RF 0.72. The metabolite W1 from both fungi was recognised as a dark quenching band at RF 0.63 (under UV light 254nm). After elution with 2 x 50ml $CHCl_3$ followed by 1 x 50ml Et_2O, c 6mg of W1 were recovered from cultures of both fungi. The UV absorption spectra of W1 from both B. cinerea and B. fabae cultures and of wyerol synthesised from wyerone (obtained from R.O. Cain, Chemistry Dept., Stirling,)were identical (Fig.35). W2 was not detected in B. cinerea cultures and as only low levels (0.17 AU/ml, λmax 300nm) were present in B. fabae cultures, this substance was not investigated further.

Samples of W1 from both fungi were examined by IR, NMR, and mass spectrometry (MS). The metabolite W1 from cultures of both fungi was found to be identical to the hydroxy ester, wyerol, prepared by sodium borohydride reduction of wyerone.

Mass spectra gave M^+ 260.1066 ($C_{15}H_{16}O_{14}$ 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 wyerone and W1 (Fig.36) clearly indicates the loss of the strong ketone absorption (1634 cm^{-1}) and

Figure 35

An example of the UV absorption spectra of the wyerone metabolite (W1) from *Botrytis* cultures incubated with wyerone, and of synthetic wyerol.

Figure 35

An example of the UV absorption spectra of the wyerone metabolite (W1) from *Botrytis* cultures incubated with wyerone, and of synthetic wyerol.

Fig.35

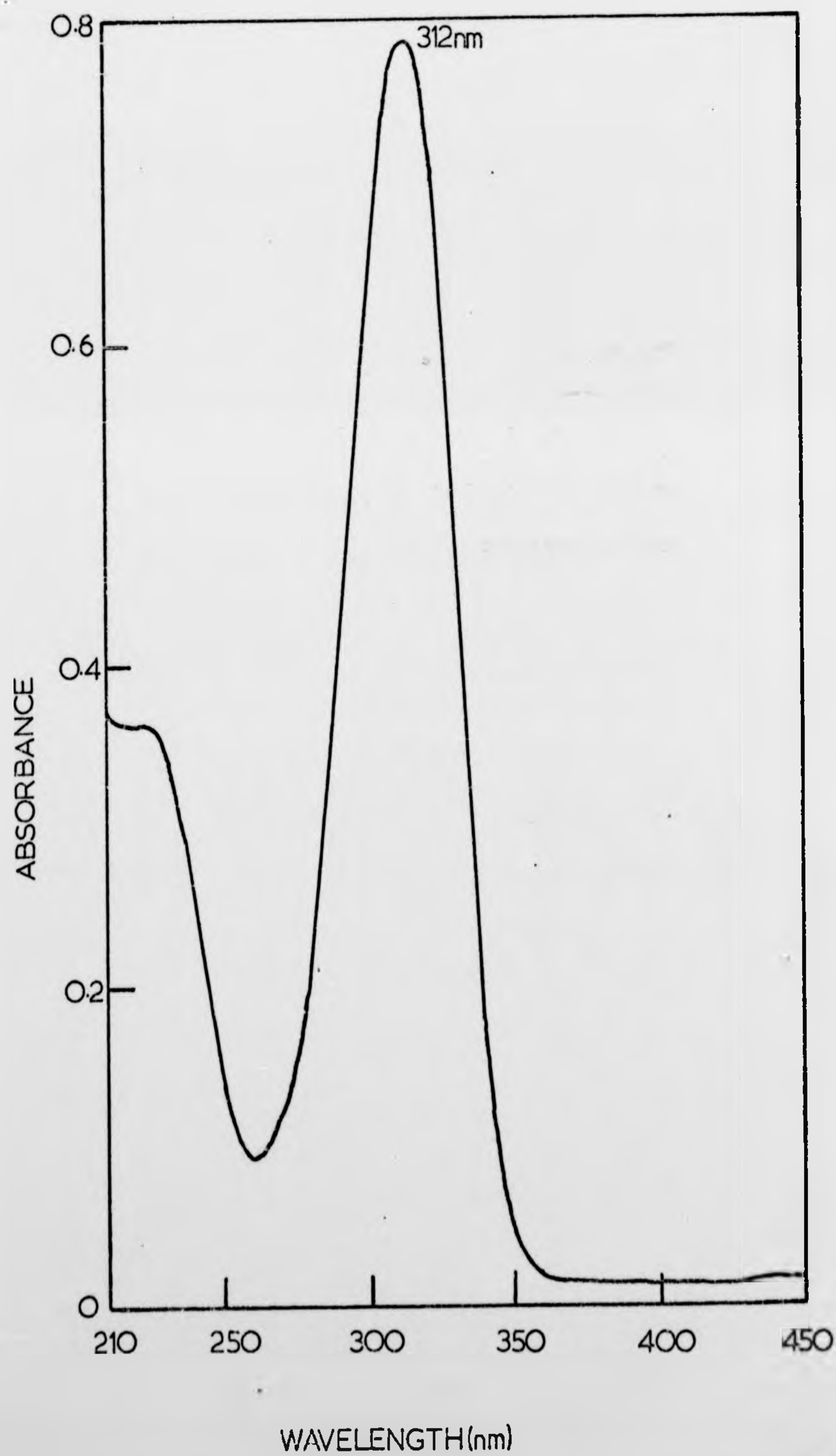
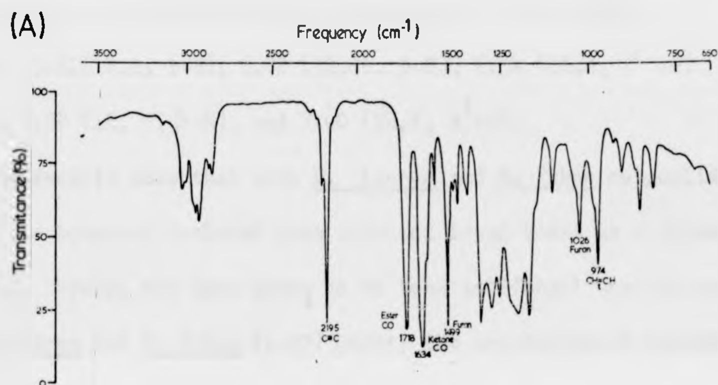


Figure 36

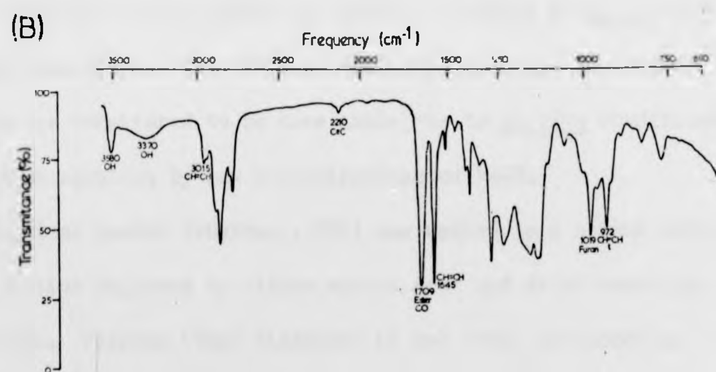
IR spectra of wyerone (A) and wyerol (B).

Fig.36

(A)



(B)



appearance of absorption due to the hydroxyl group (3580, 3370 cm^{-1}). Disappearance of the keto group adjacent to the acetylenic function resulted in drastically reduced intensity of the C≡C stretching absorption (2210cm^{-1}) in the IR spectrum of wyerol and W1. The identity of the hydroxy ester was confirmed by NMR spectroscopy (Fig.37). 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, $-OCH_3$), 5.49 (1H,dt, 4-H), 5.62(1H,d, 1-H), 6.02 (1H,dt, 5-H), 6.34 (1H,d, 2^1 -H) 6.52 (1H,d,β^1-H), 6.58 (1H, d, β-H), and 7.40 (1H,d, 1^1-H).

Thus these results show that both B. cinerea and B. fabae metabolize wyerone to wyerol, a compound isolated from infected broad beans as a phytoalexin (PA2) in chapter 2. Wyerol has been shown to be less antifungal than wyerone against both B. cinerea and B. fabae (p.57) hence, the metabolism of wyerone to wyerol can be considered as a detoxification mechanism.

C Metabolism of wyerone deposited on cellulose powder.

As wyerone appeares to be deposited on cell walls in the infected plant (p.51) an experiment was designed to study the ability of B. cinerea and B. fabae to metabolize wyerone deposited on cellulose powder. The use of deposited wyerone was considered to be more analogous to in vivo conditions than wyerone held in solution by low concentrations of MeOH.

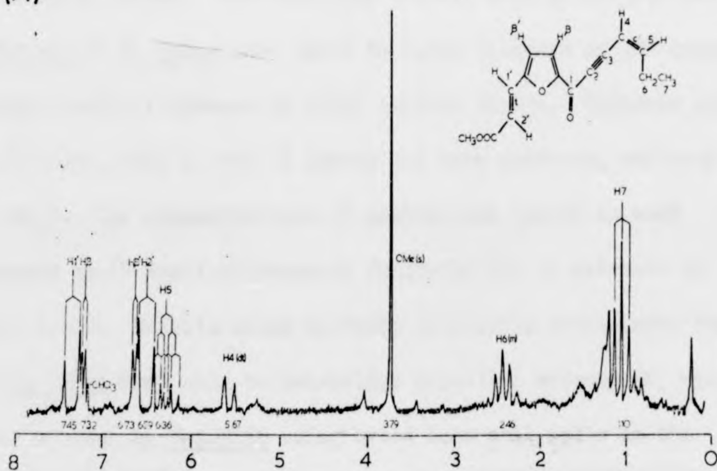
Ten g.cellulose powder (Whatman . CFI) was washed in a column with dilute ammonia solution followed by dilute acetic acid and dried overnight at room temperature. Wyerone (5mg) dissolved in 1ml $CHCl_3$ was added as 0.1ml aliquots to 5g dried cellulose powder in a 50ml conical flask. After the addition of each aliquot the flask was rotated slowly and $CHCl_3$ allowed to evaporate before subsequent additions. This procedure was found to give an even distribution of wyerone throughout the powder. Samples (0.1g) of powder + wyerone were soaked in MeOH (5ml) for 5 minutes. The wyerone

Figure 37

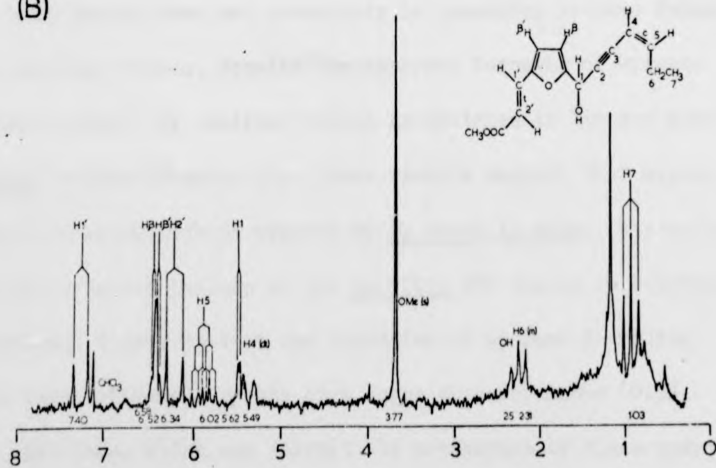
NMR spectra of wyerone (A) and wyerol (B).

Fig.37

(A)



(B)



extracted into MeOH from three separate samples, measured by UV spectrophotometry, was found to be 44.3, 44.8 and 47.4 μg; a mean recovery of 45.5 μg/0.1g cellulose powder. SPN solutions (0.5ml) with or without conidia of either B. cinerea or B. fabae were added to 4.5ml aliquots of SPN containing 0.1g cellulose powder + wyerone in 100ml conical flasks. Cultures were incubated for 24 hours, when a mesh of hyphae had been produced, and then extracted with Et_2O. The concentrations of wyerone and wyerol in each flask were measured by UV spectrophotometry following TLC of extracts in hexane : acetone (2:1). Results shown in Table 17 clearly demonstrate that B. cinerea and B. fabae were able to metabolize deposited wyerone to wyerol.

D Metabolism of wyerone by Botrytis using broad bean cell walls as the carbon source.

In previous experiments it has been demonstrated that both B. cinerea and B. fabae metabolize wyerone to the corresponding hydroxyester, wyerol in vitro However in the plant wyerol does not accumulate in spreading lesions formed by B. fabae in pod endocarp tissue, despite the apparent turnover of wyerone within the invaded tissue. By contrast wyerol accumulates in tissues bearing limited B. cinerea lesions (Chapter 3). These results suggest that wyerol is not the product of metabolism of wyerone by B. fabae in vivo. A possible explanation for the apparent failure of the in vitro SPN system to reproduce conditions in infected tissue is that the induction of wyerone degrading enzymes may have been influenced by the high sugar concentrations (0.5% Sucrose) in SPN solutions, which may inhibit the production of these enzymes by catabolite repression, as described for the effect of glucose and sucrose on pisatin breakdown by pea pathogens (de Wit - Elshove and Fuchs, 1971). To investigate this hypothesis a series of experiments were carried out

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TABLE 17 Yields (μg/flask) of wyerone and wyerol recovered after incubating wyerone (45.5μg) deposited on 0.1g cellulose powder in 5ml SPN solutions in the presence of absence of *B. fabae* or *B. cinerea* conidia for 24h.

	WYERONE	WYEROL
Wyerone incubated without conidia	35.8	-
Wyerone incubated with *B. fabae* conidia	4.1	19.9
Wyerone incubated with *B. cinerea* conidia	3.4	21.5

using isolated bean stem cell walls, prepared by the method of English et al. (1971), as the sole source of carbon apart from wyerone, for germinating conidia.

(i) Metabolism of solutions of wyerone.

Conical flasks (100ml) each containing 20ml sterile distilled water (adjusted to pH_4 with H_3PO_4) and 0.2g cell walls were inoculated with conidial suspensions (about 12,500 conidia/ml) or distilled water in the usual way. Duplicate flasks were prepared for each treatment. After incubation for 24 hours, 200µg wyerone in 50µl MeOH was added to each culture to give a final concentration of 10µg/ml cell wall suspension and 0.25% MeOH. The flasks were incubated for a further 18 hours then extracted with Et_2O. Duplicate extracts were combined and the equivalent of 10ml culture solution subjected to TLC with marker spots of wyerol and wyerone acid using Et_2O : MeOH (8:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm) as solvents. Two chromatograms from each treatment were prepared in this way; one was bioassayed with C. herbarum, the other visualized by spraying with vanillin-sulphuric acid reagent (Holloway and Challen, 1966) Results (Plate 12) indicate that the loss of wyerone from cultures of both fungi was associated with the marked appearance of wyerol. In addition wyerone acid and an unidentified compound were detected in extracts of cultures of B. cinerea and B. fabae respectively.

After TLC of extracts equivalent to 10ml of culture solution, yields of the wyerone derivatives were measured by UV spectrophotometry following elution in MeOH (Table 18). Approximately 18% of the wyerone was converted to wyerone acid by B. cinerea. It was not possible to determine whether or not B. fabae carries out this conversion since the acid itself may have been metabolized as it was produced. Substance 1 detected in B. fabae and water extracts gave no UV absorption spectrum, however substance 2 in extracts of B. fabae cultures had a λmax at 300nm. It was not possible to determine

Plate 12

TLC plate of Et_2O extracts from cell wall suspensions (10ml) incubated in the presence of wyerone (10 g/ml) alone (WC) or with either <u>B. cinerea</u> (Bc) or <u>B. fabae</u> (Bf) for 18h. Two replicate chromatograms of each extract were prepared, one was sprayed with <u>C. herbarum</u> and the other visualised with Vanillin/sulphuric acid reagent. Pure wyerone acid (WA) and wyerol (WO) were included as marker spots. Solvents: Et_2O MeOH (8:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm). W, wyerone; WO, wyerol; WA, wyerone acid; Sub 1, substance 1; Sub.2, substance 2.

Plate 12

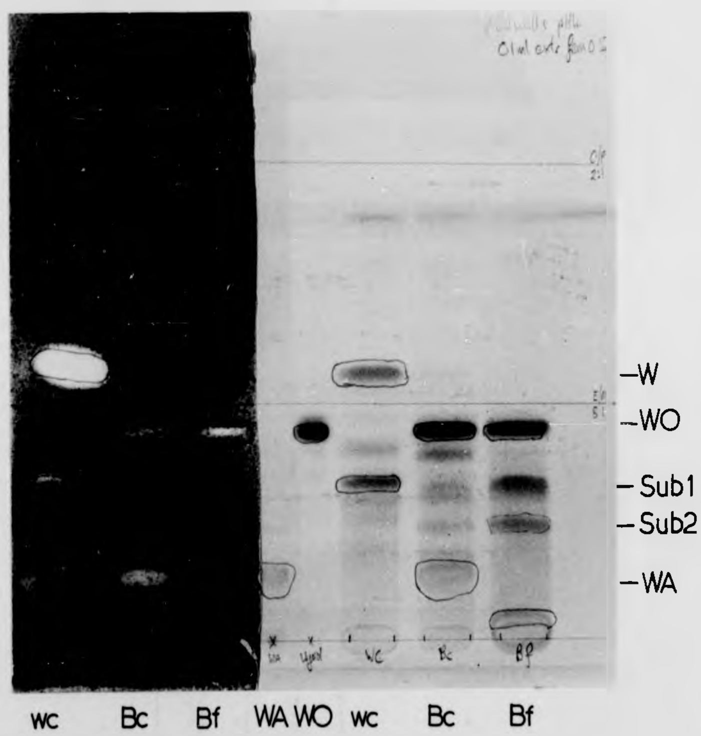


TABLE 18 Yields (μg/ml) of wyerone, wyerol and wyerone acid recovered from soultions of 0.5% cell wall suspensions containing 10 μg/ml wyerone incubated with and without germinating conidia of *B. cinerea* or *B. fabae*

	WATER CONTROL	*B. CINEREA*	*B. FABAE*
WYERONE	7.0	-	-
WYEROL	-	5.4	5.1
WYERONE ACID	-	1.3	-

if this substance was W2, the wyerone metabolite or reduced wyerone acid.

Attempts to increase the yield of wyerone acid from wyerone by altering the incubation times before the addition of wyerone to B. cinerea cultures were unsuccessful. When wyerone (200μg) was added at the same time as, or at 12 and 48 hours after inoculating cell wall suspensions with B. cinerea, 16:2 (8.1%), 25.5 (12.75%) and 20.4 (10.2%), μg (percentage conversion) wyerone acid was recovered from the respective treatments.

(ii) Metabolism of wyerone deposited on cell walls by B. cinerea

The possibility that B. cinerea could metabolize wyerone to wyerone acid at a greater rate when wyerone was deposited on cell walls was investigated.

Conical flasks (100ml) each containing 20mls cell walls suspension (0.5%) were inoculated with B. cinerea conidia as previously described. After incubating for 24 hours, 0.1g cell walls + 50μg deposited wyerone (prepared as previously described for cellulose powder p.62) were added. After further incubation for 18 hours the cultures were extracted with Et_2O and wyerone acid isolated by TLC in the usual way. Conversion of wyerone to wyerone acid was only marginally increased by the deposition of the ester on cell walls, 8.2 μg wyerone acid/flask were recovered (16.4 percentage conversion).

(iii) Detection of extracellular enzymes converting wyerone to wyerone acid

It was considered that the enzymes metabolizing wyerone to wyerol may be associated with the fungal hyphae whereas those converting wyerone to wyerone acid may perhaps be produced extracellularly. The following experiment was carried out to test this hypothesis.

Suspensions of 0.5% cell walls (20ml) were prepared and incubated with B. cinerea conidia for 24 hours as previously described. Wyerone

(200μg) was then added in 50μl MeOH and the cultures incubated for another 18 hours. After the incubation period triplicate cultures were filtered through glass wool to remove the cell walls and then through a 'millipore' membrane filter (pore size 0.45 m) to remove any remaining fungus. Two 20ml aliquots of the filtrate were dispensed into sterile 100ml conical flasks containing 200μg wyerone deposited on the bottom of the flask. After incubating for 24 hours at 18°c the filtrates were combined, extracted with Et_2O and subjected to TLC; 74% (148μg) of the wyerone was recovered and only 5.4μg of wyerone acid detected. Similar levels of wyerone acid (6.8μg) were recovered from the filtrate before incubation with wyerone. These results suggest that the conversion of wyerone to wyerone acid does not occur extracellularly.

2 Metabolism of wyerone epoxide.

A Metabolism of wyerone epoxide by germinating conidia.

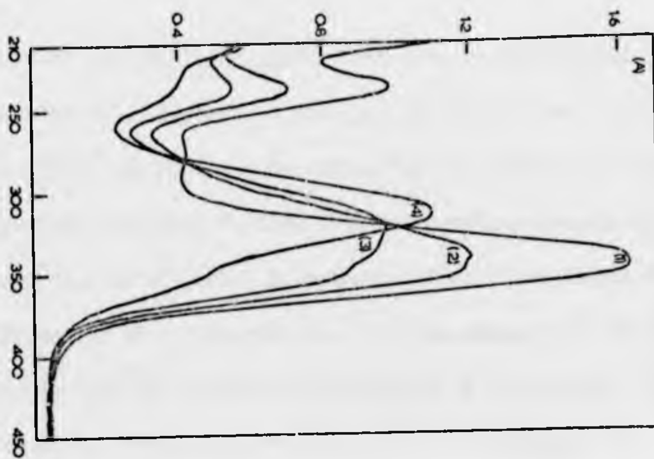
The metabolism of wyerone epoxide (19μg/ml SPN) by *Botrytis* conidia was examined as previously described for wyerone. Duplicate culture flasks were harvested at 18, 30 and 42 hour intervals after inoculation and extracted with Et_2O. Examples of the UV absorption spectra of Et_2O extracts after incubation with either *B. fabae* or *B. cinerea* conidia are illustrated in Fig. 38.

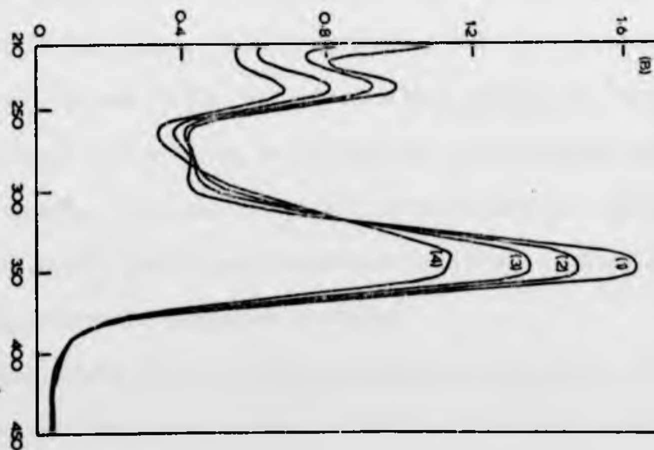
The loss of wyerone epoxide (λmax 347nm) from solutions incubated with *B. fabae* conidia was associated with the appearance of UV absorbing substances having a distinct peak at 310nm after 42 hours. In contrast, wyerone epoxide disappeared from solutions incubated with *B. cinerea* conidia much slower, after 42 hours a distinct peak was still present at 347nm. Examination of the incubation flask after extraction of the cultures showed that *B. fabae* had produced a hyphal mesh by 30 hours after inoculation,

Figure 38

UV absorption spectra of Et_2O extracts, in 5ml MeOH, from SPN solutions containing wyerone epoxide (19 μg/ml) incubated alone for 42 hours (1) or with either *B. fabae* (A) or *B. cinerea* (B) conidia for 18 (2), 30 (3) and 42h (4).

Fig.38





however B. cinerea had achieved only sparse growth by 42 hours after inoculation. On the basis of UV absorbance it was estimated that 82% of the wyerone epoxide added was recovered from solutions incubated without conidia for 42 hours.

Et_2O extracts of two replicate flasks from each treatment were combined and examined by TLC. Extracts (equivalent to 3ml culture solution) were applied to 2cm origins in MeOH and the chromatograms developed in hexane : acetone (2:1). Visualization under UV light (366nm) revealed wyerone epoxide as a blue fluorescent band at RF 0.45. In extracts of solutions incubated with conidia, the intensity of the wyerone epoxide band decreased with time, but did so at a greater rate in cultures of B. fabae than B. cinerea. The disapperance of the epoxide from solutions incubated with B. fabae was associated with the appearance of two quenching bands under UV light (254nm) at RF 0.26 (WE1) and 0.06 (WE3) and a single faint blue fluorescent band under UV light (366nm) at RF 0.14 (WE2). Besides wyerone epoxide only one other substance, which corresponded to WE1, was detected in B. cinerea cultures. None of these substances were detected in extracts of wyerone epoxide solutions incubated without conidia. Developed chromatograms bioassayed with C.herbarum showed that wyerone epoxide was the only substance possessing substantial antifungal activity present in incubation mixtures.

Extracts (equivalent to 5ml culture solution were subjected to TLC in hexane : acetone (2:1) and after elution in MeOH the UV absorption spectra of each band detected was recorded. WE1 and WE3 from cultures of B. fabae had identical spectra, with a maximum absorbance at 310nm (Fig. 39A), WE2 showed maximum absorbance at 347nm. WE1 from B. fabae and B. cinerea cultures were identical (Fig.39B). The yields of wyerone epoxide and its metabolites with time after inoculation, with and without conidia are illustrated in Fig. 40.

Figure 39

UV absorption spectra of the wyerone epoxide metabolites, WE1 (1) and WE3 (2) produced after incubating wyerone epoxide with *B. fabae* (A) conidia for 30 and 42h respectively, and WE1 (3) produced after incubating wyerone epoxide with *B. cinerea* (B) conidia for 42h.

Fig.39

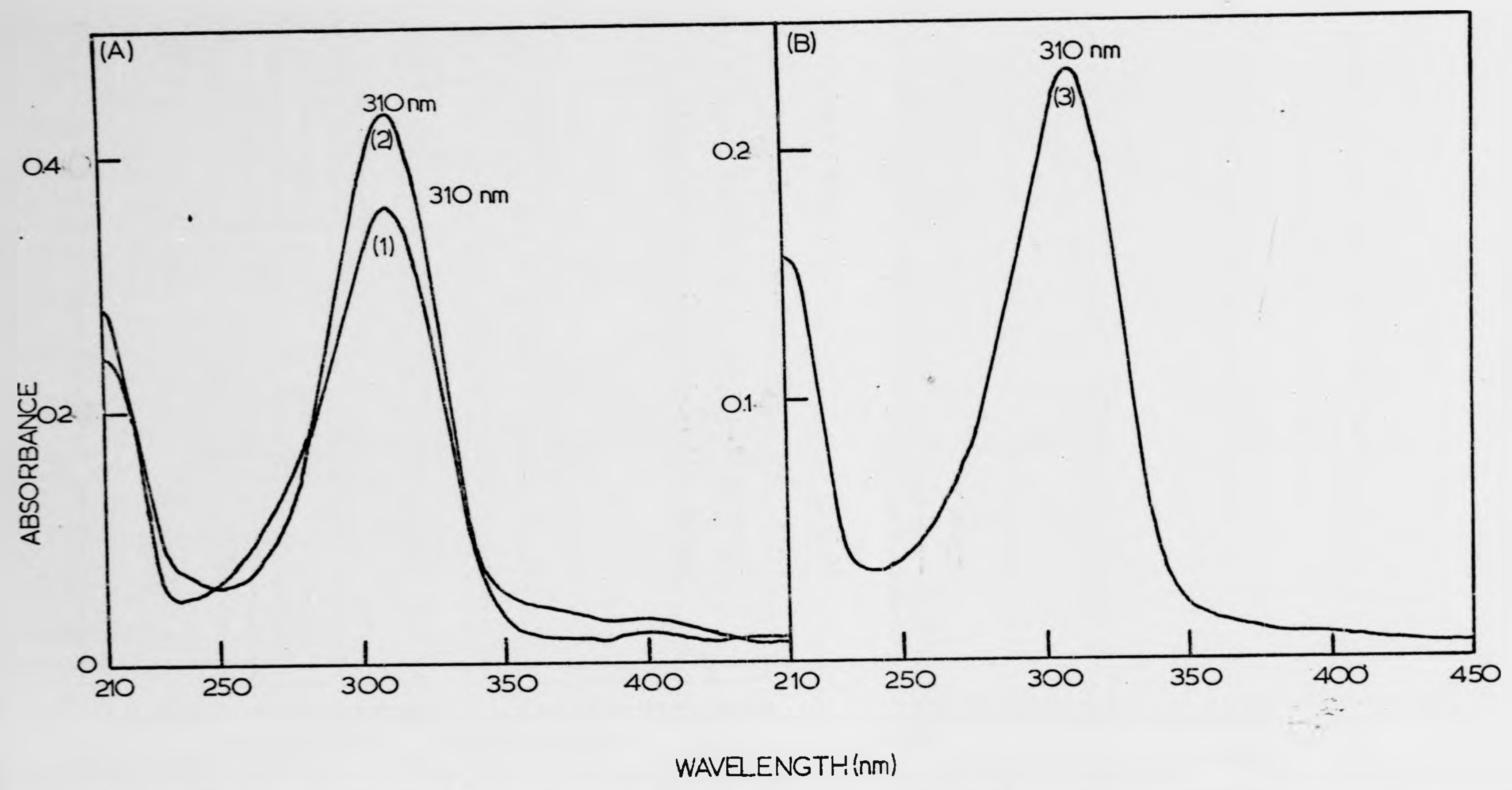
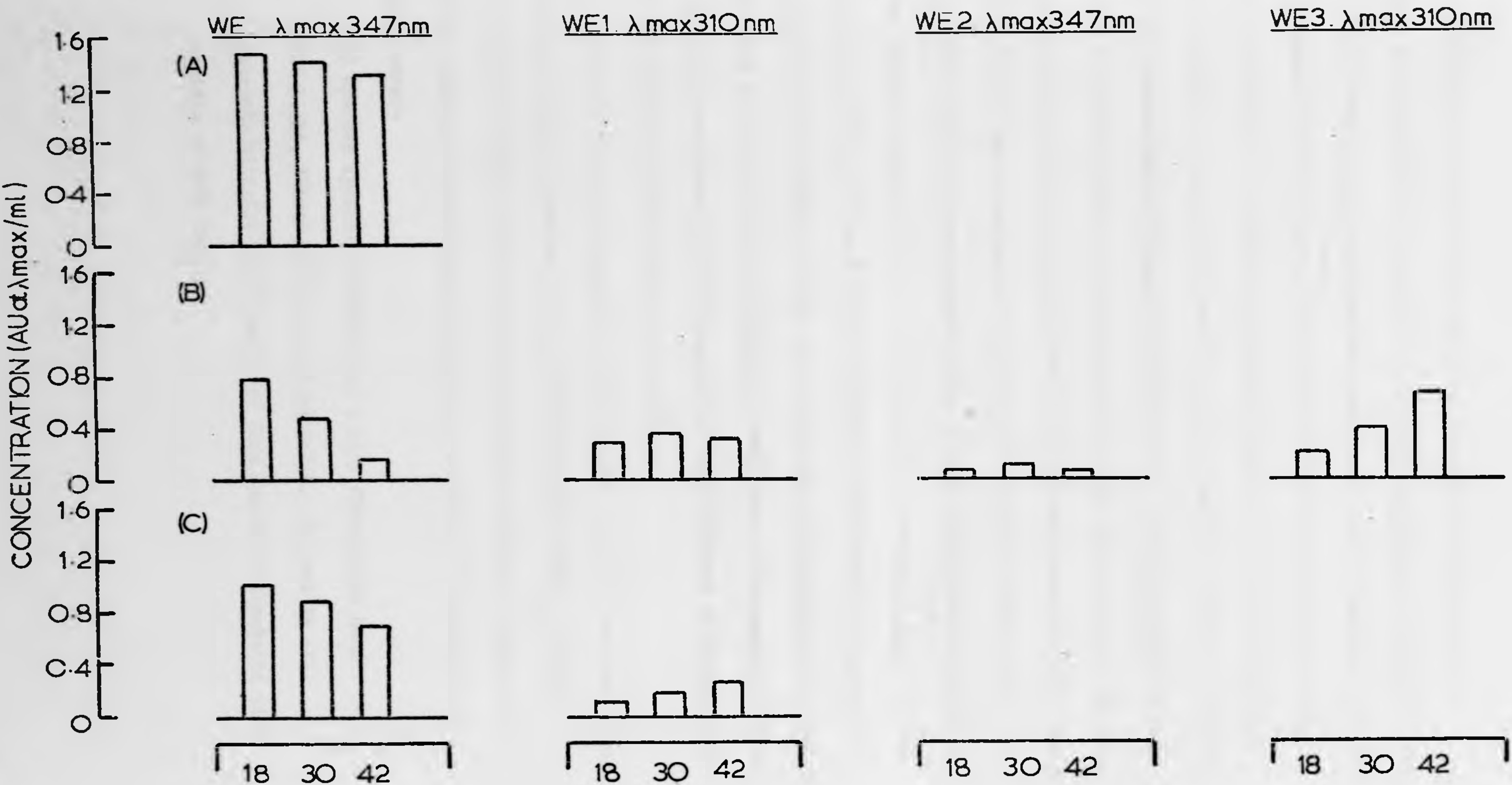


Figure 40

Changes in the concentration of wyerone epoxide (WE) and its metabolites (WE1, WE2 and WE3) after incubating wyerone epoxide (19μg/ml) in SPN solutions alone (A) or with conidia of either B. fabae (B) or B. cinerea (C).

Fig. 40



Wyerone epoxide disappeared from solutions incubated with conidia of B. fabae at a greater rate than with conidia of B. cinerea. The loss of the phytoalexin from B. fabae cultures was associated with an increase, followed by a decrease in WE1, which was then followed by an accumulation of WE3, suggesting that WE1 is further metabolized to WE3. The yield of WE2 was lower than that of WE1 and WE3, but this metabolite also followed a pattern of initial accumulation followed by disappearance. In B. cinerea cultures the loss of wyerone epoxide was associated with the appearance of WE1 only. The slight loss of wyerone epoxide from SPN solutions incubated alone was not associated with the appearance of any other UV absorbing substances.

This experiment was repeated using a lower initial concentration of wyerone epoxide (10μg/ml SPN). The UV absorption spectra of Et_2O extracts collected 18, 30 and 42 hours after incubation with either B. cinerea or B. fabae conidia are shown in Fig. 41. Both fungi completely metabolized the phytoalexin by 42 hours after inoculation. The loss of the epoxide (347nm) was associated with a shift in absorbance of the Et_2O extracts to 310nm, from solutions incubated with conidia of both fungi.

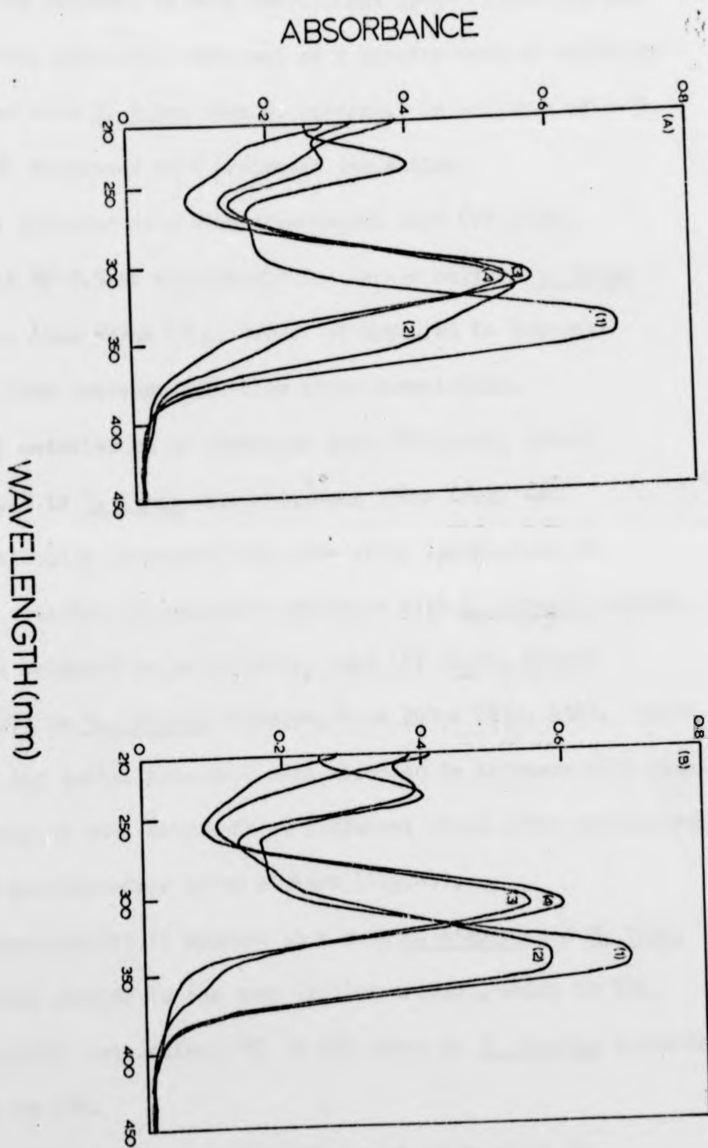
After extracts (equivalent to 5ml culture solution) had been separated by TLC using hexane : acetone (2:1, 15cm) followed by $CHCl_3$: petrol (2:1, 15cm) as solvents five distinct bands were detected. These were eluted in MeOH and the UV absorption spectra recorded. The substances detected were:-

1) Wyerone epoxide : detected as a blue fluorescent band (UV light, 366nm) at RF 0.54, λmax 347nm. Present in all extracts initially but disappeared from solutions incubated with conidia of both fungi.

Figure 41

UV absorption spectra of Et_2O extracts, in 5ml MeOH, from SPK solutions containing wyerone epoxide (10µg/ml) incubated alone for 42h (1) or with either B. fabae (A) or B. cinerea (B) conidia for 18 (2), 30 (3), and 42 h (4).

Fig.41



2) WE1 : detected as an absorbing band (UV light, 254nm) at RF 0.42 in cultures of both fungi, λmax 310nm (Figs. 42A and 43A). This substance increased at a greater rate in solutions incubated with B. fabae than B. cinerea. In cultures of both fungi WE1 decreased with prolonged incubation.

3) WE2 : detected as a blue fluorescent band (UV light, 366nm) at RF 0.3 at consistenly low levels only in B. fabae cultures, λmax 347nm (Fig. 42B). It appeared to increase rapidly then decrease with time after inoculation.

4) WE3 : detected as an absorbing band (UV light, 254nm) at RF 0.13 in B. fabae cultures, λmax 310nm (Fig. 42C) This metabolite increased with time after inoculation but was not detected in solutions incubated with B. cinerea conidia.

5) WE4 : detected as an absorbing band (UV light, 254nm) at RF 0.05 in B. cinerea cultures, λmax 294nm (Fig. 43B). After initial lag period this substance appeared to increase with time.

The levels of each substance at different times after inoculation were calculated as absorbance units at λmax (Fig.44).

From these results it appears that both B. cinerea and B. fabae metabolize wyerone epoxide to the same initial product, which is WE1, B. fabae then rapidly metabolizes WE1 to WE3 where as B. cinerea converts WE1 more slowly to WE4.

B Identification and antifungal activity of metabolites of wyerone epoxide produced by Botrytis

Wyerone epoxide in 0.1ml MeOH was added to twelve flasks containing 100ml SPN solutions, to give a final concentration of 19µg/ml SPN in each flask. One disc (5mm diameter) of medium X agar bearing actively growing

Figure 42

UV absorption spectra of the wyerone epoxide metabolites, WE1 (A), WE2 (B) and WE3 (C) produced by *B. fabae* .

Fig.42

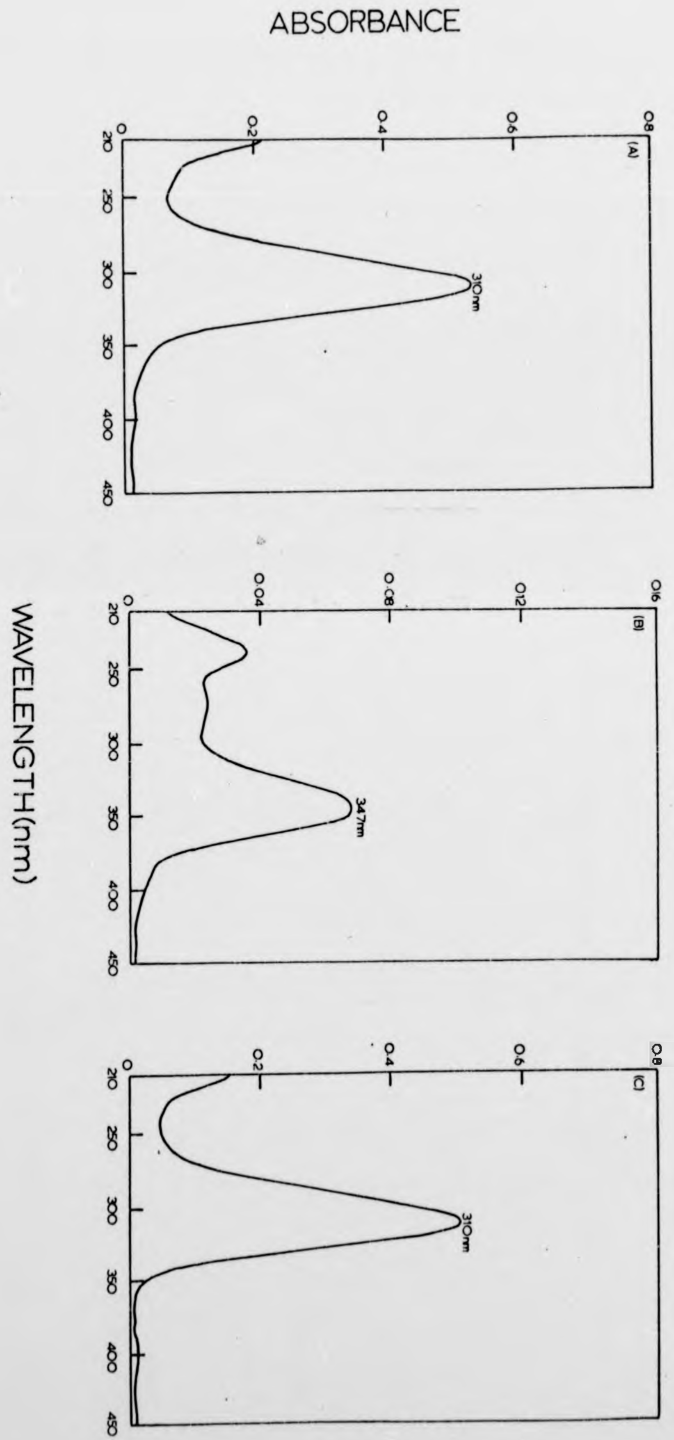
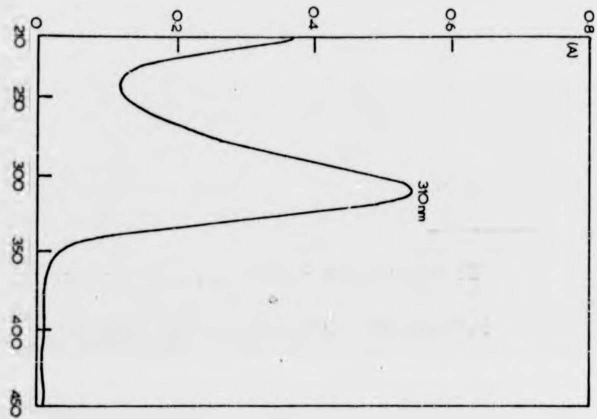


Figure 43

UV absorption spectra of the wyerone epoxide metabolites, WE1 (A) and WE4 (B) produced by *B. cinerea*.

Fig.43



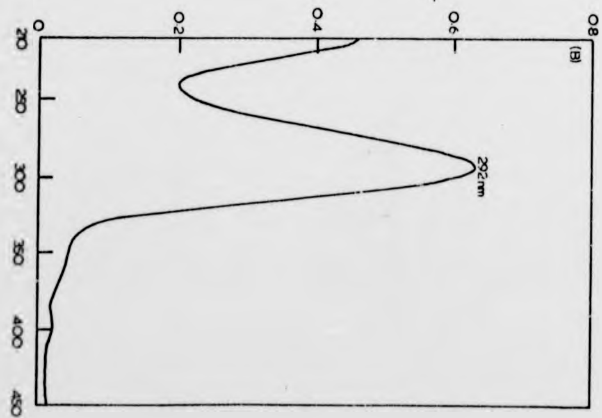
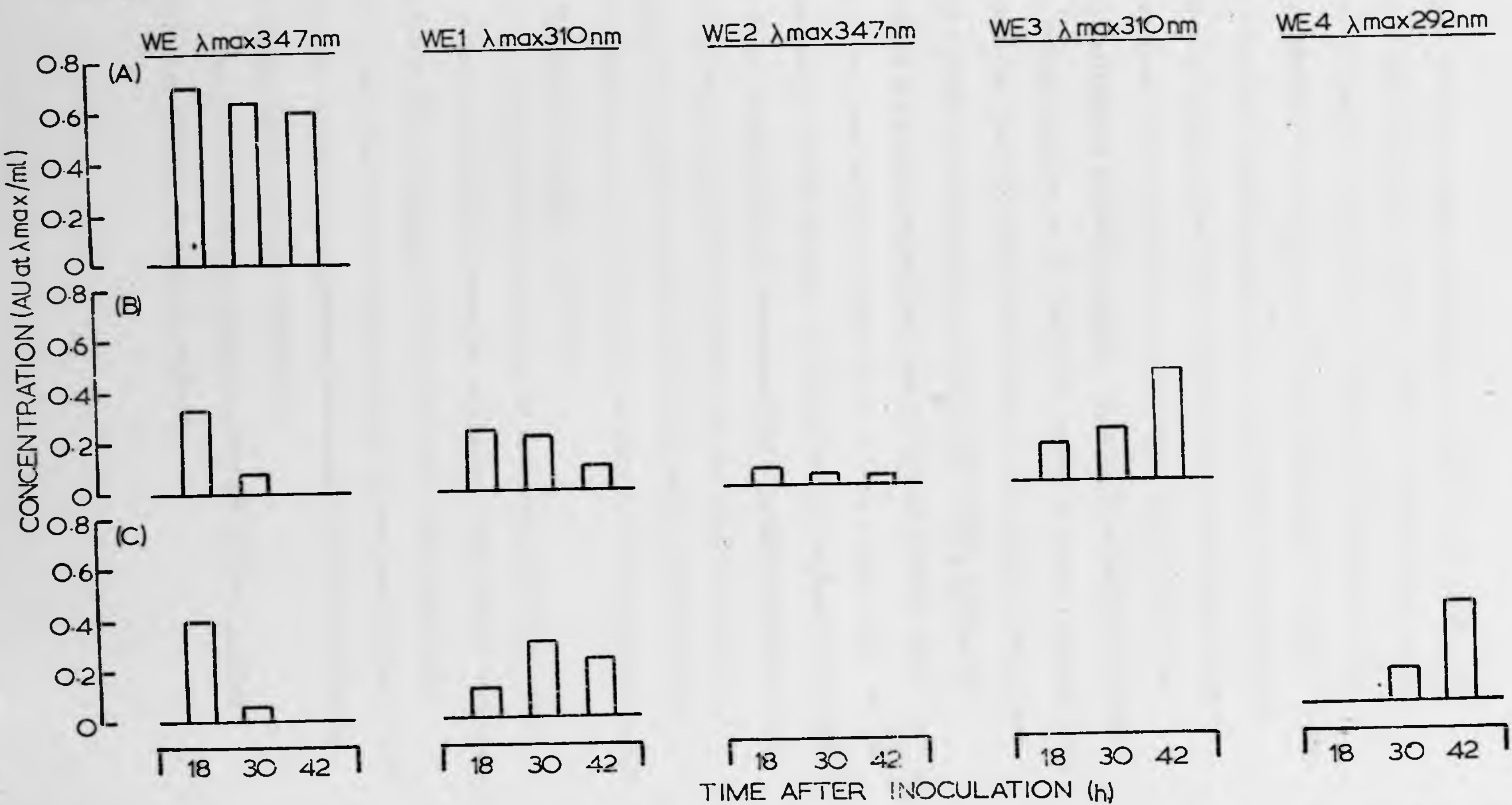


Figure 44

Changes in the concentration of wyerone epoxide (WE) and its metabolites (WE1, WE2, WE3 and WE4) after incubating wyerone epoxide (10μg/ml) in SPN solutions alone (A) or with conidia of either B. fabae (B) or B. cinerea (C).

Fig.44



mycelium of either B. cinerea or B. fabae was added to each flask. Six cultures of each fungus were incubated at 18°c in the dark on an orbital incubator (200rev/min). Loss of the phytoalexin and appearance of 310nm absorbing substances was monitored by UV spectrophotometry of aliquots of the bathing solutions. When absorbance due to the epoxide had disappeared in cultures of both fungi, three days after inoculation, individual cultures were partitioned twice with equal volumes of Et_2O. Et_2O extracts from replicate cultures of either B. cinerea or B. fabae were combined and after solvent removal separated by PLC (1mm thick layers) in hexane : acetone (2:1). The metabolites from both fungi were detected as dark quenching bands under UV light (254nm) and recovered after elution (2x50ml $CHCl_3$ followed by 1x50ml Et_2O) and solvent evaporation. Besides wyerone epoxide, three prominent bands were detected in extracts of cultures of both fungi, but the relative amounts of each calculated as absorbance units at λmax/ml differed (Table 19). The major products of wyerone epoxide metabolism characterized by their RF values and absorption spectra were WE1 and WE3 in cultures of B. cinerea and B. fabae respectively (Fig. 45 A and C). Yields of 5.5mg WE1 and 7.0mg WE3 were recovered from the metabolism of 11.4mg wyerone epoxide by B. cinerea and B. fabae respectively.

A substance detected in cultures of both fungi at RF 0.35, with λmax at 294nm, had not been recognised previously. This compound cochromatographed with WE2 from B. fabae cultures (Fig. 45B). Since both these substances were detected at low concentrations, further investigation was not carried out. No UV absorbing substance corresponding to WE4 were detected in B. cinerea cultures in this experiment.

Samples of WE1 from B. cinerea cultures and WE3 from B. fabae cultures were subjected to spectral analysis.

TABLE 19 Yields (AU at $\lambda max/ml$) of wyerone epoxide and its metabolites after PLC of Et_2O extracts from mycelium cultures of *B. cinerea* and *B. fabae* incubated with wyerone epoxide (19 $\mu g/ml$) for 3 days.

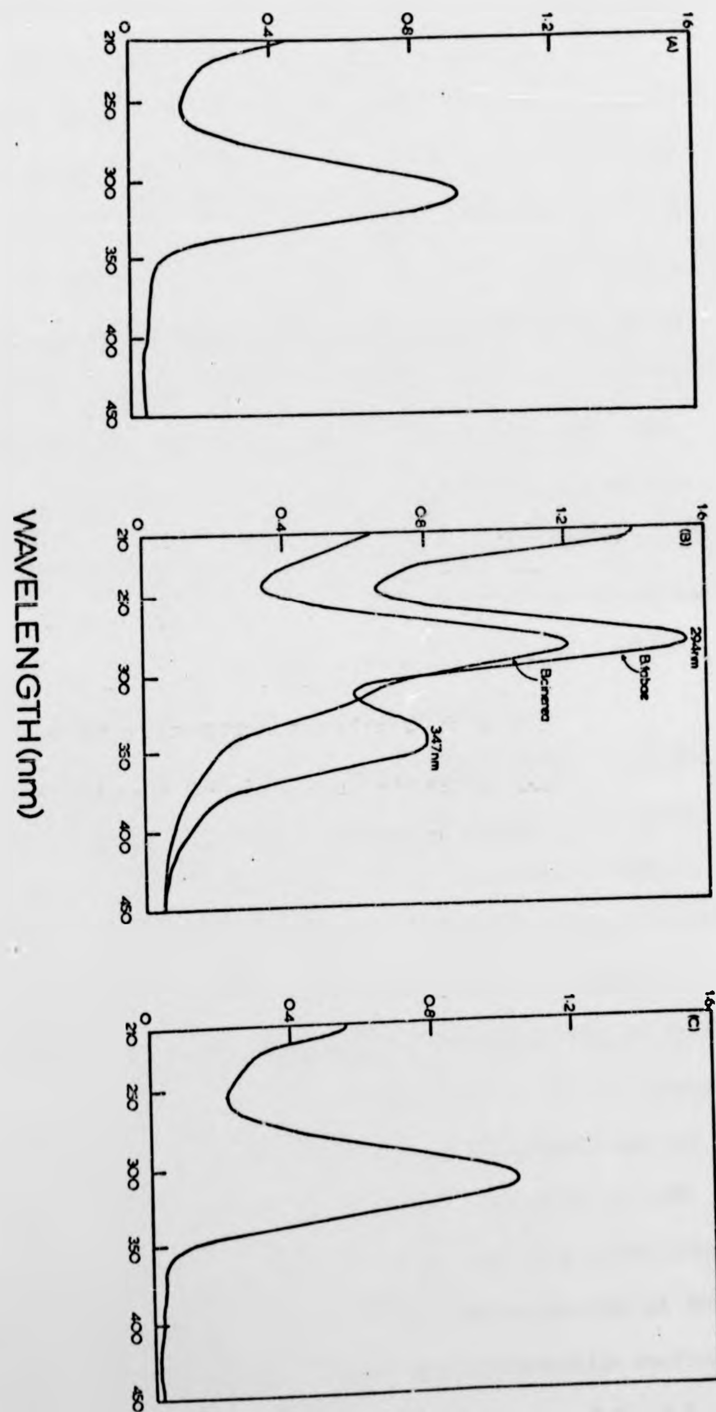
	RF*	$\lambda max(nm)$	ABSORBANCE UNITS	
			B. CINEREA	B. FABAE
WYERONE EPOXIDE	0.57	347	0.06	0.05
WE1	0.49	310	0.62	0.16
WE2(A)	0.35	284	0.08	0.12
(B)		347	-	0.06
WE3	0.23	310	-	0.74

* - Solvent system : Hexane : acetone (2:1)

Figure 45

UV absorption spectra of purified WE1 (A) and WE3 (C) from B. cinerea and B. fabae cultures respectively and of the substances corresponding to WE2 (B) from both B. cinerea and B. fabae cultures incubated with wycrone epoxide solutions.

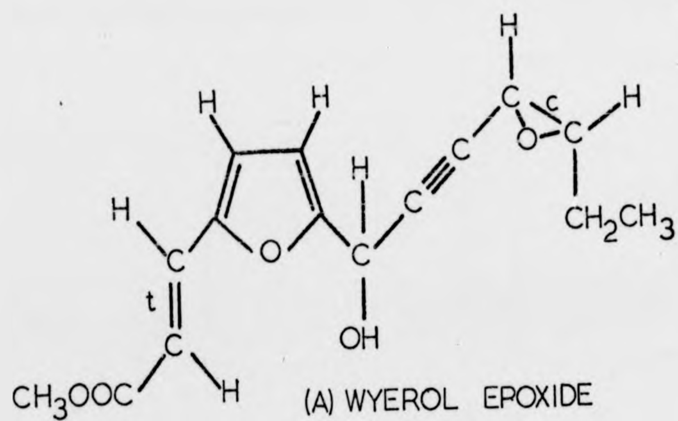
Fig.45



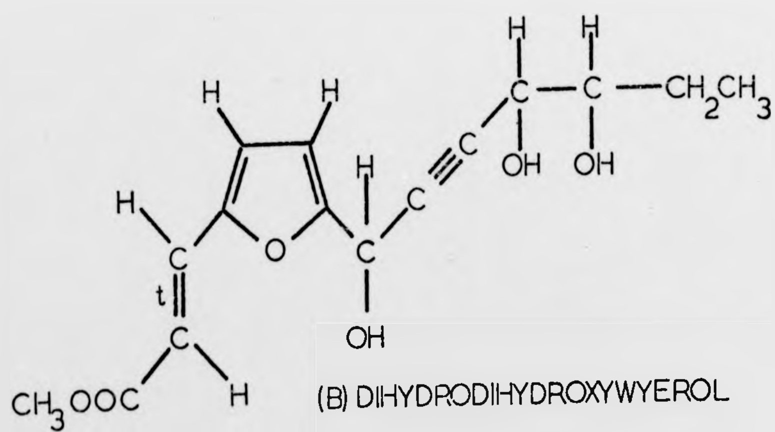
The molecular formula of the metabolite produced by <u>B. cinerea</u> (WE1) as determined by mass spectrometry was $C_{15}H_{16}O_5$ (M+ 279.0972) and was identified as wyerol epoxide (Fig.46A) on the basis of the following evidence. The UV absorption spectrum was similar to that of wyerol with λmax 310nm (ϵ20,000). The NMR shifts (Fig. 47B) of the furan protons 6.53 (1H,d, H) and 6.60 (1H,d, - H) and the presence of a methine proton 5.53 (1H,br,s, 1-H) also indicate that the ketone function had disappeared. IR absorption was present at 3570, 3350 (-OH) and 868 cm^{-1} (epoxide) (Fig. 48B). NMR signals due to the epoxide protons were at δ 3.09 (1H,dt, 5-H) and 3.54 (1H,dd, 4-H). 5-H appeared as a double triplet as in wyerone epoxide (Fig.47A) but 4-H showed further long range coupling to 1-H ($J_{1,4} \sim$ 1Hz) giving rise to a double doublet in the spectrum of wyerol epoxide. Wyerol epoxide was visualized on thin layer chromatograms as an orange spot on a yellow background after treatment with the picric acid spray reagent for epoxides.

The more polar metabolite produced by <u>B. fabae</u> (WE3) was visualized on chromatograms as a deep pink spot on a white background after treatment with lead tetra-acetate roseaniline reagent. It had a molecular formula $C_{15}H_{18}O_6$ as determined by mass spectrometry (M^+ 294.1107) and was identified as 4,5 - dihydro - 4,5 - dihydroxy wyerol (Fig.46B) from the following spectral data. The UV absorption spectrum was virtually identical to that of wyerol epoxide with λmax 310 nm (ϵ 18,000). The NMR spectrum of dihydrodihydroxy wyerol (Fig.47C) showed that the epoxide protons had disappeared and new multiplets centered at δ 3.65 and 4.3 had appeared. Above δ5.0 the NMR spectrum was virtually identical to wyerol epoxide. The IR spectrum (Fig.48C) showed very strong hydroxyl absorption at 3380 cm^{-1} with a shoulder at 3560 cm^{-1}. This information together with the lead tetra-acetate roseaniline confirmatory colour test for a vicinal diol was strong evidence in favour of the 4,5 -

Fig. 46



(A) WYEROL EPOXIDE



(B) DIHYDRODIHYDROXYWYEROL

Figure 47

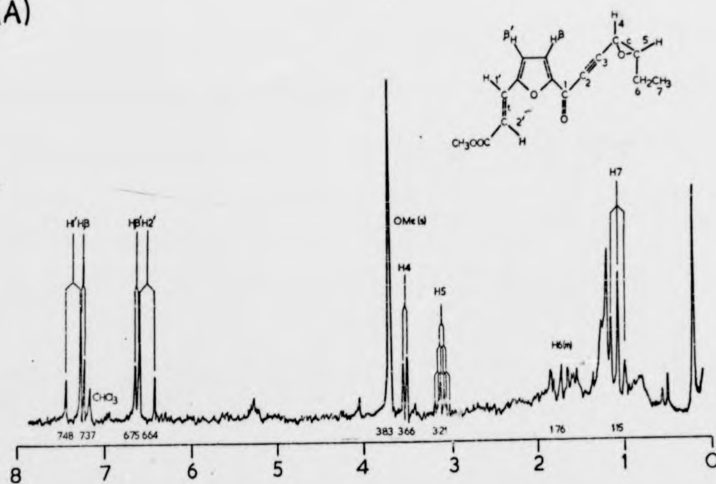
NMR spectra of wyerone epoxide (A) wyerol epoxide (B) and dihydrodihydroxy wyerol (C).

Figure 47

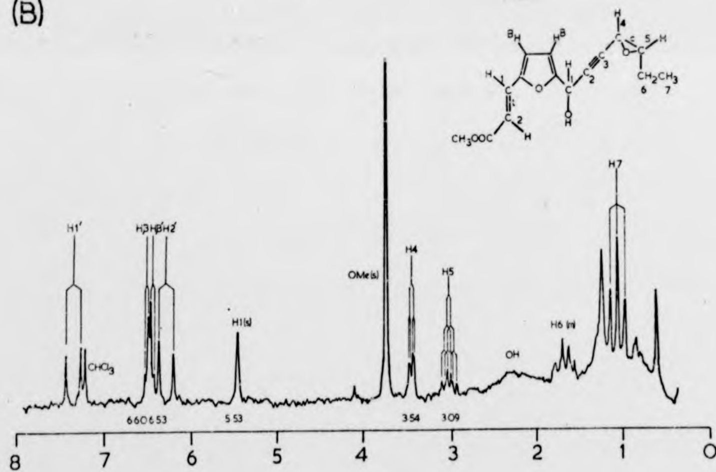
NMR spectra of wyerone epoxide (A) wyerol epoxide (B) and dihydrodihydroxy wyerol (C).

Fig.47

(A)



(B)



(C)

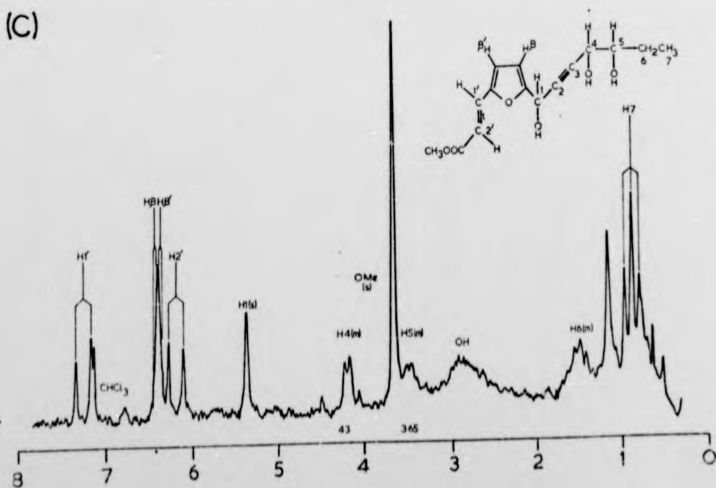
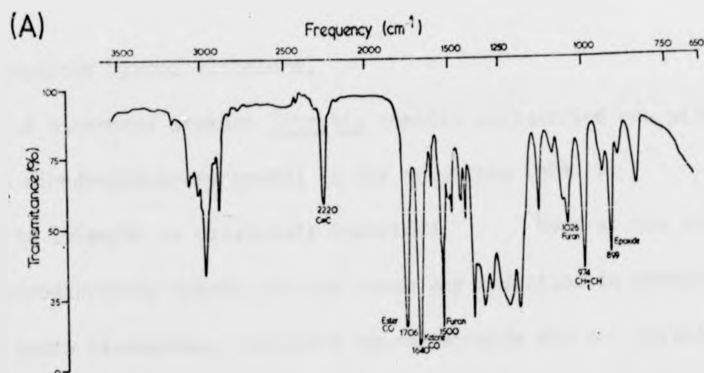


Figure 48

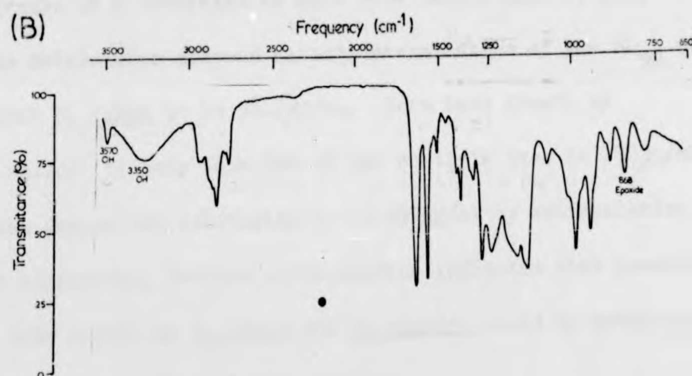
IR spectra of wyerone epoxide (A) wyerol epoxide (B) and dihydrodihydroxy wyerol (C).

Fig. 48

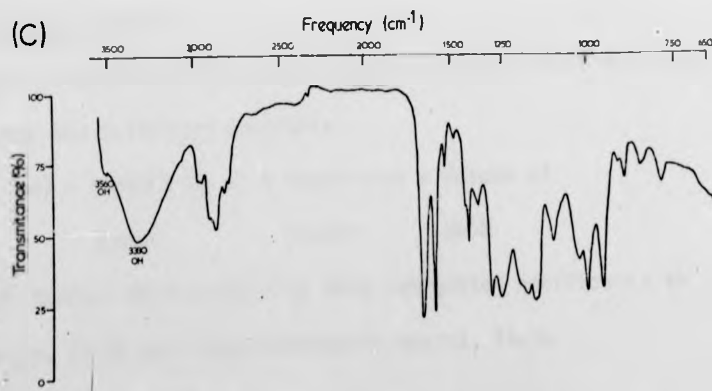
(A)



(B)



(C)



dihydro - 4,5 - dihydroxy wyerol structure.

A series of bioassays against Botrytis conidia were carried out with wyerol epoxide and dihydrodihydroxy wyerol in SPN solutions (pH4) at concentrations up to 150μg/ml as previously described. Even at the highest concentration dihydrodihydroxy wyerol did not cause any reduction in germination recorded after 18 hours incubation, similarly wyerol epoxide did not affect the germination of B. cinerea. However, there was a marked reduction in the germination of B. fabae conidia at wyerol epoxide concentrations above 100 μg/ml (Table 20).

Plotting graphs of % reduction in germ tube length against log. concentration of the metabolites allowed direct determination of the ED_{50} of wyerol epoxide against B. fabae to be 38.2μg/ml. Germ tube growth by B. cinerea was not reduced to less than 50% of the controls even in 150μg/ml. The ED_{50} against this fungus was calculated to be 597μg/ml by extrapolation of the dosage/response regression, further extrapolation indicated that complete inhibition of germ tube growth by B. fabae and B. cinerea would be achieved by 213 and 1.4×10^4μg wyerol epoxide /ml respectively.

Germ tube growth by both B. cinerea and B. fabae was not affected by dihydrodihydroxy wyerol even at the highest concentration tested (120μg/ml)

C Calculation of concentrations of wyerone epoxide and dihydrodihydroxy wyerol

The amounts of each metabolite was calculated from the UV absorption spectra in MeOH using the following formula:-

$$\text{Yield } (\mu g) = \text{Absorbance at } \lambda_{max} \times \text{Conversion factor} \times \text{Volume of MeOH}$$

Conversion factors were calculated from extinction coefficents to be for wyerol epoxide, 13.8; and dihydroxydihydro wyerol, 16.3.

TABLE 20 Antifungal activity of wyerol epoxide against germination of *B. fabae* conidia

WYEROL EPOXIDE (μg/ml)	REDUCTION IN GERMINATION (%)
12.5	0
25	0
50	40.4
100	91.1
150	98

In conclusion, it appears that both B. cinerea and B. fabae are able to inactivate wyerone epoxide. However, whereas both fungi can metabolize the phytoalexin to wyerol epoxide only B. fabae converts wyerol epoxide to dihydro-dihydroxy wyerol. The different patterns of wyerone epoxide metabolism by these fungi may be related to the differential sensitivity of these fungi to wyerol epoxide.

3 Metabolism of wyerone acid

The metabolites of wyerone and wyerone epoxide produced by B. cinerea and B. fabae in vitro, and reduced wyerone acid, a reported metabolite of wyerone acid produced by B. fabae (Mansfield and Widdowson, 1973) are reduced forms of the original phytoalexins. However, in contrast to reduced wyerone acid, the metabolites of wyerone and wyerone epoxide retain the acetylenic function. Reduced wyerone acid was originally isolated and identified from B. fabae infected pod seed cavities (Mansfield and Widdowson,1973; Mansfield et al., 1973). A metabolite of wyerone acid produced B. fabae after incubation with the phytoalexin for 4 days in vitro was identified as reduced wyerone acid on the basis of their identical RF on paper chromatograms and identical UV absorption spectra (Mansfield and Widdowson, 1973). However, the hydroxy forms of the wyerone derivatives all have λmax c 300-310nm, even though the degree of saturation of the $CH_3 CH_2.CH=CH.C\equiv C.$ side chain may differ (Fawcett et al., 1968) and the paper chromatographic systems used in earlier work may not have allowed separation of closely related compounds such as wyerol acid and reduced wyerone acid. It was therefore decided to reinvestigate the metabolism of wyerone acid by B. cinerea and B. fabae in vitro.

A Metabolism of wyerone acid by germinating conidia

Wyerone acid solution (10µg/ml SFM) were incubated with or without

conidia of B. cinerea or B. fabae. After 24 hours triplicate flasks of each treatment were extracted with Et_2O and the extracts resuspended in MeOH for UV spectrophotometry.

Typical examples of UV absorption spectra of the Et_2O extracts are shown in Fig. 49. The loss of wyerone acid (λmax 354nm) from solutions incubated with B. fabae conidia was associated with the appearance of a distinct peak of absorption at 310nm. In contrast, extracts from solutions incubated with B. cinerea showed that wyerone acid was still present in cultures but at a lower concentration than in solutions of wyerone acid incubated without conidia. The loss of wyerone acid was not associated with a distinct peak shift as detected in B. fabae cultures, instead a number of UV absorbing substances appeared to be present. Examination of the incubation flasks after Et_2O extraction showed that a mesh of hyphae had developed from conidia of B. fabae but only c 50% of B. cinerea conidia had germinated and these had produced germ tubes of about 20µm in length.

The triplicate extracts of each treatment were combined for TLC. Extracts (equivalent to 5ml culture solution) were applied to 2.5cm origins in MeOH and the chromatograms developed in Et_2O : MeOH (6:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm), Wyerone acid was detected as a blue fluorescent band under UV light (366nm) at RF 0.26 in extracts of solutions of the phytoalexin incubated with B. cinerea or alone. No wyerone acid was detected in extracts of solutions incubated with B. fabae conidia, disappearance of the phytoalexin in B. fabae cultures was associated with the appearance of a quenching band under UV light (254nm) at RF 0.45. No other substances were detected by examination of chromatograms under UV light. Developed chromatograms bioassayed with C. herbarum showed wyerone acid to be the only inhibitor present.

Figure 49

UV absorption spectra of Et_2O extracts, in 5ml MeOH, from SPN solutions incubated for 24h in the presence or absence of wyerone acid (10μg/ml) and conidia of either *B. cinerea* or *B. fabae*.

(1) Wyerone acid solutions incbuated without conidia.

(2) " " " " with *B. fabae* conidia.

(3) " " " " " *B. cinerea* conidia.

(4) SPN solutions " " *B. fabae* " .

(5) " " " " *B. cinerea* " .

Figure 49

UV absorption spectra of Et_2O extracts, in 5ml MeOH, from SPN solutions incubated for 24h in the presence or absence of wyerone acid (10µg/ml) and conidia of either *B. cinerea* or *B. fabae*.

(1) Wyerone acid solutions incbuated without conidia.

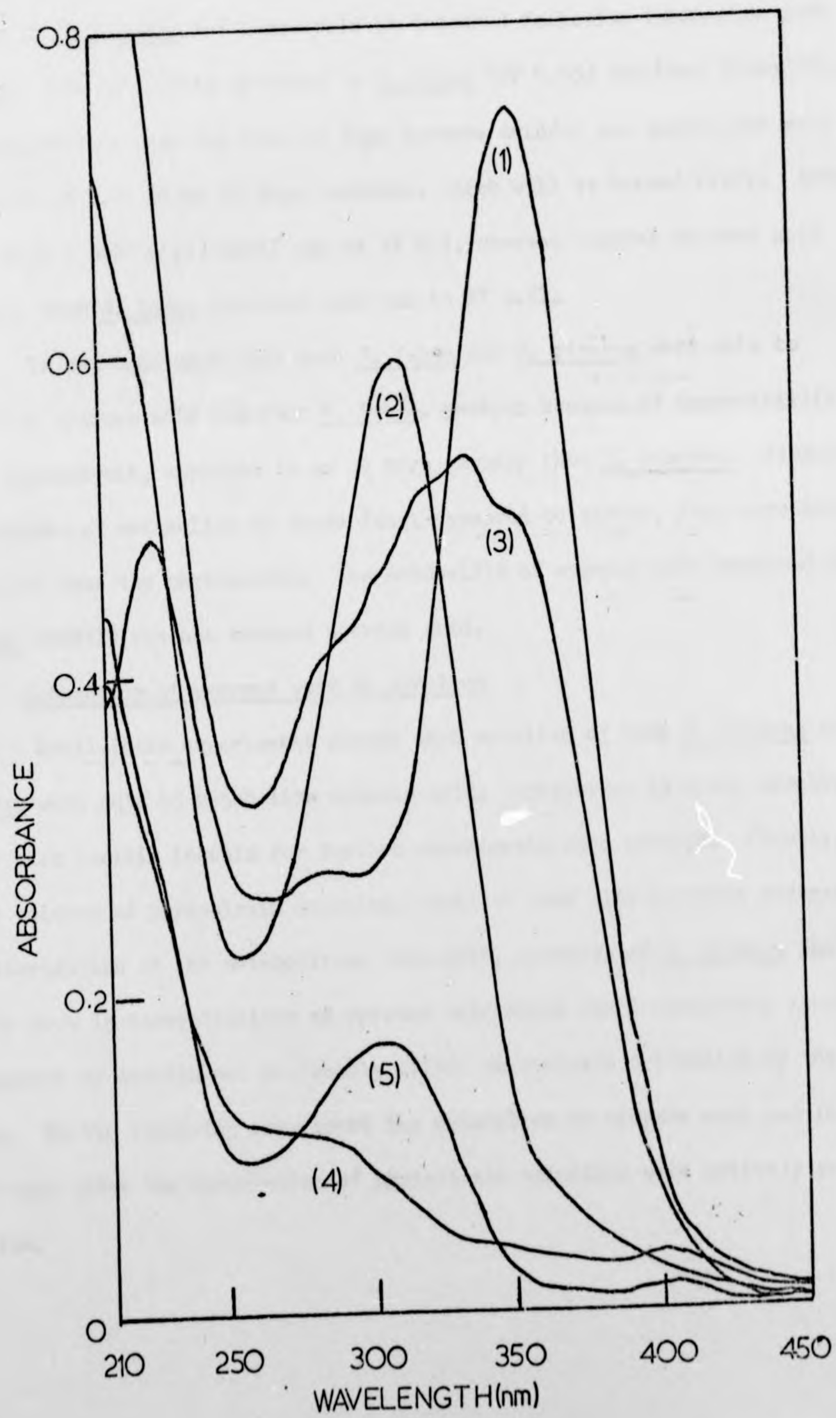
(2) " " " " with *B. fabae* conidia.

(3) " " " " " *B. cinerea* conidia.

(4) SPN solutions " " *B. fabae* " .

(5) " " " " *B. cinerea* " .

Fig.49



Similar chromatograms of extracts (equivalent to 5ml culture solution) were prepared and developed as above for elution and UV spectrophotometry of the substances detected under UV light. In the absence of conidia 80% of the original wyerone acid was recovered after TLC, only 42.2% remained in cultures of B. cinerea and none could be detected following incubation with B. fabae. The metabolite produced by B. fabae (RF 0.45) had λmax 300nm (Fig.50). It was calculated that the loss of 10µg wyerone acid/ml was associated with the appearance of 0.42 AU/ml of this compound, which will be termed WABfl. After TLC in Et_2O : MeOH (6:1) WABfl ran at RF 0.9, whereas reduced wyerone acid recovered from B. fabae infected pods ran to RF 0.61.

It was concluded that both B. fabae and B. cinerea were able to metabolize wyerone acid and that B. fabae, perhaps because of insensitivity to the phytoalexin, appeared to so so more rapidly than B. cinerea. Although the products of metabolism by these fungi appeared to differ, they were less inhibitory than the phytoalexin. The metabolite of wyerone acid produced by B. fabae (WABfl) was not reduced wyerone acid.

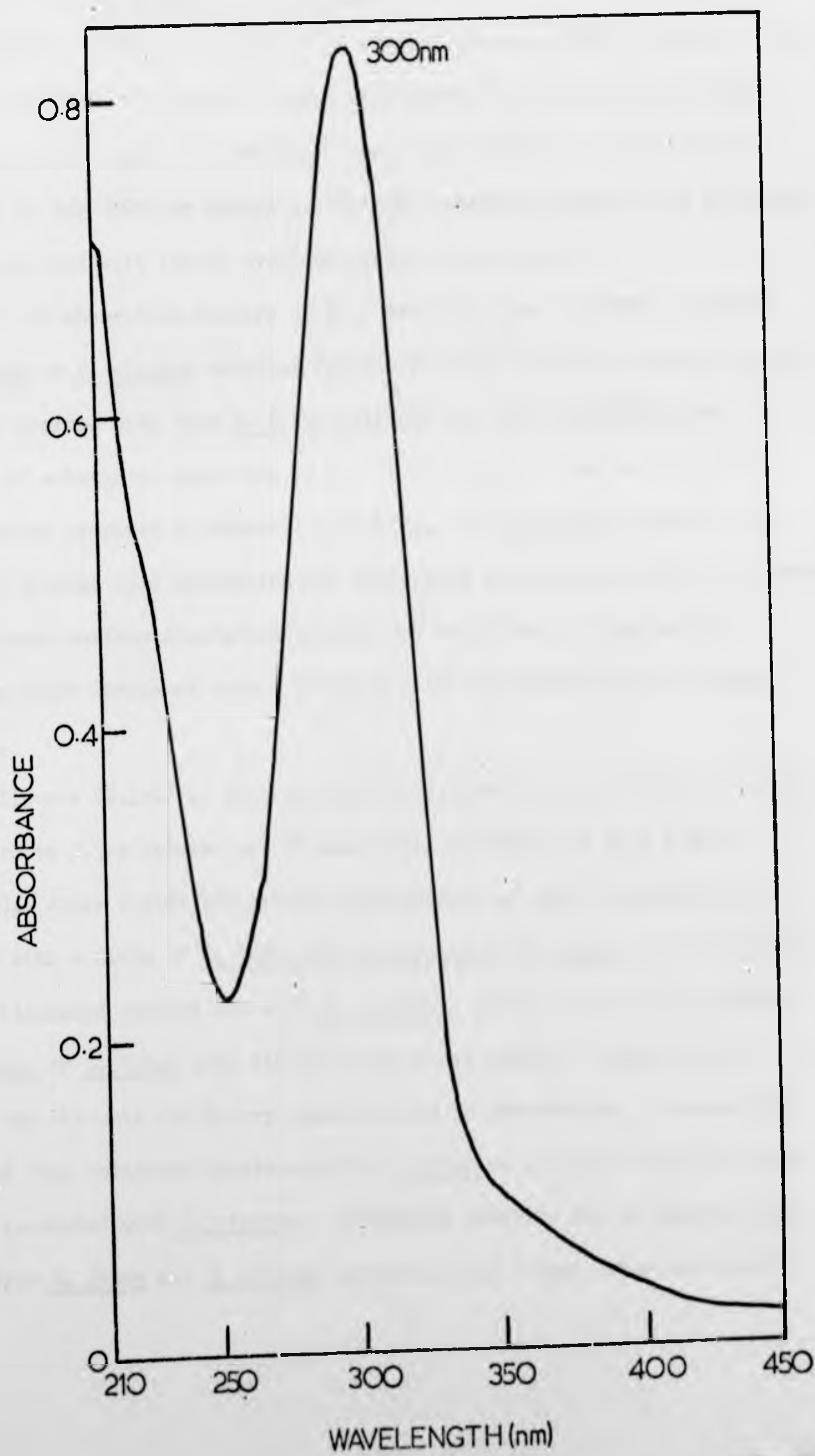
3 Metabolism of wyerone acid by mycelium

Preliminary experiments showed that mycelium of both B. cinerea and B. fabae were able to metabolize wyerone acid. Advantages in using mycelial rather than conidia inocula for further experiments were twofold. Firstly, larger volumes of phytoalexin solutions could be used allowing more accurate characterization of the metabolites. Secondly, mycelium of B. cinerea was able to grow in concentrations of wyerone acid which would completely inhibit germination of conidia and profoundly affect phytoalexin metabolism by this fungus. In the following experiment the metabolism of wyerone acid was followed for 5 days after the inoculation of phytoalexin solutions with actively growing mycelium.

Figure 50

UV absorption spectrum of the metabolite (WABf1) of wycrone acid produced by *B. fabae*.

Fig.50



Wyerone acid (730μg) in 50μl MeOH was added to flasks containing 50ml SPN solution giving a final concentration of 14.6 μg wyerone acid/ml and 0.1% MeOH. A disc (5mm diameter) of medium x agar bearing actively growing mycelium of either B. cinerea or B. fabae was added to each flask. The flasks were incubated at 18°c in the dark on an orbital incubator (200 rev/min). Each day for 5 days after inoculation flasks were removed and extracted with Et_2O and after recording their UV absorption spectra in MeOH, the extracts were redissolved in 1ml MeOH for analysis. Flasks containing wyerone acid solutions but not inoculated with fungal mycelium served as controls.

The UV absorption spectra of Et_2O extracts from solutions incubated with B. fabae or B. cinerea mycelium for the various times are shown in Fig.51. The loss of wyerone acid from B. fabae cultures was associated with the appearance of substances absorbing in the 310nm region of the spectrum, and this absorbance appeared to decrease with time. In B. cinerea cultures, the decrease in wyerone acid absorption was associated with an increase in substances showing a broad maximum absorption between 290 and 330nm two days after inoculation which developed into a distinct peak (315-320nm) with prolonged incubation.

Aliquots (0.1ml) of each extract (equivalent to 5ml culture solution) were applied to 2.5cm origins and chromatograms developed in Et_2O : MeOH (8:1, 15cm). After drying triplicate chromatograms of each treatment were bioassayed with conidia of C. herbarum, B. cinerea or B. fabae. Plate 13 shows TLC plate bioassays carried out with C. herbarum, plates sprayed with conidia of B. cinerea or B. fabae gave virtually identical results. Wyerone acid (RF 0.29) was the only inhibitory band detected in the extracts. Wyerone acid disappeared from solutions incubated with B. fabae at a greater rate than from solutions incubated with B. cinerea. Inhibitory activity due to wyerone acid was lost from B. fabae and B. cinerea cultures 2 and 3 days after inoculation

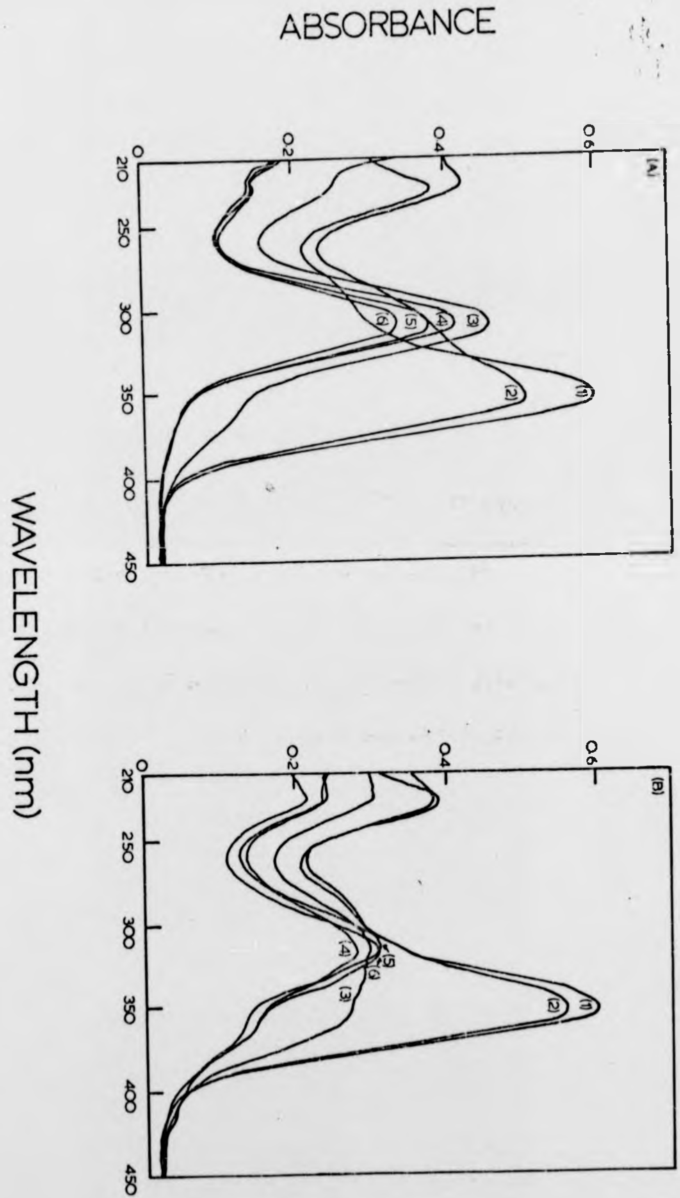
Figure 51

UV absorption spectra of Et_2O extracts, from SPN solutions containing wyerone acid (15 μg/ml) incubated alone for 4 days (1) or with either B. fabae (A) or B. cinerea (B) mycelium for 1 (2), 2 (3), 3 (4), 4 (5) and 5 days (6).

Figure 51

UV absorption spectra of Et_2O extracts, from SPN solutions containing wyerone acid (15 µg/ml) incubated alone for 4 days (1) or with either *B. fabae* (A) or *B. cinerea* (B) mycelium for 1 (2), 2 (3), 3 (4), 4 (5) and 5 days (6).

Fig. 51



ons

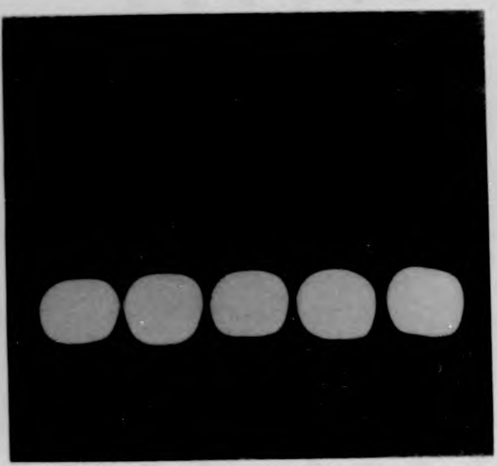
s (1)

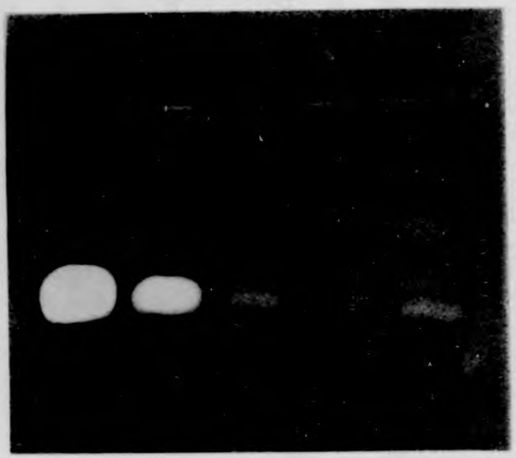
(2), 2 (3),

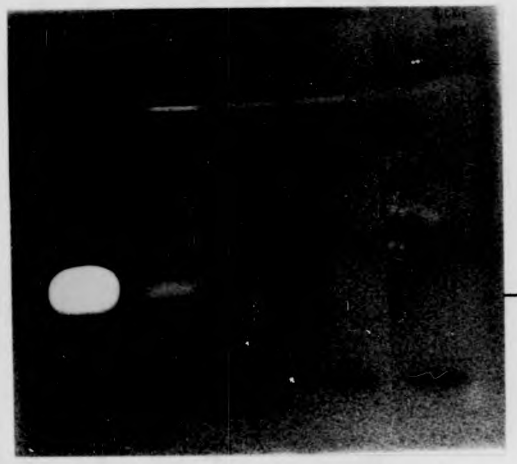
Plate 13

TLC plate bioassays of extracts from 5ml culture solution containing wyerone acid (14.6 $\mu g/ml$) collected 1,2,3,4 and 5 days after incubating alone (control) or with either B. cinerea or B. fabae mycelium. Solvent: Et_2O : MeOH (8:1) WA, wyerone acid.

Plate 13







TIME AFTER INOCULATION (days)

respectively, but remained constant throughout the experiment in solutions of the phytoalexin incubated without conidia.

Similar chromatograms of extracts (equivalent to 10ml culture solution) were prepared and developed as above for detection of the metabolites under UV light and characterization by UV spectrophotometry. The amounts of wyerone acid (μg/ml) and its metabolites (AU λmax /ml) recovered after incubating solutions of the phytoalexin with or without B. cinerea and B. fabae mycelium are illustrated in Fig. 52. Three substances which quenched the fluorescence of silica gel appeared to be associated with the disappearance of wyerone acid from extracts of B. fabae cultures.

1) WABf1 : RF 0.7, λmax 300nm (Fig.53A,1) which increased rapidly after inoculation, but decreased with prolonged incubation. This metabolite had the same RF in Et_2O : MeOH (8:1) as WABf1 detected in the previous experiment.

2) WABf2 : RF 0.52, λmax 300nm (Fig.53A,2) this metabolite increased less rapidly and reached much lower levels than WABf1, but also decreased after prolonged incubation.

3) WABf3 : RF 0.44, λmax 300nm (Fig.53A,3) which increased rapidly after an initial lag. Increase in WABf3 was coincident with the decrease observed in WABf1 and WABf2. This substance had an identical RF to reduced wyerone acid in Et_2O : MeOH (8:1) and hexane : acetone (2:1, RF 0.09).

In contrast, the loss of wyerone acid from cultures of B. cinerea was associated with the appearance of two major metabolites, both of which quenched the fluorescence of silica gel under UV light (254nm)

1) WABc1 : RF 0.7, λmax 300nm (Fig 53B, 1) which accumulated slowly with time after inoculation and appeared to be identical to WABf1.

Figure 52

Changes in the concentrations of wyerone acid (WA) and its metabolites (WABf1,2,3 and WABc1, 2) after incubating wyerone acid (15µg/ml) in SPN solution either alone (A) or with B. fabae (B) or B. cinerea (C) mycelium. The dotted lines indicates that these two substances were estimated from mixtures.

Fig.52

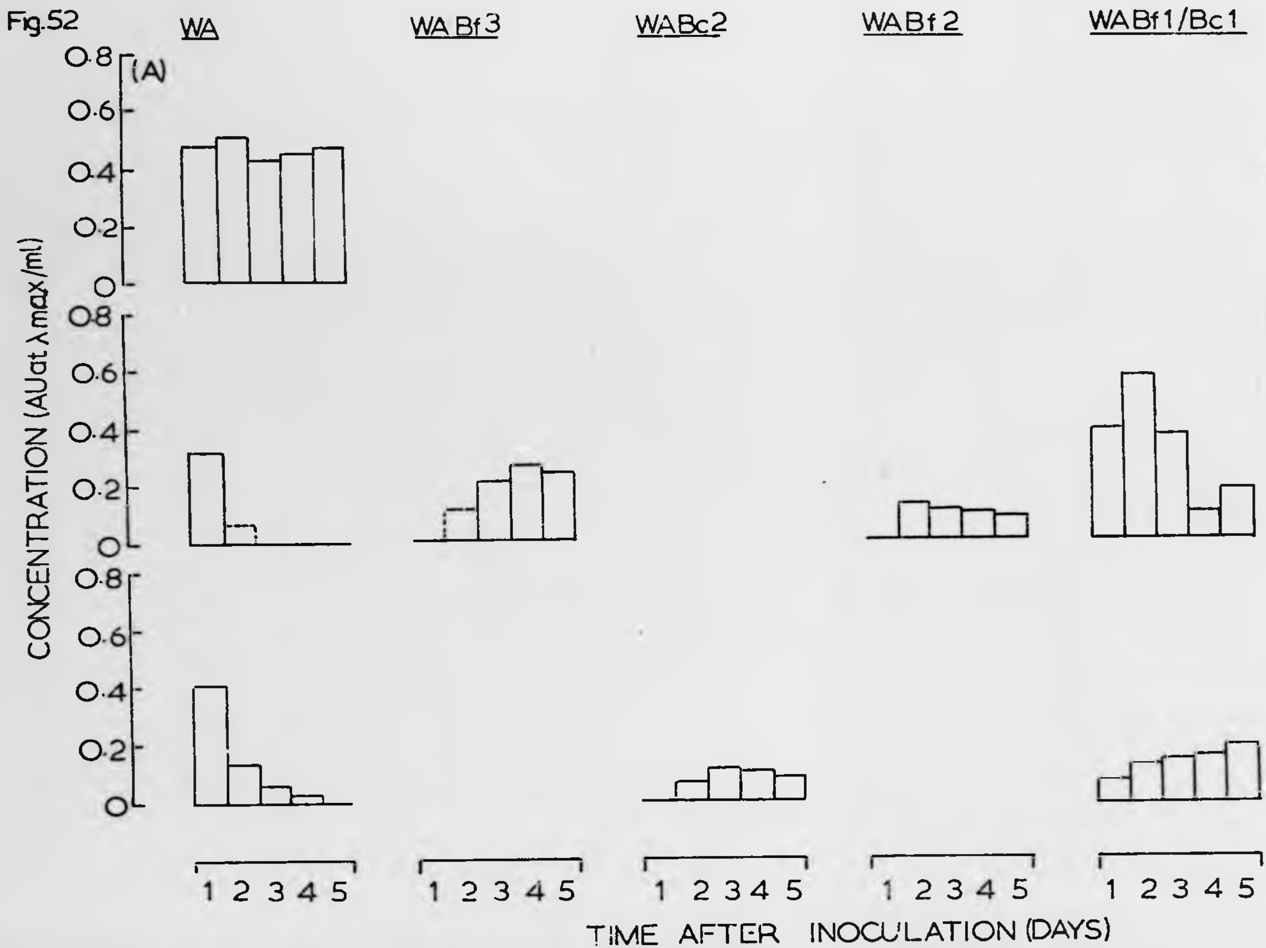


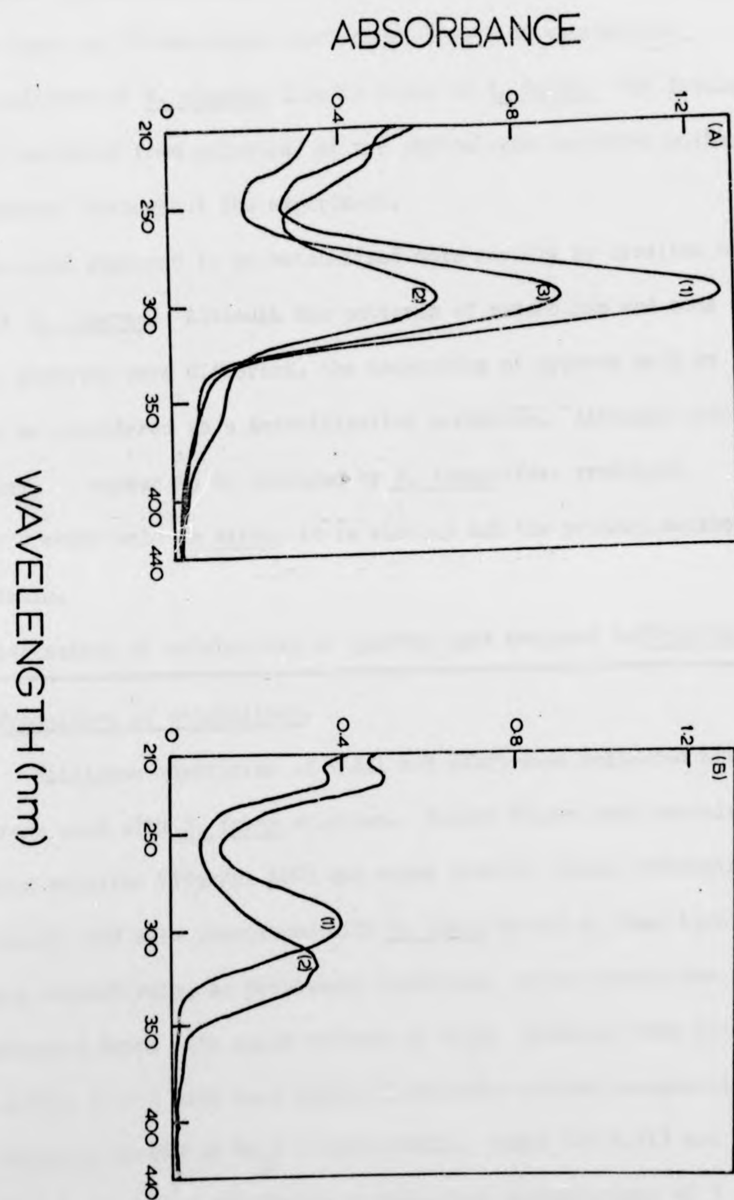
Figure 53

UV absorption spectra of the metabolites of wyerone acid produced by *B. fabae* (A) (WABf1(1) WABf2(2)) and WABF3(3)) and by *B. cinerea* (B) (WABc1(1) and WABc2(2)).

Figure 53

UV absorption spectra of the metabolites of wyerone acid produced by B. fabae (A) (WABf1(1) WABf2(2)) and WABF3(3)) and by B. cinerea (B) (WABc1(1) and WABc2(2)).

Fig.53



2) WABc2 : RF 0.43 λmax 318 shoulder 330 (Fig 53B, 2). This metabolite showed a similar pattern of accumulation to that of WABc1.

On the basis of UV absorbance much lower levels of metabolites accumulated in cultures of B. cinerea than in those of B. fabae. The levels of wyerone acid recovered from solutions of the phytoalexin incubated without mycelium was constant throughout the experiment.

Wyerone acid appeared to be metabolized more rapidly by mycelium of B. fabae than of B. cinerea. Although the patterns of metabolism and some of the metabolites produced were different, the conversion of wyerone acid by these fungi may be considered as a detoxification mechanism. Although reduced wyerone acid does appear to be produced by B. fabae after prolonged incubation with wyerone acid in vitro, it is clearly not the primary metabolite of this phytoalexin.

C Characterization of metabolites of wyerone acid produced by Botrytis.

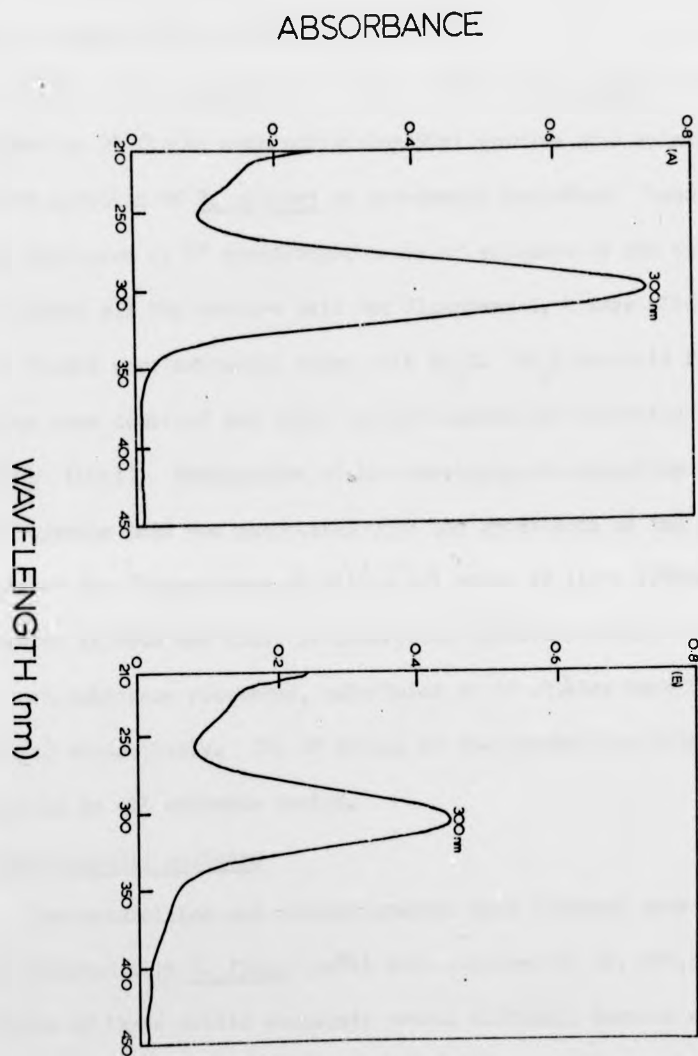
(i) Production of metabolites.

Milligram quantities of WABf1 and WABf3 were collected after incubating wyerone acid with B. fabae mycelium. Twelve flasks each containing 50ml wyerone acid solution (19μg/ml SPN) and eight similar flasks containing 18μg wyerone acid/ml SPN were inoculated with B. fabae mycelium, then incubated for 3 and 5 days respectively, as previously described. After incubation, flasks were extracted twice with equal volumes of Et_2O. Extracts from flasks incubated for either 3 or 5 days were combined and after solvent evaporation each extract subjected to PLC in Et_2O : MeOH (10:1). WABf1 (RF 0.71) and WABf3 (RF 0.47) were recovered by elution in MeOH from chromatograms of 3 and 5 day extracts respectively and their UV absorption spectra recorded (Fig.54). Yields of c 3.4mg WABf1 and 2.5mg WABf3 were recovered from the metabolism of 11.5 and 7.2mg wyerone acid respectively. WABf3 had identical RF values

Figure 54

UV absorption spectra of purified *B. fabae* metabolites of wyerone acid, WABf1 (A) and WABf3 (B).

Fig. 54



to reduced wyerone acid in Et_2O : MeOH (6:1), 0.65; Et_2O : MeOH (10:1), 0.42 and hexane : acetone (1:1), 0.18.

The products of the metabolism of wyerone acid by B. cinerea were obtained by incubating 24 flasks each containing 50ml wyerone acid solution (19µg/ml SPN) with mycelium of B. cinerea as previously described. Loss of wyerone acid was monitored by UV spectrophotometry of aliquots of the culture solution. When almost all the wyerone acid had disappeared, 4 days after inoculation, the flasks were extracted twice with Et_2O. Et_2O extracts from replicate cultures were combined and after solvent evaporation separated by PLC in Et_2O : MeOH (10:1). Examination of the developed chromatograms revealed that the loss of wyerone acid was associated with the appearance of two major bands which quenched the fluorescence of silica gel under UV light (254nm). Each band was eluted in MeOH and their UV absorption spectra recorded (Fig.55). The amounts of each substance recovered, calculated as AU at λmax were 119 and 113 for WABc1 and WABc2 respectively. The RF values of the metabolites WABf1 and WABc1 were identical in all solvents tested.

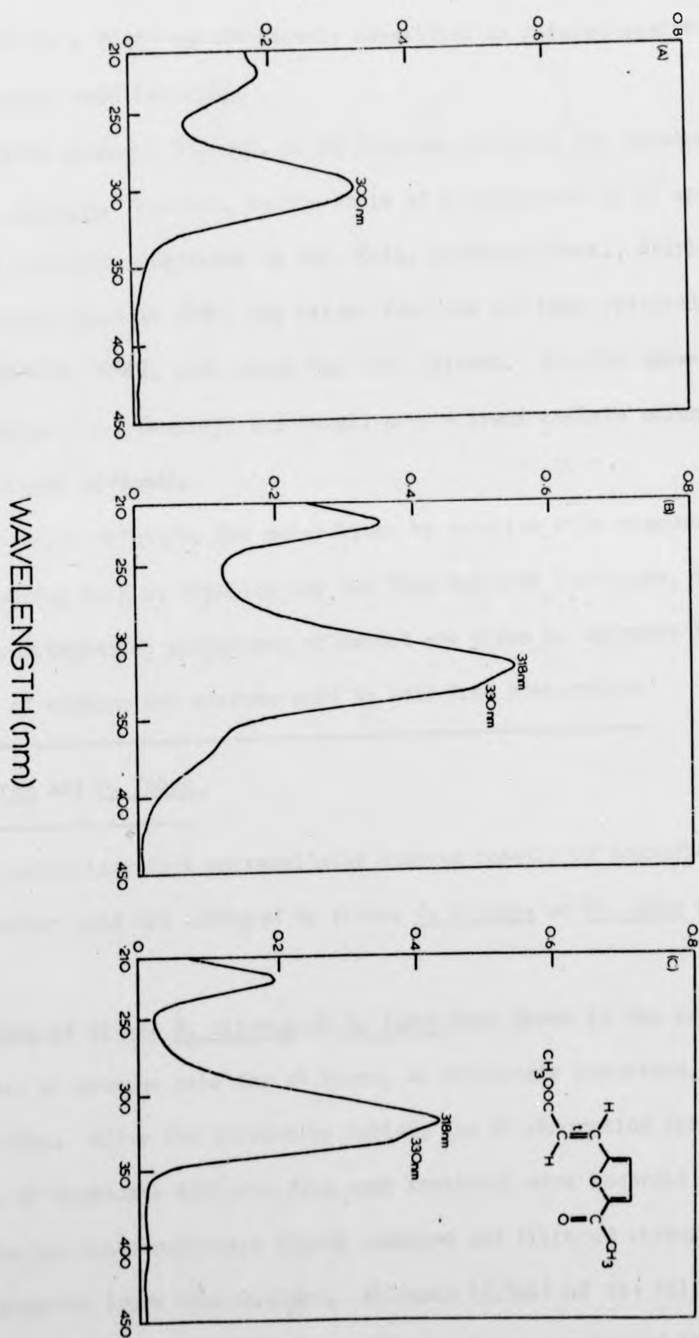
(ii) Spectroscopic analysis

The metabolites and reduced wyerone acid isolated from pod endocarp tissue infected with B. fabae (p.86) were examined by IR, NMR, and MS. NMR and IR analysis of these acidic compounds proved difficult because of their insolu bility in less polar solvents such as $CHCl_3$ and no useful MS data was obtained because of their low volatilities. NMR spectra of reduced wyerone acid, WABf1 and WABf3 were obtained in deuteriomethanol but were difficult to interpret because of large peaks at δ 3.36 and 4.77 due to deuterium/hydrogen exchange. Nevertheless, spectra of WABf3 and reduced wyerone acid were virtually identical, both showed a broad multiplet centered at 1.9 - 2.1 assigned to

Figure 55

UV absorption spectra of purified *B. cinerea* metabolites of wyerone acid, WABc1 (A) and WABc2 (B) and of synthetic Methyl 3 - (5 acetyl - 2 furyl) prop. trans - 2 - enoate.

Fig.55



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($3 \times CH_2$) indicating the saturation of the C≡C function, which was not apparent in the spectrum of WABf1. Signals at δ5.12 in spectra of WABf3 and reduced wyerone acid and at 5.15 in WABf1 were assigned to the hydroxyl proton. From NMR and TLC data WABf3 was tentatively identified as reduced wyerone acid and WABf1 as wyerol acid (Fig.56).

Similarly no useful IR, NMR, or MS data was obtained for metabolites produced by B. cinerea. However, on the basis of a comparison of UV spectra with synthetic compounds (provided by R.O. Cain, Chemistry Dept., Stirling) it may be concluded that in WABc2 the ketone function has been retained but the CH_3. CH_2.CH=CH. C≡C. side chain has been altered. Fig.55c shows the UV spectrum of Methyl 3 - (-5-acetyl - 2 furyl) prop - trans enolate which is virtually identical to WABc2.

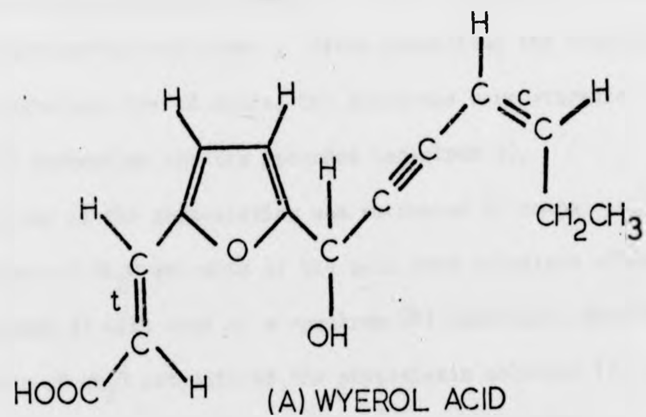
Attempts to methylate the metabolites by reaction with diazomethane proved unsuccessful both at Stirling and the Food Research Institute, Norwich. Details of the methylation procedures attempted are given in Appendix II.

4 Metabolism of wyerone and wyerone acid by cell free preparation of B. cinerea and B. fabae.

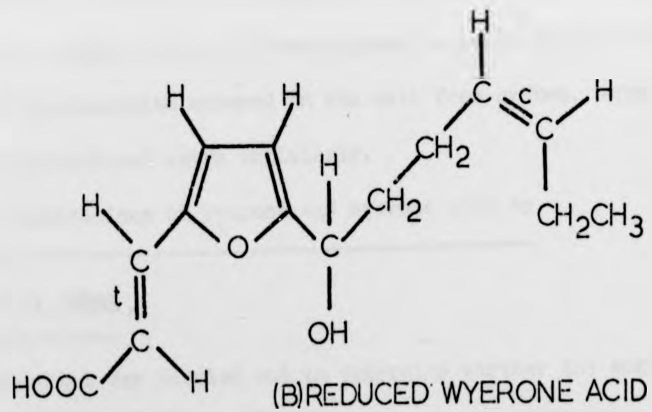
The possibility that extracellular enzymes capable of degrading wyerone or wyerone acid are produced by either B. cinerea or B. fabae was investigated.

Conidia of either B. cinerea or B. fabae were grown in the presence of 10µg/ml wyerone or wyerone acid for 24 hours, as previously described, to allow enzyme induction. After the incubation period, the UV absorption spectra of Et_2O extracts of duplicate cultures from each treatment were recorded (spectrum 1) and those from two other replicate flasks combined and filtered through a 'millipore' membrane (pore size 0.45µm). Aliquots (2.5ml) of the filtrate from each treatment were added to 2.5ml sterile distilled water, adjusted to pH4

Fig.56



(A) WYEROL ACID



(B)REDUCED WYERONE ACID

with galacturonic acid, containing 10µg/ml of either wyerone or wyerone acid (spectrum 2), understerile conditions . After incubating the filtrates with the phytoalexin solutions for 18 hours, the solutions were extracted with Et_2O and their UV absorption spectra recorded (spectrum 3).

Degradation of the phytoalexins was estimated by compa ring the UV absorption spectrum of Et_2O extracts of the cell free solutions after 18 hours incubation (spectrum 3) with that of a spectrum (4) calculated from the UV absorption spectra of Et_2O extracts of the phytoalexin solution (2) and the culture filtrate (1) assuming that no metabolism occured. An example is shown in Fig. 57. Values for the observed and expected ratio of absorbance at 310nm (due to metabolite) and 350nm (due to phytoalexin) were determined. Metabolism of either phytoalexin in cell free conditions, would have resulted in an increase in the 310 : 350nm ratio. Results given in table 21 show that no degradation of the phytoalexins occured in the cell free system, suggesting that they are not metabolized extra cellularly.

5 Metabolism of combinations of wyerone and wyerone acid by *B. cinerea* and *B. fabae*.

This experiment was carried out to determine whether the enzymes involved in the metabolism of wyerone acid are also responsible for the metabolism of wyerone acid.

Conidial suspensions of *B. fabae* or *B. cinerea*, or sterile distilled water alone were incubated with solutions of 7.5µg wyerone/ml, 7.5µg wyerone acid/ml or a combination of both phytoalexins (7.5µg/ml of each) as previously described. Twenty four hours after incubation duplicated flasks of each treatment were extracted with Et_2O and extracts separated by TLC in Et_2O : MeOH (6:1, 7cm) followed by $CHCl_3$: petrol (2:1, 14cm). Bands corresponding

Figure 57

UV absorption spectra used to investigate the metabolism of wyerone and wyerone acid by exoenzymes,

Spectrum 1 - Et_2O extract of phytoalexin solutions incubated with fungal conidia for 24 hours.

Spectrum 2 - Et_2O extract of phytoalexin solution to be incubated with fungal filtrate.

Spectrum 3 - Et_2O extract of phytoalexin solution incubated with fungal filtrate for 18 hours.

Spectrum 4 - calculated spectrum from (1) and (2) if no metabolism occurs.

Fig. 57

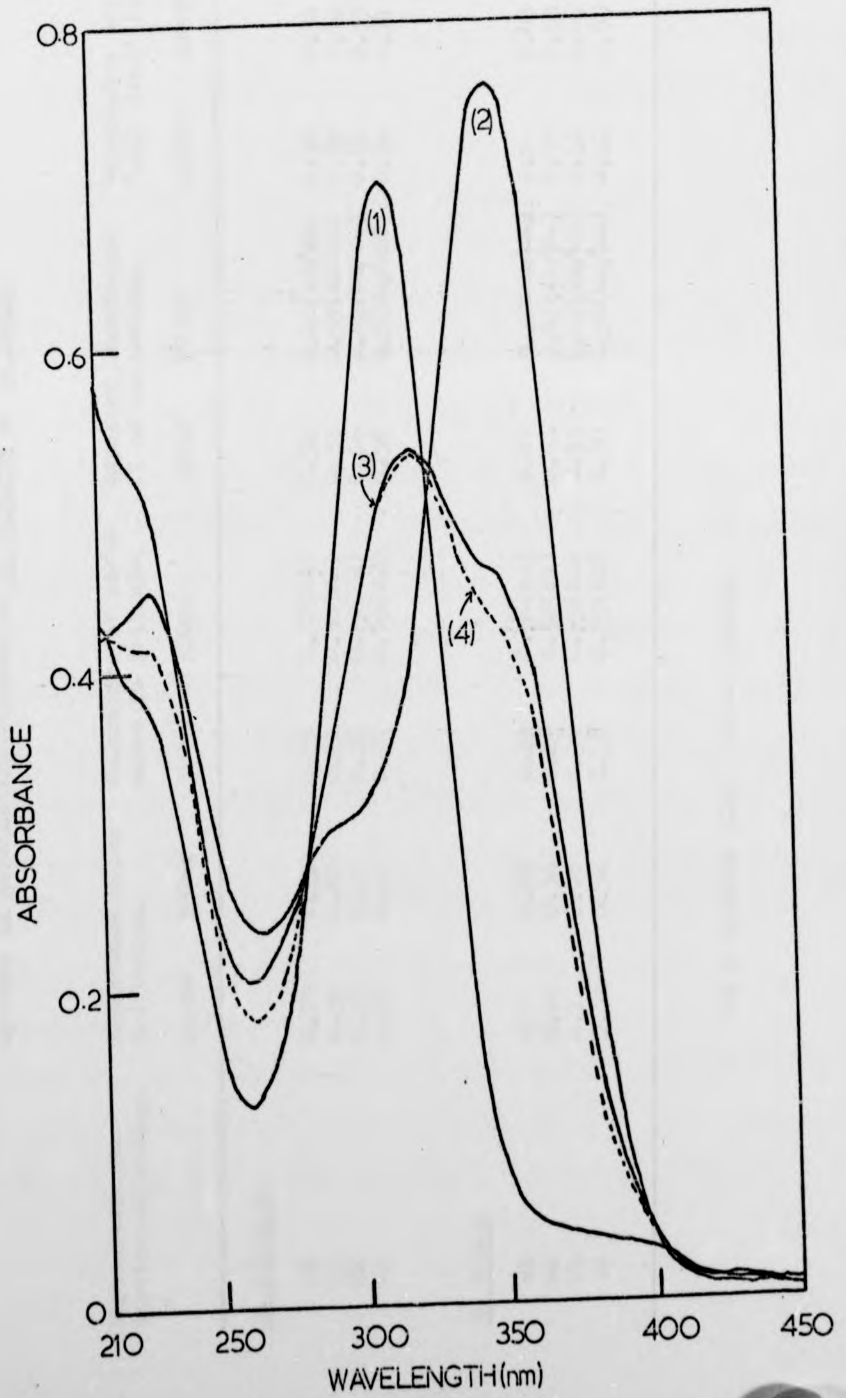


TABLE 21 Detection of wyerone acid and wyerone metabolism by exoenzymes induced with either wyerone acid or wyerone in germinating conidia of *B. cinerea* or *B. fabae*.

Phytoalexin in incubation medium.	Absorbance after incubation.		Absorbance of WA/WE added to filtrate.		Expected absorbance if no metabolism.		Absorbance after 'cell free' incubation.		310/350nm	
	310nm	350nm	310nm	350nm	310nm	350nm	310nm	350nm	Expected	Obtained
B. CINEREA										
WE	0.74	0.25	0.29	0.58(WA)	0.51	0.41(WE/WA)	0.36	0.34	1.24	1.05
WA	0.38	0.62	0.29	0.58(WA)	0.33	0.59(WA/WA)	0.39	0.76	0.55	0.51
WE	0.74	0.25	0.38	0.79(WE)	0.56	0.52(WE/WE)	0.52	0.53	1.1	0.99
WA	0.38	0.62	0.38	0.79(WE)	0.38	0.7(WA/WE)	0.36	0.71	0.53	0.50
B. FABAE										
WE	0.7	0.08	0.29	0.58(WA)	0.49	0.33(WE/WA)	0.5	0.44	1.48	1.13
WA	0.64	0.36	0.29	0.58(WA)	0.46	0.47(WA/WA)	0.45	0.55	0.97	0.81
WE	0.7	0.08	0.38	0.79(WE)	0.53	0.44(WE/WE)	0.52	0.48	1.22	1.08
WA	0.64	0.36	0.38	0.79(WE)	0.50	0.58(WA/WE)	0.52	0.53	0.86	0.99

WA - WYERONE ACID WE - WYERONE

to wyerone, wyerol, wyerone acid, wyerol acid and reduced wyerone acid were eluted in MeOH and their recovery from each treatment recorded (Table 22). Both wyerone and wyerone acid disappeared from solutions containing _B. fabae_ whether the phytoalexins were present singly or in combination. However, germinating conidia of _B. cinerea_ were able to metabolize wyerone but not wyerone acid when the phytoalexins were supplied either alone or in combination. This suggests that the enzymes metabolizing wyerone in _B. cinerea_ are different from those which metabolize wyerone acid.

6 Time course of phytoalexin metabolism and germ tube growth by _B. fabae_ and _B. cinerea_ conidia.

These experiments were carried out in order to determine if phytoalexin inactivation has a direct role in allowing fungal growth or whether the sensitivity of the fungus to the phytoalexin determines the rate of phytoalexin metabolism. The hypothetical relationship between fungal growth and phytoalexin metabolism shown in Fig. 58 illustrates two extreme situations. If fungal growth occurs at the same time as or before detoxification of phytoalexin, growth of the fungus will depend largely on its sensitivity to the inhibitor. Apparent differences in the rates of metabolism will infact merely be a reflection of differences in sensitivity (Fig58A). Alternatively, if fungal growth is prevented until the phytoalexin is inactivated and the growth rate only increases when the phytoalexin reaches non-inhibitory concentrations, then differences in rates of metabolism may be considered to be of primary importance in controlling fungal growth (Fig.58B).

Concentration of the phytoalexin may also determine which mechanism is controlling growth. At low concentrations fungal growth may be a consequence of insensitivity to the inhibitor where as at higher concentrations metabolism

TABLE 22 Yields ($\mu g/ml$) of wyerone, wyerone acid and their respective metabolites after incubating wyerone ($7.5 \mu g/ml$) and wyerone acid ($7.5 \mu g/ml$) either alone or in combination, with and without conidia of *B. cinerea* or *B. fabae* for 24h.

PHYTOALEXIN IN INCUBATING SOLUTION		WATER CONTROL	B. CINEREA	B. FABAE
WYERONE	WYERONE	5.0	0.5	-
	WYEROL	-	5.1	6.0
WYERONE ACID	WYERONE ACID	6.3	4.5	-
	WYEROL ACID	-	-	0.23 *
	REDUCED WYERONE ACID	-	-	0.18 *
WYERONE + WYERONE ACID	WYERONE	5.1	1.5	-
	WYEROL	-	3.4	5.3
	WYERONE ACID	6.6	4.3	0.1
	WYEROL ACID	-	-	0.21 *
	REDUCED WYERONE ACID	-	-	0.20 *

* - yield AU at $\lambda max/ml$.

Figure 58

The hypothetical relationship between fungal growth and phytoalexin metabolism (see text for explanation).

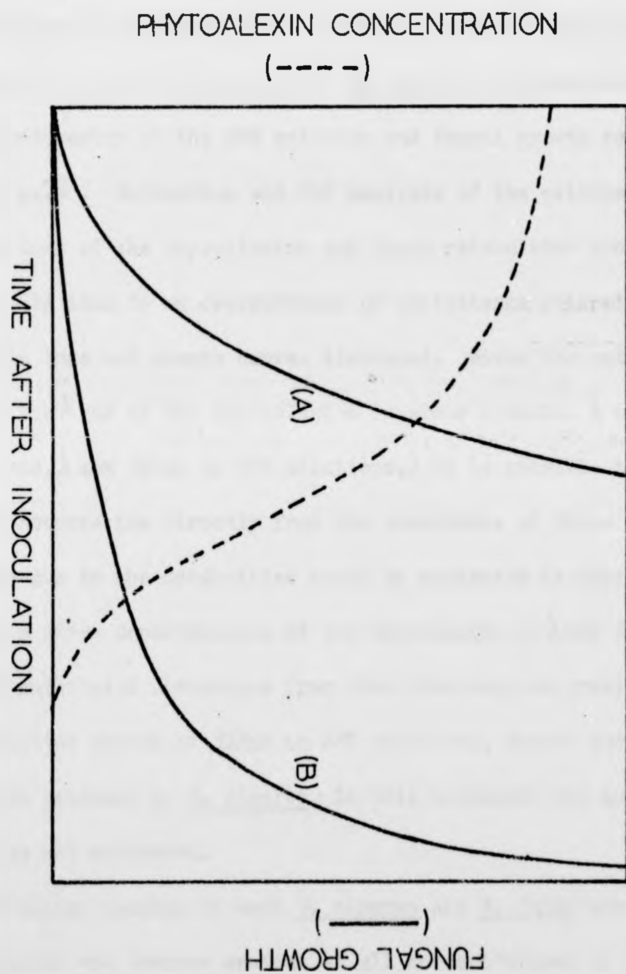


Fig.58

may assume a more important role. At an intermediate concentration both these mechanisms may be involved.

The loss of phytoalexins and appearance of metabolites in SPN solutions containing different concentrations of wyerone, wyerone epoxide and wyerone acid incubated with either B. cinerea or B. fabae conidia was monitored by UV absorption spectrometry of the SPN solution and fungal growth recorded as described on p.22. Extraction and TLC analysis of the culture solutions was avoided since loss of the phytoalexins and their metabolites encountered with this method would lead to an overestimate of phytoalexin metabolism and hence the phytoalexin loss and growth curves displaced. Since the metabolites do not absorb at the λmax of the phytoalexins (wyerone epoxide, λmax 354nm; wyerone and wyerone acid, λmax 360nm in SPN solutions,) it is possible to calculate the phytoalexin concentration directly from the absorbance at these wavelengths. The absorbance due to the metabolites could be estimated by calculating the absorbance of a given concentration of the phytoalexin at λmax 312nm and subtracting this calculated absorbance from that obtained, as previously described. All the metabolites absorb at 312nm in SPN solutions, except for the metabolites of wyerone acid produced by B. cinerea. In this treatment the accumulation of the metabolites was not estimated.

Germinating conidia of both B. cinerea and B. fabae were able to metabolize wyerone and wyerone epoxide at all concentrations of the phytoalexin tested (Fig. 59 and 60 respectively). Germination was delayed and germ tube growth reduced with increasing phytoalexin concentration and this was reflected in the loss of the phytoalexin and accumulation of metabolites in the incubating medium. In general, metabolism of the phytoalexin occured with the onset of germination and germ tube growth, growth being associated with loss of the phytoalexin and appearance of metabolites. The growth/phytoalexin loss curves

Figure 59

The relationship between growth of *B. cinerea* (A) and *B. fabae* (B) and the metabolism of wyerone.

● wyerone concentration in fungal cultures.

○ " " " solutions incubated alone.

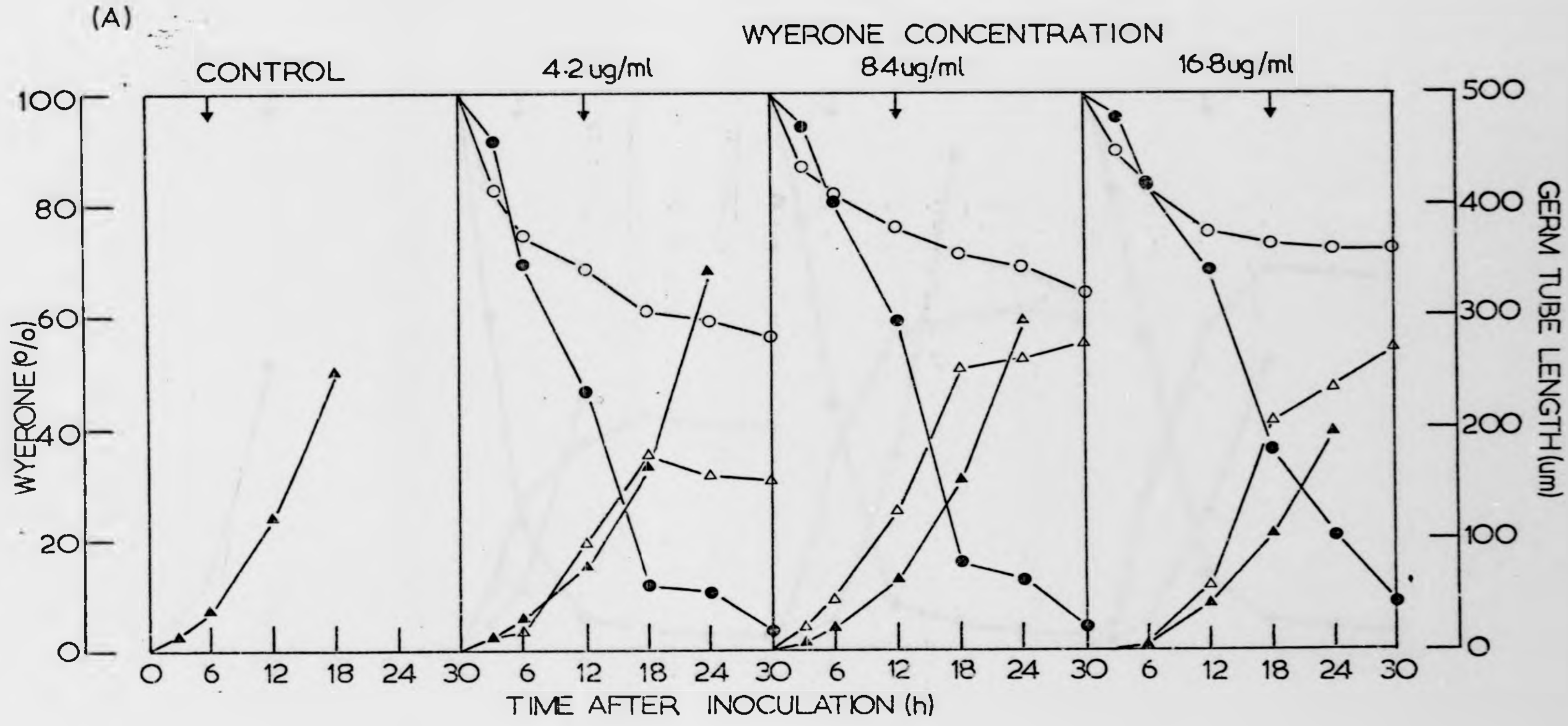
▲ germ tube growth.

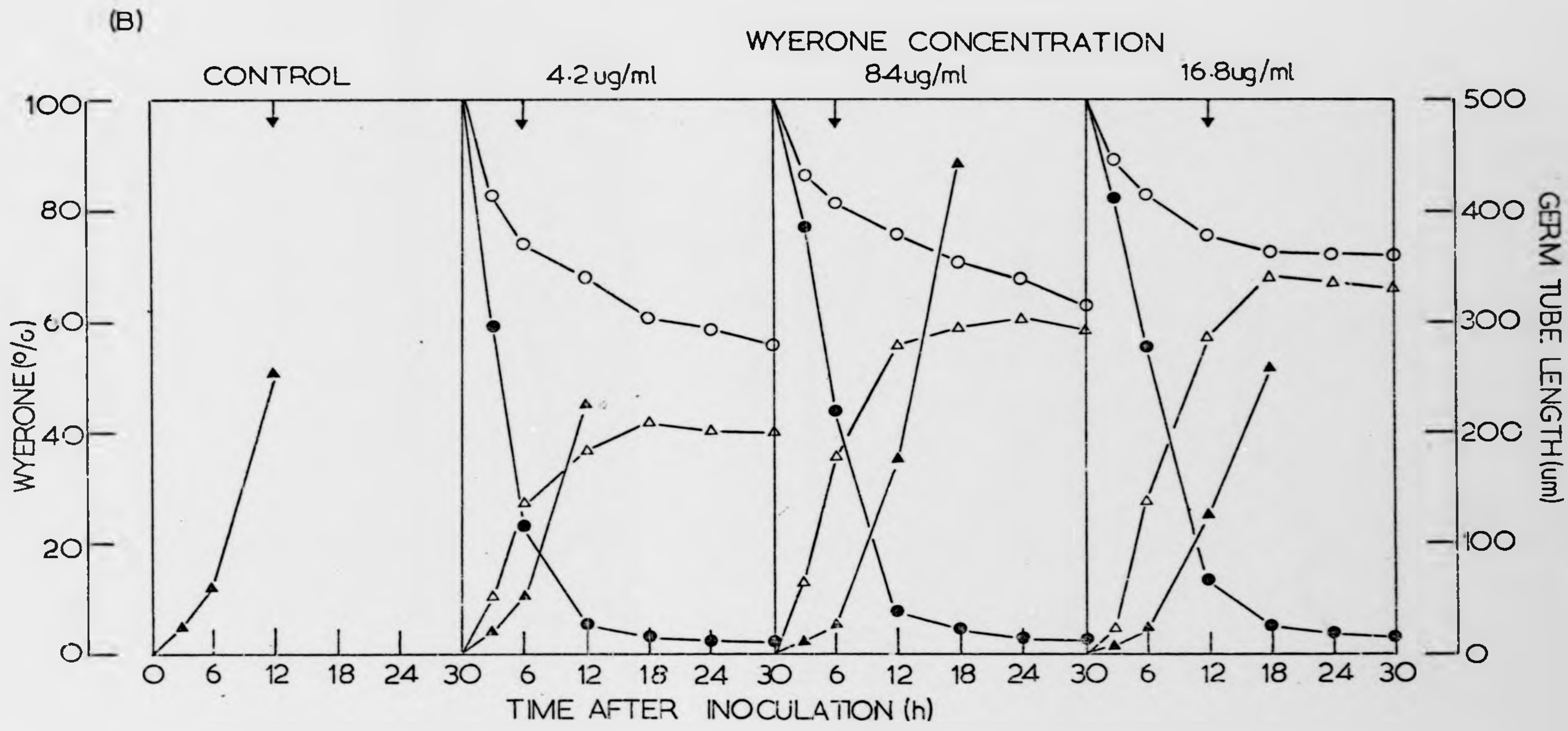
△ metabolite concentration ml^{-1} in fungal cultures.

Scales:

4.2	µg/ml	wyerone	0-0.5	Absorbance units
8.4	"	"	0-1.0	" "
16.8	"	"	0-2.0	" "

Arrows indicate when maximum (98-100%) germination attained.


(A)
WYERONE CONCENTRATION
CONTROL
4·2 ug/ml
8·4 ug/ml
16·8 ug/ml
WYERONE (%)
GERM TUBE LENGTH (um)
TIME AFTER INOCULATION (h)



(B)
WYERONE CONCENTRATION
CONTROL
4.2 ug/ml
8.4ug/ml
16.8ug/ml
WYERONE (%)
GERM TUBE LENGTH (um)
TIME AFTER INOCULATION (h)

Figure 60

The relationship between growth of B. cinerea (A) and B. fabae (B) and the metabolism of wyerone epoxide.

● wyerone epoxide concentrations in fungal cultures.

○ " " " " solutions incubated alone.

▲ germ tube growth.

△ metabolite concentration ml^{-1} in fungal cultures.

Scales:

2.7 μg/ml	wyerone epoxide	0-0.5	Absorbance units.
5.4 "	" "	0-1.0	" "
11.5 μg/ml	" "	0-1.0	" "

Arrows indicate when maximum (98-100%) germination attained.

Figure 60

The relationship between growth of B. cinerea (A) and B. fabae (B) and the metabolism of wyerone epoxide.

● wyerone epoxide concentrations in fungal cultures.

○ " " " " solutions incubated alone.

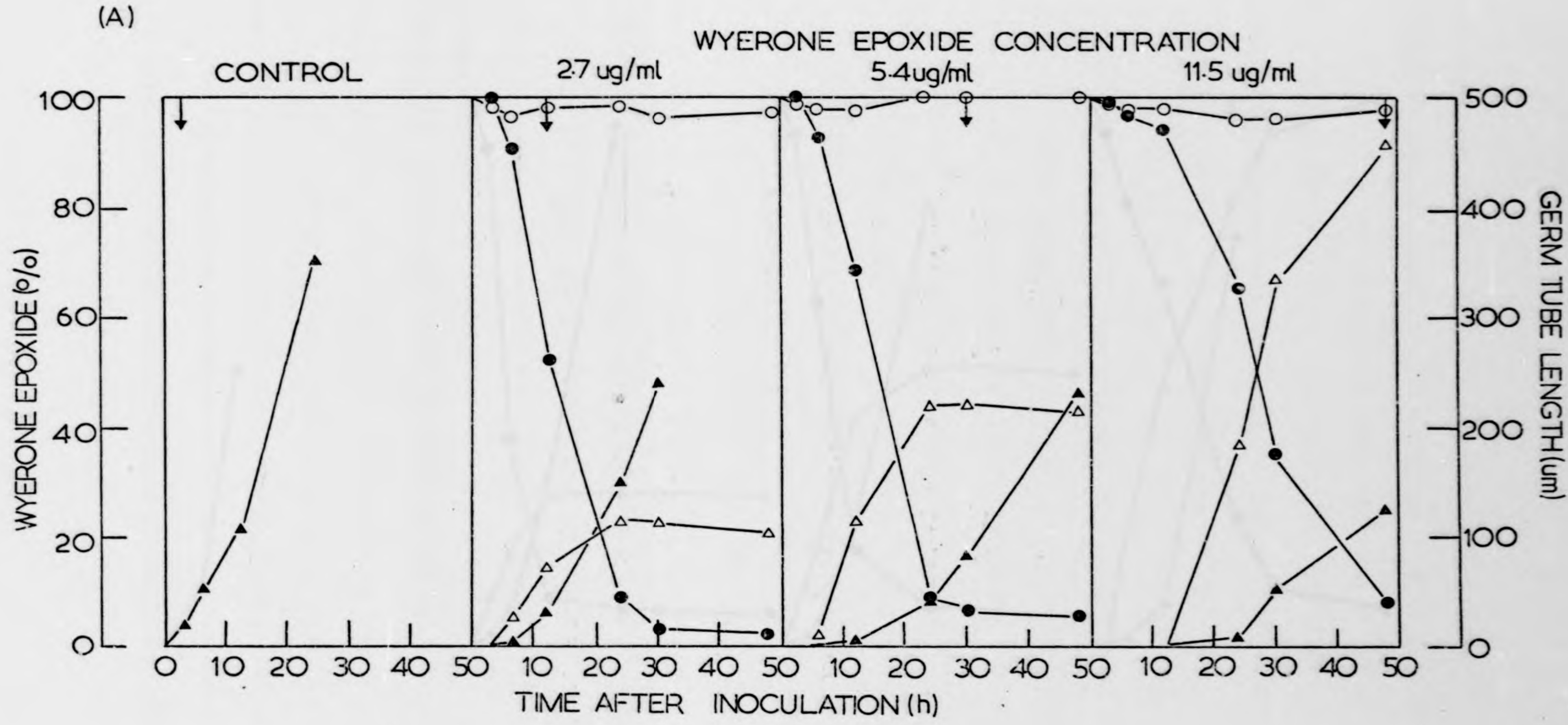
▲ germ tube growth.

△ metabolite concentration ml^{-1} in fungal cultures.

Scales:

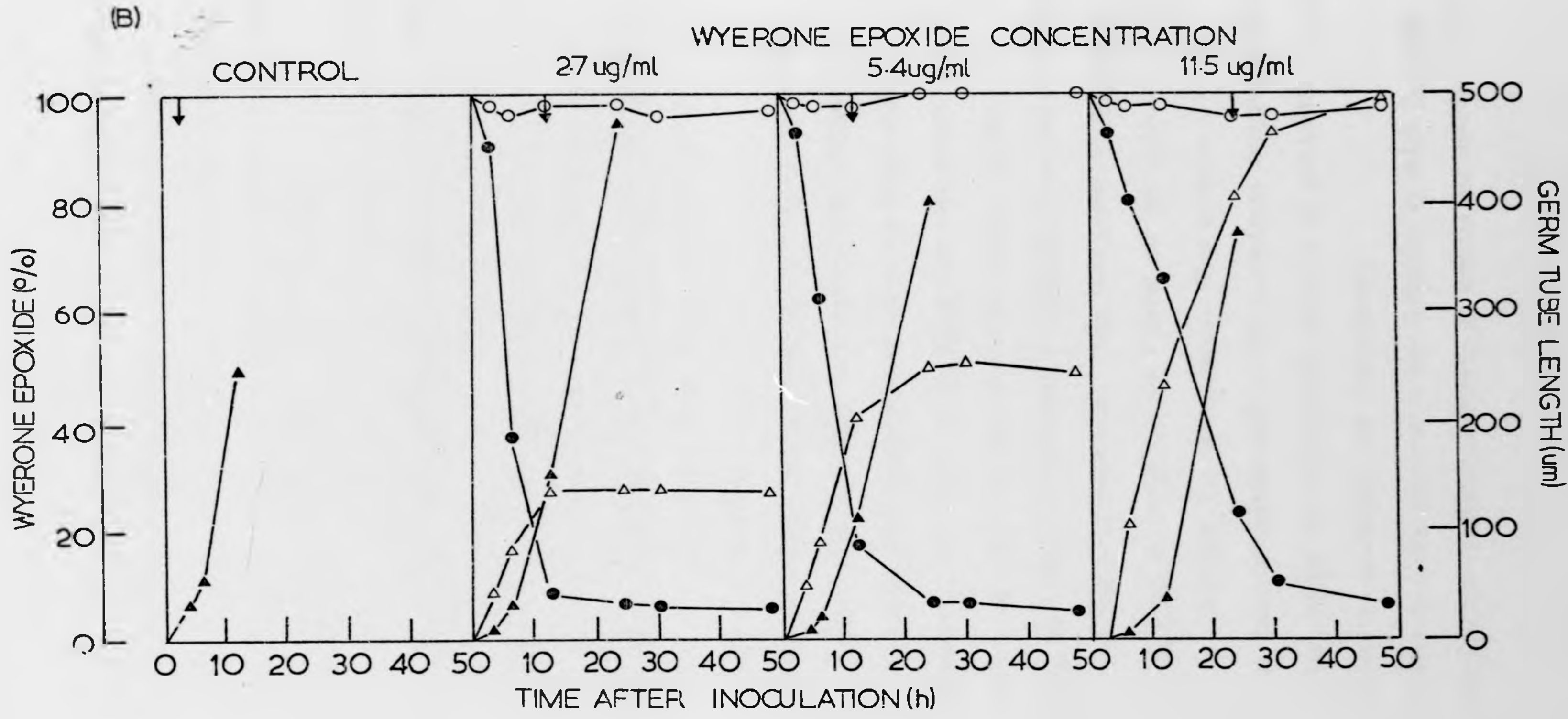
2.7 μg/ml	wyerone	epoxide	0-0.5	Absorbance units.
5.4 "	"	"	0-1.0	" "
11.5 μg/ml	"	"	0-1.0	" "

Arrows indicate when maximum (98-100%) germination attained.



alone.

ined.



(B)
WYERONE EPOXIDE CONCENTRATION
CONTROL
2.7 ug/ml
5.4ug/ml
11.5 ug/ml
WYERONE EPOXIDE (%)
100
80
60
40
20
0
0 10 20 30 40 50 10 20 30 40 50 10 20 30 40 50 10 20 30 40 50
TIME AFTER INOCULATION (h)
500
400
300
200
100
0
GERM TUBE LENGTH (um)

were intermediate between the two hypothetical cases outlined above, suggesting that fungal growth is a result of a combination of insensitivity to the phytoalexins and their metabolism. Differences in the abilities of the fungi to metabolize the phytoalexins appeared to be related to the mass of the fungus present and hence sensitivity of the fungus to the phytoalexin.

The loss of wyerone incubated without fungi in this experiment (Fig.59) could not be accounted for by metabolism, since no change in the UV spectrum was observed. It is possible that wyerone was precipitated from solutions. However, Et_2O extracts of the incubation flasks failed to recover more wyerone than was present in solution. It appears that the phytoalexin was removed abiotically in some way, a similar loss of wyerone derivatives was detected in solutions incubated with B. cinerea and B. fabae since the levels of wyerol in solution were lower than expected.

The relationships between fungal growth and metabolism of wyerone acid (Fig.61) were somewhat different from those observed with wyerone and wyerone epoxide. At wyerone acid concentrations where the fungi could metabolize the phytoalexin, the pattern of B. fabae germ tube growth and phytoalexin metabolism was similar to that described above. However, the growth of B. cinerea germ tubes at the lowest phytoalexin concentration occured at the same time as wyerone acid metabolism. Germ tube growth by B. cinerea in 8.6μg/ml and by B. fabae in 17.2μg wyerone acid/ml did not appear to relate to their abilities to metabolize the phytoalexins. This suggests that the sensitivity of these fungi to wyerone acid is the predominant factor affecting fungal growth and may explain the differential abilities of germinating B. fabae and B. cinerea conidia to metabolize this phytoalexin.

In conclusion it would appear that the mechanism by which B. fabae and B. cinerea overcome the inhibitory activity of wyerone and wyerone epoxide

Figure 61

The relationship between growth of B. cinerea (A) and B. fabae (B) and the metabolism of wyerone acid.

● wyerone acid concentrations in fungal cultures.

○ " " " " solutions incubated alone,

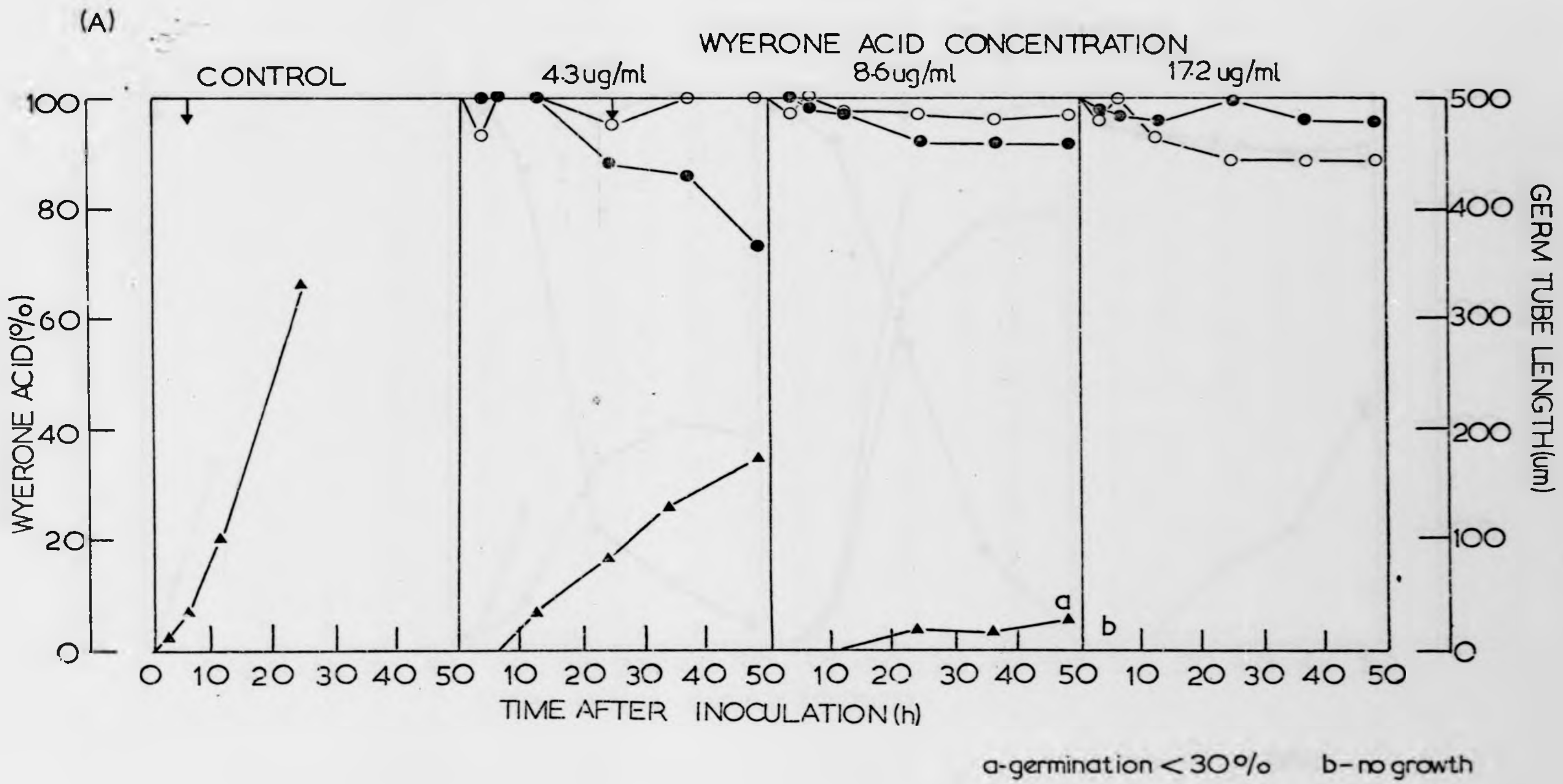
▲ germ tube growth.

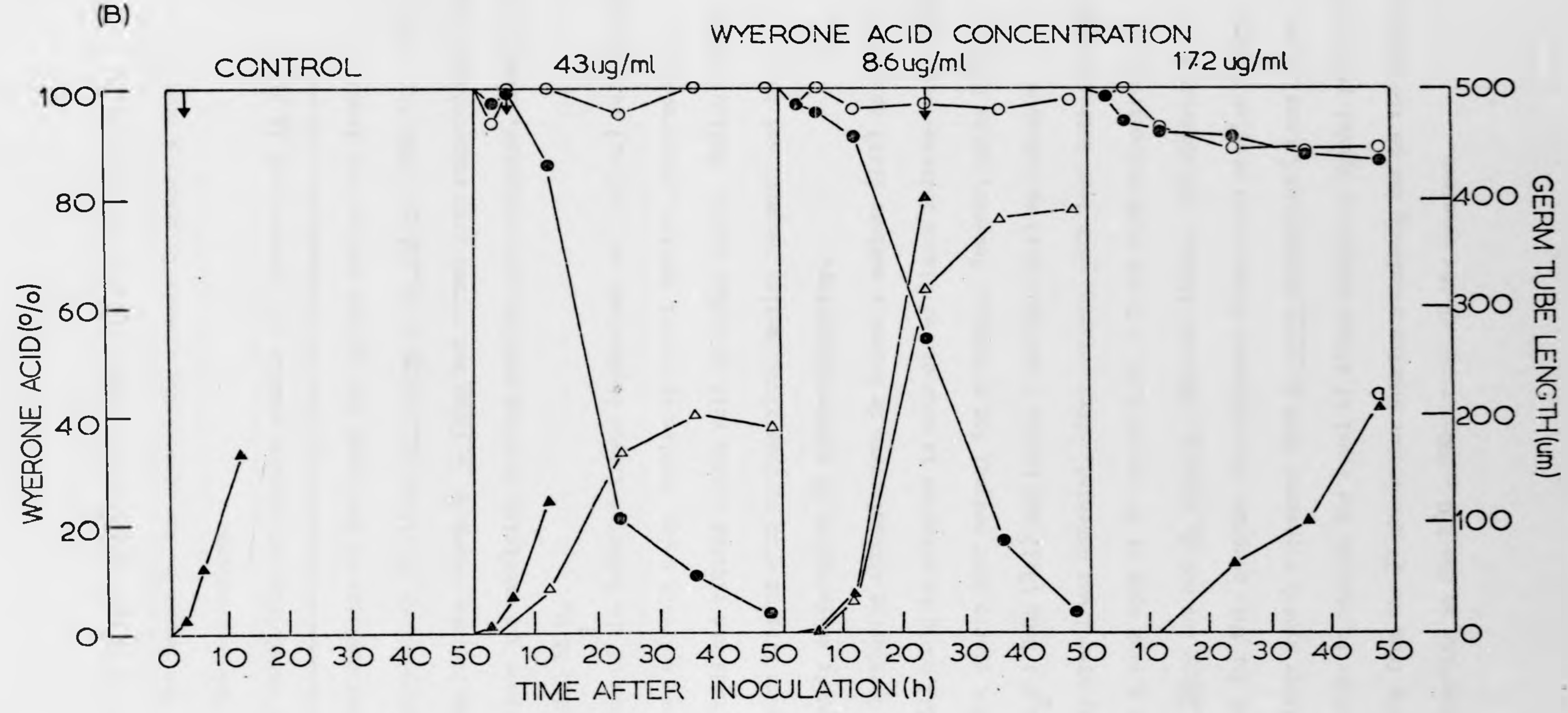
△ metabolite concentration ml⁻¹ in fungal cultures.

Scales:

4.3μg/ml	wyerone acid	0-0.5	Absorbance units
3.6 "	" "	0-0.5	" "

Arrows indicate when maximum (98-100%) germination attained.


(A)
WYERONE ACID CONCENTRATION
CONTROL
4.3ug/ml
8.6ug/ml
17.2 ug/ml
WYERONE ACID(%)
0
20
40
60
80
100
GERM TUBE LENGTH(um)
0
100
200
300
400
500
0 10 20 30 40 50 10 20 30 40 50 10 20 30 40 50 10 20 30 40 50
TIME AFTER INOCULATION (h)
a
b
a-germination < 30%
b-no growth


(B)
WYERONE ACID CONCENTRATION
CONTROL
43ug/ml
8·6ug/ml
17·2 ug/ml
WYERONE ACID (%)
GERM TUBE LENGTH (um)
TIME AFTER INOCULATION (h)
a germination < 16%

may be different from that operating for wyerone acid. In the latter, sensitivity of the fungus to the phytoalexin appears to assume a more important role than metabolism.

7 Detection of metabolites of wyerone epoxide and wyerone acid *in vivo*

Pod seed cavities of the broad bean *cv* the sutton were inoculated with conidial suspensions of either *B. cinerea* or *B. fabae*. Four days after inoculation when lesions caused by *B. fabae* had spread into uninoculated tissue, the infected tissue and overlying inoculum droplet were collected, combined and extracted with Et_2O.

Extracts of the infected tissue (equivalent to 1 g.f.w.) were subjected to TLC in various solvents using samples of wyerol epoxide, dihydrodihydroxy wyerol, wyerol acid and reduced wyerol acid as marker spots. Following TLC, chromatograms were treated with various visualization reagents and the separated compounds subjected to UV spectrophotometry.

After developing chromatograms in hexane : acetone (1:1) no wyerol epoxide (RF 0.61) could be detected in extracts of tissue infected with either fungus, using the picric acid reagent for epoxides. However, using $CHCl_3$: MeOH (10:1), Et_2O : MeOH (8:1) and hexane : acetone (2:1) as solvents dihydrodihydroxy wyerol was detected, after spraying with lead tetraacetate-rosaniline, as a pink spot at RF values 0.35, 0.7 and 0.26 respectively in extracts of *B. fabae* but not *B. cinerea* infected tissue. UV absorption spectra obtained for this compound from infected tissue were similar to that of dihydrodihydroxy wyerol recovered from *in vitro* metabolism (λ max 310nm). It was not possible to measure the level of dihydrodihydroxy wyerol in infected tissue accurately from the UV absorption spectra obtained, due to the presence of substances absorbing in the 280 - 290nm range of the spectrum.

Et_2O : MeOH (8:1) was the only solvent system in which wyerol acid and reduced wyerone acid could be clearly separated for comparison with standards in hexane : acetone (1:1) wyerol acid and reduced wyerone acid moved to RF 0.13 and 0.07 respectively. Reduced wyerone acid was only detected in extracts of tissues infected with *B. fabae* but not *B. cinerea*. Wyerol acid had a similar RF to dihydrodihydroxy wyerol (RF 0.7) using Et_2O : MeOh (8:1) thus no positive identification could be made using this solvent. However, in chromatograms developed in hexane : acetone (1:1) wyerol acid was separated from dihydrodihydroxy wyerol and detected in traces amounts in extracts from *B. fabae* but not *B. cinerea* infected tissue, after visualization with vanillin - sulphuric acid reagent.

8 Isolation of metabolites detected *in vitro* from pod tissue infected with *B. fabae*.

B. fabae infected pod endocarp tissue (*c* 200g.f.w.) and overlying inoculum droplets were collected 4 days after inoculation. Et_2O extracts of infected tissue and inoculum droplets were separated by PLC in Et_2O : MeOH (10:1) and the bands corresponding to reduced wyerone acid (RF 0.35) and to both wyerol acid and dihydrodihydroxywyerol (RF 0.74) eluted in 3 x 50ml MeOH. Fig.62 shows the UV absorption spectra obtained from these eluates from both inoculum droplets and infected tissue.

The eluates containing dihydrodihydroxy wyerol and wyerol acid were combined and rechromatographed by development twice in hexane : acetone (1:1), dihydrodihydroxy wyerol was detected at RF 0.67 and wyerol acid at RF 0.45. After elution their UV absorption spectra were recorded (Fig.63A and B respectively). Yields of 4.5mg and 25 AU at λmax 300nm of dihydrodihydroxy wyerol and wyerol acid were recovered respectively.

Figure 62

UV absorption spectra of substances isolated from *B. fabae* infected pod tissue, corresponding to the metabolites of wyerone acid and wyerone epoxide produced by *B. fabae in vitro*.

(A) reduced wyerone acid from inoculum droplets.

(B) " " " " tissue.

(C) dihydrodihydroxy wyerol from inoculum droplets.

(D) " " " " tissue.

Figure 62

UV absorption spectra of substances isolated from _B. fabae_ infected pod tissue, corresponding to the metabolites of wyerone acid and wyerone epoxide produced by _B. fabae_ _in vitro_.

(A) reduced wyerone acid from inoculum droplets.

(B) " " " " tissue.

(C) dihydrodihydroxy wyerol from inoculum droplets.

(D) " " " " tissue.

Fig 62

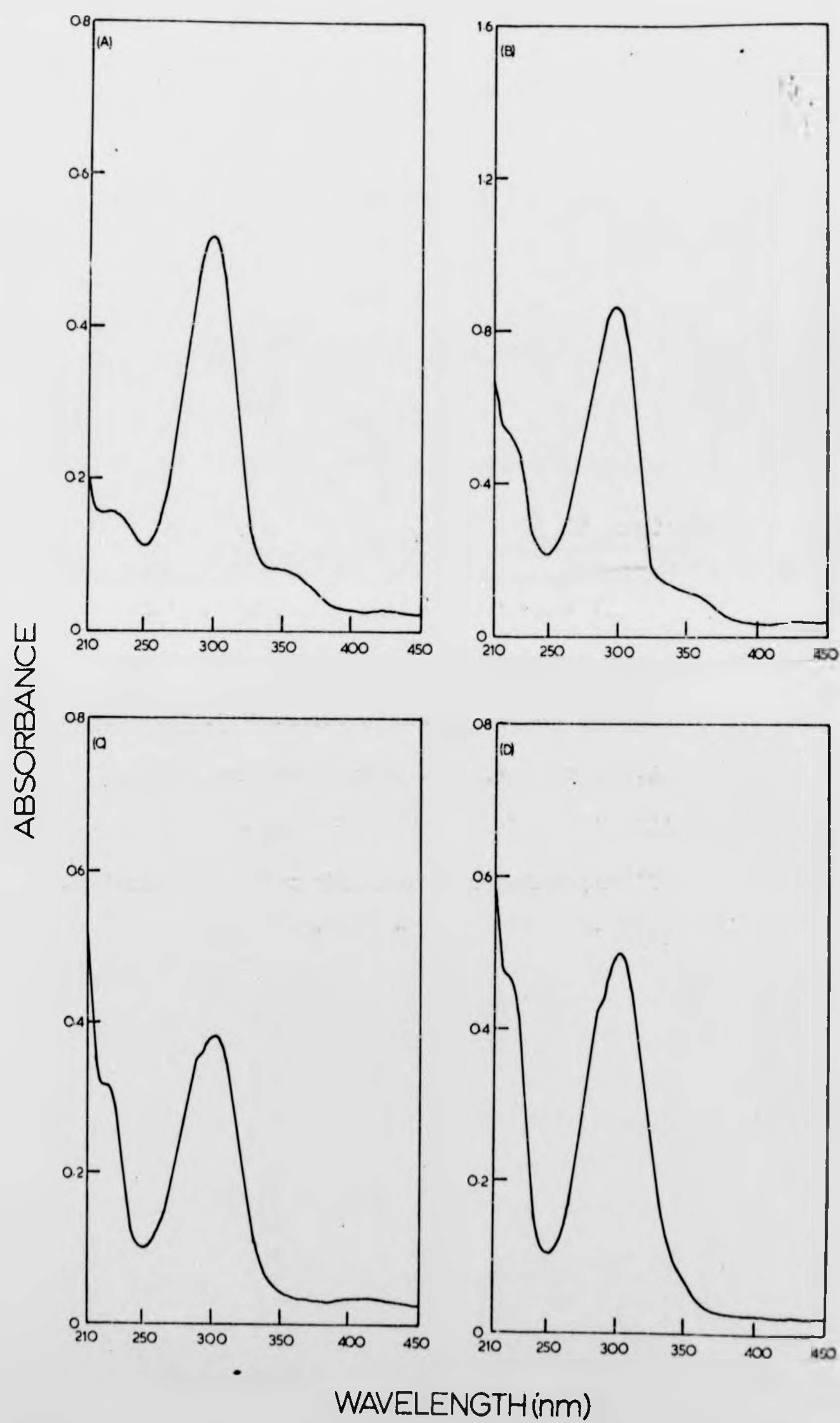
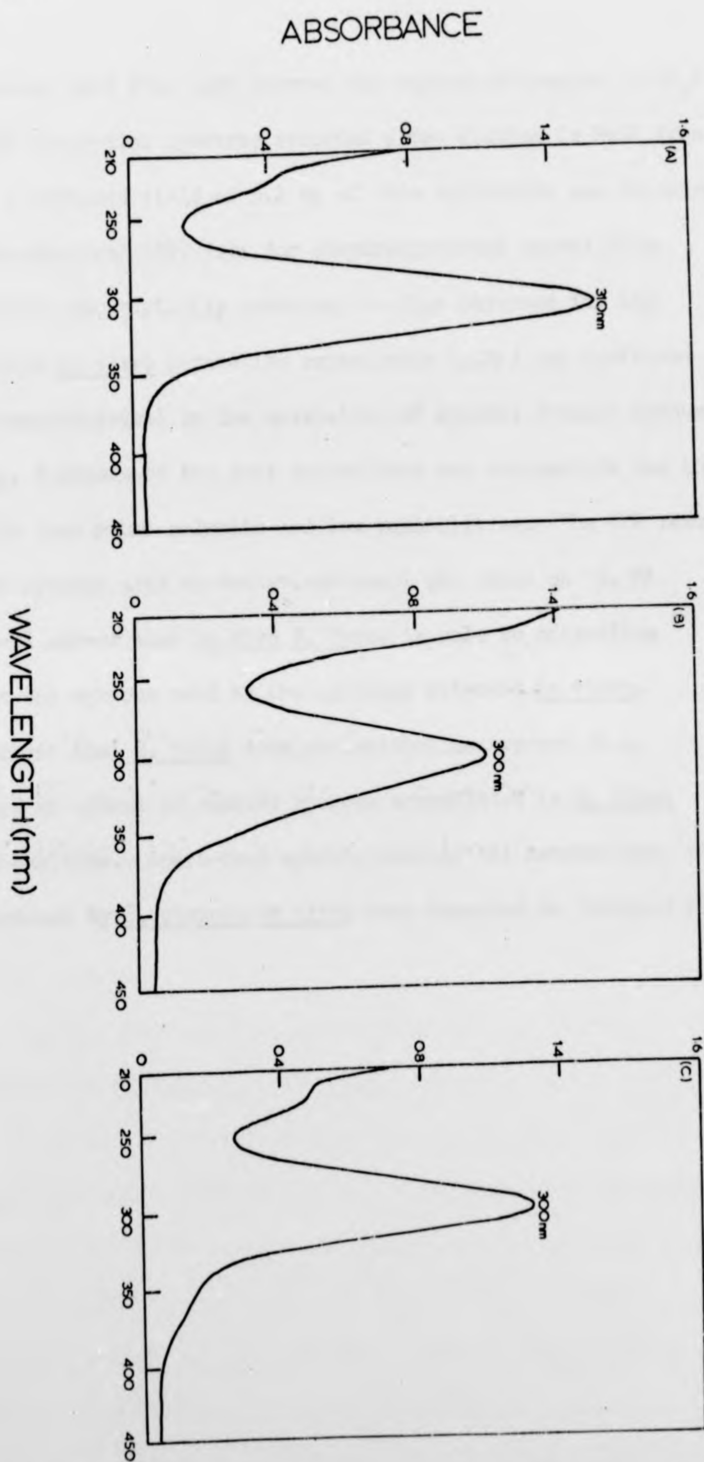


Figure 63

UV absorption spectra of substances corresponding to reduced wyerone acid (A), wyerol acid (B) and dihydrodihydroxy wyerol (C) isolated from B. fabae infected pod tissue.

Fig.63



Reduced wyerone acid from both sources was rechromatographed in Et_2O : MeOH (8:1) and the UV absorption spectrum recorded after elution in MeOH from RF 0.47 (Fig.63c). A combined yield of 5.2 mg of this metabolite was recovered.

NMR and Mass Spectral (MS) data for dihydrodihydroxy wyerol from B. fabae infected tissue was virtually identical to that obtained for the compound recovered from in vitro metabolism experiments (p.79) and confirmed that this substance was identical to the metabolite of wyerone epoxide produced by B. fabae in vitro. Analysis of the acid metabolites was not possible due to their insolubility in less polar solvents and low volatilities. The NMR results obtained for reduced wyerone acid in deuteriomethanol are shown on p. 79

Thus it would appear that in vivo B. fabae is able to metabolize both wyerone epoxide and wyerone acid by the pathways detected in vitro. However, it would appear that B. fabae does not metabolize wyerone in a similar manner, since no wyerol or reduced wyerone accumulated in B. fabae lesions in pod seed cavities. Apart from wyerol, none of the metabolites of the phytoalexins produced by B. cinerea in vitro were detected in infected tissue.

DISCUSSION

A The multicomponent phytoalexin response of *Vicia faba*.

In earlier investigations (Deverall, 1967; Deverall and Vessey, 1969; Mansfield, 1972; Mansfield and Deverall, 1974b), it was reported that the resistance of *V. faba* to infection by *B. cinerea* was characterized by the formation of a single phytoalexin identified as wyerone acid by Letcher *et al.* (1970). Additional research showed that wyerone, the methyl ester of wyerone acid accumulates in leaves (Fawcett *et al.*,1971) and shoots (Keen, 1972) of the broad bean after infection by *B. fabae* and *Phytophthora megasperma* var *sojae* respectively.

In addition to wyerone acid and wyerone, four other phytoalexins have been characterized in tissues of *V. faba* challenged by *B. cinerea*. Three of these inhibitors were shown to be wyerone epoxide, wyerol, and medicarpin (demethylhomoterocarpin) and the fourth (PA3b) tentatively identified as 4, hydrohydroxy - 5, Keto - wyerone. The multicomponent phytoalexin response of *V. faba* is summarized in Fig.64.

In contrast to these findings Keen (1972) detected only wyerone in broad bean shoots inoculated with *P. megasperma* var *sojae*. However, it has been shown that all these inhibitors are produced in shoots after inoculation with suspensions of *B. cinerea* conidia (J.W. Mansfield, pers. comm.) An explanation of these differences may lie in the inoculation techniques employed, Keen used mycelial inocula whereas most other results were obtained from tissues inoculated with suspensions of *B. cinerea* conidia in sterile distilled water. However, the possibility that different fungi induce different patterns of phytoalexin accumulation should not be overlooked. Similar discrepancies have been reported in the phytoalexin response of peas to *Fusarium solani* f.sp *pisi*,

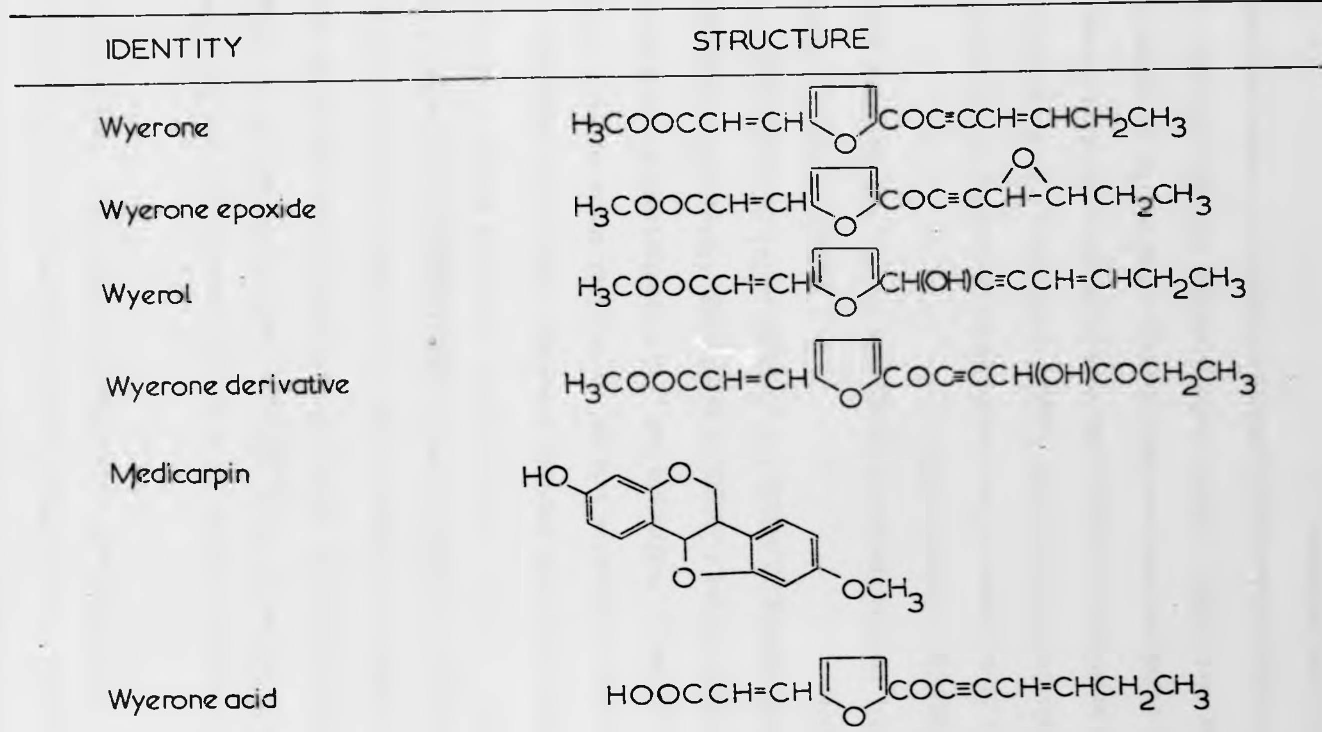


Fig. 64

Aphanomyces euteiches and Rhizoctonia solani (Pueppke and Van Etten, 1974; 1976)

The production of five furanoacetylenic phytoalexins by V. faba parallels the accumulation in other plants of several structurally related phytoalexins; for example the isoflavanoids phaseollin, phaseollidin, phaseollinisoflavan, 2[1] - methoxyphaseollinisoflavan and kievitone in French bean (see Table 1).

The detection of medicarpin as a phytoalexin from the broad bean confirms the view that members of the Leguminosae are characterized by the production of isoflavanoid phytoalexins. This pterocarpanoid phytoalexin may be the same as that described by Cruickshank (1963) as 'vicatin' which accumulated in inoculum droplets containing spores of Monilinia fructicola incubated on pod seed cavities. However, this is unlikely since only traces of medicarpin were detected in inoculum droplets containing conidia of B. cinerea. Unfortunately no qualitative data on the chemical characteristics of vicatin have been published. Medicarpin has been characterized as a phytoalexin in a number of plant species : Canavalia ensiformis, (Keen, 1972); Cicer arietinum (Keen, 1975a); Medicago sativa (Smith et al.,1971) Melilotus alba,(Ingham and Millar 1973); Trifolium pratense (Higgins and Smith, 1972); various species of Trigonella (Ingham and Harborne, 1976); Vigna unguiculata (Lampard, 1974)

The significance of the production of medicarpin as well as the furanoacetylenic phytoalexins by V. faba is unclear. It is possible that medicarpin production represents the expression of a primitive disease resistance mechanism which has been largely superseded by the accumulation of the wyerone derivatives. Such an interpretation may apply not only to V. faba but also to other legumes which produce medicarpin as a minor component of their phytoalexin response.

In this context it would be of particular interest to carry out a systematic survey of the genus *Vicia* for the ability of different species to produce the wyerone compounds and medicarpin.

The presence of two induced phytoalexin systems in a single species appears to be unique at present. Although a number of compounds have been implicated in the disease resistance of potato tubers to *Phytophthora fructicola*, only the isoprenoid derivatives can be considered as phytoalexins (Kuc, 1972).

B Induction of phytoalexin biosynthesis in *V. faba*.

As previously reported for wyerone acid (Mansfield and Deverall, 1974b) the production of all the phytoalexins by *V. faba* was correlated with the degree of necrosis within limited lesions. The inhibitors were not detected at symptomless inoculation sites, but increased rapidly when fungal induced cell damage became macroscopically visible. This suggests that phytoalexin synthesis is closely associated with tissue necrosis and browning.

Two basic hypotheses have been advanced to explain the induction of phytoalexins following fungal infection. One envisages that phytoalexins are produced by live cells in response to the presence of fungal metabolites, and the other, as first formulated by Müller and Börger (1940), that phytoalexin synthesis is associated with necrobiosis of host cells.

Support for the induction of phytoalexins by fungal metabolites independent of host cell death (Fig.65A) is provided by Cruickshank and Perrin (1968). They isolated a peptide, monilicolin A, from mycelium of *Monilinia fruticola*, which did not cause necrosis but specifically induced the formation of phaseollin in French beans. Subsequently Paxton *et al.* (1974) confirmed that monilicolin A induced phaseollin formation in live cells of pod endocarp. Rathmell and Bendall (1971) have also indicated that more than one control

Figure 65

Possible mechanisms of phytoalexin induction.

Fig.65

(A) FUNGAL METABOLITES → PHYTOALEXIN INDUCTION

PATHOENZYMES/ TOXINS → CELL DEATH

(B) PATHOENZYMES/ TOXINS → CELL DEATH → PHYTOALEXIN INDUCTION

(C) FUNGAL METABOLITES → [PHYTOALEXIN INDUCTION] → CELL DEATH

(D) PATHOENZYMES/ TOXINS → PHYTOALEXIN INDUCTION ; CELL DEATH

factor may operate in the changes of phenol metabolism in French bean during disease and that phytoalexin formation may represent a specific stimulation of isoflavanoid metabolism which is separate from any general increase in phenol metabolism associated with cell death. The inability to detect phaseollin in extracts of bean leaves which had undergone a hypersensitive response to Pseudomonas mors-prunorum (Stholasuta et al., 1971) also suggests that phytoalexin formation and necrosis are under separate control. However, Bailey (1973a) showed that phaseollin production and necrosis occured in French bean leaves following inoculation with tobacco necrosis virus (TNV) and he argues that since TNV is unlikely to produce compounds such as monilicolin A, it is highly improbable that phaseollin production is directly controlled by diffusible fungal metabolites. Although a number of workers have shown that inducers or elicitors, isolated from fungi grown in vitro, are capable of causing phytoalexin synthesis (Anderson - Prouty and Albersheim, 1975; Frank and Paxton, 1971; Keen, 1975b) their role in the disease situation still remains to be clarified.

Much evidence exists for the second hypothesis. A close relationship between necrosis and phytoalexin production has been demonstrated in a number of host/parasite interactions (Bailey, 1973; Bailey and Deverall, 1971; Bailey and Ingham, 1971; Mansfield and Deverall, 1974b; Sato et al.,1969). The suppression of both necrosis and the accumulation of rishitin and phytuberin in potato tubers also suggests that necrosis and phytoalexin synthesis are closely linked (Varns and Kuc 1971). Additional evidence supporting this cell-death hypothesis has been provided by the finding that necrosis in bean hypocotyls caused by freezing resulted in the formation of phaseollin (Rahe and Arnold, 1975). Sato et al.(1971) suggest that cell death may be a possible trigger for the synthesis of phytoalexins (Fig.65B). It is

possible that metabolism which can occur in dying plant cells is all that is required for the synthesis of phytoalexins as suggested by Bailey et al. (1976). However it is likely that this metabolism is involved in catabolic rather than anabolic processes that would be expected to be associated with phytoalexin synthesis. Alternatively, metabolites released from dead cells may induce phytoalexin synthesis in adjacent living cells as suggested by Mansfield et al. (1974)

Another possible explanation of the close association between phytoalexin production and necrosis is that fungal metabolites act as specific inducers of phytoalexin formation, which may be confined to live cells, where the phytoalexins may ultimately reach phytotoxic concentrations and cause host cell death (Fig.65C). The possibility that factors responsible for cell death also induce phytoalexin production (Fig 65D) is another mechanism which cannot be overlooked. For example, if cell wall degrading enzymes cause host cell death it is possible that independent of their activity against the host cell wall they may induce phytoalexin synthesis directly by binding to sites, perhaps on cell membranes in a similar manner to that described for lectins (Callow, 1975). The formation of an enzyme protein - receptorsite complex may trigger off events which lead to phytoalexin synthesis. A discussion of the nature of such receptor sites would be highly speculative at present. However since cell wall degrading enzymes are substrate specific it would be expected that receptors either mimic the substrate for the enzyme or bind to sites on the protein not associated with the active site.

At present it is not possible to draw any firm conclusions on the induction of phytoalexins in the disease situation. If fungal metabolites are to be considered as inducers or elicitors of phytoalexin synthesis these compounds must be shown to occur *in vivo*. The major problems preventing a

solution to this enigma is the inability of present techniques to determine if the process of necrobiosis and phytoalexin synthesis are separate or closely linked. In this respect the use of fluorescence microscopy (Mansfield et al., 1974) coupled with improved biochemical assay techniques for phytoalexins in the broad bean/Botrytis system should provide some interesting results pertaining to the localization of phytoalexin synthesis and the sequence of events leading to necrosis and phytoalexin production.

C Changes in phytoalexin concentrations in tissue of *V. faba* following inoculation with *Botrytis*.

There appears to be a causal relationship between phytoalexin accumulation and restriction of *Botrytis* in broad bean tissues. Higher yields of all phytoalexins were recovered from tissues undergoing a resistant response than from those in which a susceptible reaction developed. However, cotyledon and pod tissue although exhibiting a resistant response to fungal invasion showed distinctly different patterns of phytoalexin accumulation.

(i) In cotyledons

A similar pattern of phytoalexin accumulation occurred in cotyledon tissue after inoculation with either *B. cinerea* or *B. fabae*, and both fungi were restricted to the inoculation site. However, *B. fabae* consistently induced higher levels of all phytoalexins and this may have been related to the greater symptom development caused by this fungus than by *B. cinerea*.

Wyerone was the predominant phytoalexin in cotyledons. Although the other phytoalexins were present at much lower levels, the concentrations of wyerone acid and wyerone epoxide recovered from infected tissue were sufficient to account for the total inhibition of fungal growth. The inability of both *B. cinerea* and *B. fabae* to grow and colonize cotyledon

tissue may be a consequence of the rapid accumulation of wyerone. There are two possible mechanisms by which accumulation of wyerone may lead to the inhibition of these fungi. Firstly, wyerone itself, perhaps deposited on cell walls may come into contact with the advancing fungal hyphae and inhibit growth directly. Alternatively, deposited wyerone may not come into direct contact with the fungal hyphae, but acts as a precursor pool for the more active and polar phytoalexins, wyerone acid and wyerone epoxide. These phytoalexins may then generate an antifungal environment around invading fungal hyphae.

The reason why the pattern of phytoalexin accumulation in cotyledons differs from that in pods and leaves remains to be determined. It is possible, however, that the rapid accumulation of wyerone in this tissue is a consequence of the availability of large quantities of storage materials in cotyledon cells, which provide precursors and energy for synthesis of this phytoalexin. The wyerone formed may be localized at sites which are not readily available to the enzymes which convert it to the other phytoalexins.

(ii) In pods

Wyerone acid and wyerone epoxide were the predominant phytoalexins in inoculum droplets recovered from limited lesions caused by avirulent species of *Botrytis*, the acid being present at far higher concentrations than the epoxide. Since very little of any phytoalexin was detected in *B. allii* infected tissue (p.50) it would appear that wyerone acid is perhaps the major phytoalexin preventing fungal growth.

As relatively low concentrations of wyerone epoxide, wyerol, medicarpin and PA3b were detected at any stage after the infection of pod endocarp with *B. cinerea* it seems unlikely that they contribute significantly to the restriction of the fungus. Wyerone acid and wyerone appear to be the major phytoalexins, however their patterns of accumulation differed. Wyerone acid

increased rapidly reaching maximum concentrations 2 days after inoculation, coinciding with the inhibition of fungal growth. Whereas wyerone increased relatively slowly but finally reached similar levels to wyerone acid, six days after inoculation.

The significance of the different patterns of accumulation of wyerone acid and wyerone is difficult to interpret. The rapid increase in wyerone acid suggests that it is likely to be the only phytoalexin present during the early stages of the disease reaction which could contribute towards creating an antifungal environment in the lesion. This explanation is supported by the fact that the pH of inoculum droplets dropped rapidly to 4.0 - 4.5, within the range at which wyerone acid is most active. However, if wyerone is deposited on cell walls adjacent to invading fungal hyphae it may play a more important role in the direct inhibition of the fungus than is suggested by its low concentration during the early stages of infection. Localized deposited wyerone would be an effective mechanism in preventing fungal growth.

Alternatively wyerone may act as a precursor pool for wyerone acid and perhaps wyerone epoxide, as suggested for the cotyledon system. This explanation has support from the demonstration of the conversion of wyerone to wyerone acid during the fractionation of pod tissue, and if this were the case would explain the relatively low rates of wyerone accumulation during the early stages of the disease reaction when there is a rapid increase in wyerone acid concentration.

In order to delimit the actual role of wyerone in the disease resistance of the broad bean more conclusive results relating to the localization of wyerone in infected tissue with respect to the invading hyphae and to the conversion of wyerone to the other phytoalexins should be obtained.

A similar situation to that discussed here has been observed in the <u>Rhizoctonia solani/Phaseolus vulgaris</u> interaction (Smith <u>et al</u>.,1975) Kievitone was the predominant phytoalexin in the early stages of infection,

wheras phaseollin did not accumulate until the latter stages of the disease reaction. The phytoalexins phaseollidin and phaseollinisoflavan were present at much lower levels at all stages of lesion development. It was concluded that Kievitone played a primary role in lesion limitation. In the broad bean wyerone acid would appear to be the primary factor in the resistance of pod endocarp to invading fungi.

Changes in the concentrations of phytoalexins in pod tissue after infection with B. fabae suggest that although this fungus induces phytoalexin synthesis, it is able to inactivate all the phytoalexins at a greater rate than they are produced and hence prevent their accumulation. The inability to detect the inhibitors in inoculum droplets at anytime after inoculation with B. fabae in this work and elsewhere (Deverall et al. 1969) supports this conclusion.

Since phytoalexins were only detected in infected tissue, it is probable that they are produced by cells affected but not yet killed by the advancing hyphae as suggested by Deverall et al., (1969); Deverall and Vessey (1969); and Mansfield et al., (1974) and that invading hyphae are exposed to phytoalexins released from these cells. Whether or not hyphal tips of B. fabae ever come into contact with inhibitory concentrations of the phytoalexins cannot be determined from the data available.

D Antifungal activities of the phytoalexins from V. faba.

(i) Differential sensitivities of B. cinerea and B. fabae to wyerone derivatives.

The tolerance of B. cinerea and B. fabae to the wyerone derivatives in vitro correlates with their pathogenicity towards the broad bean. Germ tubes produced by B. fabae conidia were less sensitive than those of B. cinerea to each phytoalexin (Table 23). Although the basis of the differential

TABLE 23 ED_{50}'s for wyerone, wyerone epoxide, wyerol and wyerone acid in SPN solutions at pH4 against germ tube growth by *B. cinerea* and *B. fabae*

(μg/ml)	B. CINEREA	B. FABAE
WYERONE	18.1	23.8
WYERONE EPOXIDE	6.4	20.0
WYEROL	75.5	>1000
WYERONE ACID	6.1	23.3

sensitivities of the fungi to these phytoalexins is unclear , it may be explained at least in part by differing abilities of the fungi to metabolize the phytoalexins to inactive forms as suggested for wyerone acid by Deverall and Vessey (1969) and Mansfield and Widdowson (1973) However, differential uptake of the phytoalexins, hence concentration at the site of action or alternatively differences in the number and/or types of inhibitor receptor or sensitive sites may also afford an explanation of the observed activities. The latter has been suggested as an explanation of the differential sensitivity of plant cultivars to host specific fungal toxins (Scheffer and Yoder, 1972).

(ii) Effect of pH on the activity of wyerone derivatives

During the development of a medium to investigate the interaction of phytoalexins and fungi *in vitro* it was shown that the pH of the incubating medium markedly affected the inhibitory activity of wyerone acid, but not of wyerone or wyerone epoxide, towards both fungi. Earlier work (Deverall and Rodgers, 1972) had already shown the effect of pH on wyerone acid activity under more controlled pH con ditions. Similar pH effects have been reported on the antifungal activity of benzoic acid towards *Nectria galligena* (Brown and Swinburne, 1971).

The action of low pH on the activity of wyerone acid is probably through the suppression of dissociation of wyerone acid. The undissociated molecule may be readily taken up by the fungi as discussed for fungicides by Byrde (1965) and for organic acids by Rothstein (1965). In studies on the effect of pH on the toxicity of benzoic acid to bacteria and yeasts, it has been shown that only the undissociated molecule, which has greater lipoid solubility than the ionised form, enters the cytoplasm (Bosund, 1962).

Although it can be suggested that only uncharged wyerone acid can pass into fungal cells without hindrance, the ionic form may be prevented from entering the cell, perhaps by either absorption onto the

cell wall or repulsion by groups of similar charge. It must be stressed that it is not known whether wyerone acid must pass into the cytoplasm to produce growth inhibitory effects. Bosund (1962) points out that there is evidence that benzoic acid and related inhibitors act by interfering with reactions connected with the cell membrane, also it has been suggested that phaseollin acts either on the plasma membrane or affects some process required for membrane function (Van Etten and Bateman, 1971). As an alternative explanation for the effect of pH on wyerone acid activity, Deverall and Rodgers (1972) suggest that pH may affect fungal membranes directly and facilitate the inhibitory activity of wyerone acid.

The relationship between pH and sensitivity of B. cinerea germ tubes to wyerone acid appears to be a Log. linear relationship whereas B. fabae becomes increasingly tolerant of wyerone acid with increasing pH. This suggests that the mechanism effected by pH may be the same as that responsible for the differential sensitivity of the two fungi to the phytoalexin.

A knowledge of the mode of action of wyerone acid and of the location of the receptor sites for the phytoalexin within the cytoplasm or perhaps on the fungal cell membrane is required before a conclusive explanation of the effect of pH on wyerone acid activity can be reached.

(iii) Comparative activities of wyerone derivatives

At pH4, wyerone acid and wyerone epoxide had similar activities against both fungi and were more active than wyerone. Wyerol was the least effective inhibitor of all the phytoalexins tested (Table 23).

Since these phytoalexins (except wyerol) have the Keto - acetylenic moiety, which is probably responsible for the antifungal activity of these compounds (Fawcett et al., 1969; Mansfield and Widdowson, 1973) and assuming that the sites of phytoalexin action are within the fungal cell, it would be

expected that the rate of phytoalexin uptake is the primary factor determining antifungal activity. Because both wyerone and wyerone epoxide are relatively lipoid soluble, due to the presence of methyl ester, they should move through the fungal cell membrane without hindrance and it would therefore be expected that wyerone would be at least as active as wyerone epoxide and wyerone acid. However, this is not the case suggesting that factors other than rate of phytoalexin uptake are involved. Differences in the activities of wyerone and of wyerone acid and wyerone epoxide may be due to differences in the rates of metabolism of the phytoalexins, or differences in the mode of action of the phytoalexins. The activity of each inhibitor may differ at a specific site, or alternatively each phytoalexin may affect different sites causing different degrees of growth inhibition. The effects of wyerone acid and wyerone epoxide against _Botrytis in vitro_ appear to be additive suggesting that these inhibitors have a similar mode of action. If they had effects at different sites their activities would probably have been manifested synergistically. Recently Smith _et al._ (1975) have shown that phaseollin and Kievitone from French bean hypocotyls challenged by _Rhizoctonia solani_ exhibit a similar additive interaction.

Wyerol was much less active than the other phytoalexins, presumably due to the reduction of the keto - group to an alcohol. Since wyerol is only present at low levels in infected tissue it would appear that it does not play a significant role in the disease resistance of the broad bean.

(iv) Antifungal activity in relation to inhibition of fungal growth _in vivo_

Although wyerone acid and wyerone epoxide are the predominant phytoalexins in inoculum droplets, wyerone epoxide was present at much lower concentrations than wyerone acid and is unlikely alone to be completely anti-

fungal in vivo. In view of the additive effect of wyerone acid and epoxide it is probable that compared with the acid, the epoxide has relatively little influence on the growth of B. cinerea in pod tissue. However, in cotyledon tissue infected with either B. cinerea or B. fabae the epoxide may be as or more important than wyerone acid in preventing fungal growth. If wyerone is deposited in cell walls it may also play an important role in the restriction of the fungus to the inoculation site, by preventing the growth of hyphae which come into contact with it.

It is interesting to compare the phytoalexin response of the broad bean with that of Phaseolus vulgaris. The pattern of phaseollin accumulation in hypocotyls infected by R. solani (Smith et al., 1975) is similar to that found for wyerone in this work. Also Muller (1958) showed that phaseollin was adsorbed onto living and dead tissue. As phaseollin was only present at low concentrations during the early stages of the disease reaction, when the fungus is inhibited Smith et al. (1975) suggest that phaseollin does not play a significant part in the resistance phenomenon. However, if phaseollin is adsorbed onto cell walls it may have a similar mode of action to that suggested for wyerone.

In conclusion it would appear that wyerone acid and wyerone are the major inhibitors contributing to the antifungal environment in restricted lesions caused by Botrytis in the broad bean.

E Metabolism of the broad bean phytoalexins by Botrytis.

(i) Metabolism of wyerone derivatives by Botrytis in vitro.

The predominant metabolite of wyerone detected in cultures of both fungi was wyerol, the acetylenic alcohol derivative of wyerone, which was also identified as a phytoalexin, PA2 (Chapter 2). No reduced wyerone, the methyl ester of reduced wyerone acid could be detected in cultures of

either fungus. Although wyerol is the initial conversion product of wyerone metabolism, it may be further metabolized after prolonged incubation by saturation of the acetyleric band, however, since no reduced wyerone could be detected in _B. fabae_ infected pod tissue at any time after inoculation, this conversion would appear to have little significance _in situ_.

The data presented for the metabolism of wyerone epoxide by _B. fabae_ and _B. cinerea_ are compatible with the scheme illustrated in Fig.66B. As with the metabolism of wyerone by these fungi, the first detectable product formed during the degradation of wyerone epoxide was the acetylenic alcohol derivative, wyerol epoxide. However, _B. fabae_ rapidly metabolized the wyerol epoxide formed by converting the epoxide group to the 1,2 diol forming dihydrodihydroxy-wyerol. _B. cinerea_ also metabolized wyerol epoxide, at a much slower rate than _B. fabae_, to an unidentified substance.

Wyerone acid was rapidly metabolized by _B. fabae_ to a substance tentatively identified as wyerol acid, However, with prolonged incubation this substance was converted to reduced wyerone acid. Another substance (WABf2) was also detected at relatively low levels and had a similar UV absorption spectrum to wyerol acid and reduced wyerone acid. This substance may be an intermediate in the conversion of wyerol acid to reduced wyerone acid Fig.66C. In contrast, the metabolism of wyerone acid by _B. cinerea_ was associated with the appearance of two substances which were not sequentially related. One of these substances appeared to be wyerol acid and the other had UV charactristics similar to Methyl 3 - (5 formyl - 2 furyl) prop trans - 2 - enoate (Fawcett _et al_, 1968) and Methyl 3 - (5 acetyl - 2 furyl) prop trans - 2 - enoate suggesting the acetylenic side chain had been altered, but not the keto group.

The initial conversion product of each phytoalexin produced by both fungi would appear to be the acetylenic alcohol derivative (Fig.66). Although the similar nature of the initial conversion products suggests that the same

Figure 66

Pathways of phytoalexin metabolism by *Botrytis*

Bc - *B. cinerea*

Bf - *B. fabae*

Fig.66

(A)

$CH_3OOCCH=CH$–[furan]–$COC≡CCH=CHCH_2CH_3$
WYERONE

↓ Bf/Bc

$CH_3OOCCH=CH$–[furan]–$CH(OH)C≡CCH=CHCH_2CH_3$
WYEROL

↓ ?

(B)

$CH_3OOCCH=CH$–[furan]–$COC≡C\text{-}CH(O)CH\text{-}CHCH_2CH_3$
WYERONE EPOXIDE

↓ Bf/Bc

$CH_3OOCCH=CH$–[furan]–$CH(OH)C≡C\text{-}CH(O)CH\text{-}CHCH_2CH_3$
WYEROL EPOXIDE

Bc → ?

↓ Bf

$CH_3OOCCH=CH$–[furan]–$CH(OH)C≡CCH(OH)CH(OH)CH_2CH_3$
DIHYDRODIHYDROXYWYEROL

(C)

$HOOCCH=CH$–[furan]–$COC≡CCH=CHCH_2CH_3$
WYERONE ACID

Bc → $HOOCCH=CH$–[furan]–CO_2
WABc 2

↓ Bf/Bc

$HOOCCH=CH$–[furan]–$CH(OH)C≡CCH=CHCH_2CH_3$
WYEROL ACID

↓ Bf

$HOOCCH=CH$–[furan]–$CH(OH)CH=CHCH=CHCH_2CH_3$
WABf 2

↓ Bf

$HOOCCH=CH$–[furan]–$CH(OH)CH_2CH_2CH=CHCH_2CH_3$
REDUCED WYERONE ACID

enzyme system is involved in the metabolism of the wyerone derivatives by Botrytis, this may not be the case for B. cinerea. In experiments with mixtures of wyerone and wyerone acid, B. cinerea metabolized wyerone to wyerol but did not metabolize the acid. Although B. fabae readily metabolized mixtures of wyerone and wyerone acid it was not possible to determine if this was carried out by the same or different enzyme systems. However, the accumulation of reduced wyerone acid in B. fabae lesions contrasts with the failure to detect reduced wyerone at any stage after inoculation and also implies that the enzymes converting wyerone acid do not attack wyerone. By contrast Stemphylium botryosum, a pathogen of alfalfa, which metabolizes medicarpin to vestitol (Steiner and Millar, 1974) is also able to metabolize the other closely related pterocarpanoid phytoalexins, phaseollin and pisatin (Heath and Higgins, 1973) and maackiain (Higgins, 1975) to their respective 2' hydroxyisoflavan derivatives.

It is possible that the final metabolites of the metabolism of each of the phytoalexins is CO_2 and water, as reported for pisatin breakdown by pea pathogens (De Wit-Elshove, 1969; De Wit - Elshove and Fuchs, 1971). Hence, a stoichiometric consideration of the metabolism of the phytoalexins must await the application of radiotracer techniques to this problem.

(ii) Metabolism of phytoalexins as a detoxification process.

All the metabolites of the wyerone derivatives were less active against germ tube growth of B. cinerea and B. fabae than the parent phytoalexins (Table 24). These conversions may therefore be considered as detoxification mechanisms. However, whereas wyerone epoxide was more active against B. cinerea than B. fabae, wyerol epoxide had greater activity against B. fabae than B. cinerea. Dihydrodihydroxy wyerol had virtually no activity against the growth of either fungus. Thus, it appears that the conversion of wyerol epoxide to dihydrodihydroxy wyerol by B. fabae represents a further detoxification. Since B. cinerea does not convert wyerol epoxide to dihydrodihydroxy-

TABLE 24 $ED_{50}S$ for phytoalexins and metabolites against germ tube growth by B. cinerea and B. fabae

(μg/ml)	B. CINEREA	B. FABAE
WYERONE	18.1	28.8
WYEROL	75.9	1000
WYERONE EPOXIDE	6.4	20.0
WYEROL EPOXIDE	500	38.5
DIHYDRODIHYDROXYWYEROL	>1000	>1000
WYERONE ACID	6.1	23.3
REDUCED WYERONE ACID	>1000*	>1000*

* - from J. W. Mansfield, Personal communication

wyerol it is probable that the enzyme responsible for this conversion is not present in this fungus. However, it is possible that this conversion is somehow 'triggered' by the inhibitory action of wyerol epoxide. Thus, because of its sensitivity to wyerol epoxide these enzymes are induced in _B. fabae_ but not in the less sensitive _B. cinerea_. A similar situation may exist for the conversion of phaseollin by _C. lindemuthianium_ and _B. cinerea_. Both fungi are able to convert phaseollin to 6a-hydroxy phaseollin (Burden _et al_.,1974; Van den Heuvel and Glazner, 1975.) which is as antifungal as the phytoalexin towards _C. lindemuthianium_ but less active against _B. cinerea_. However, _C. lindemuthianium_ but not _B. cinerea_ was able to metabolize 6a-hydroxy-phaseollin further to an unidentified substance termed CL_2. Unfortunately no antifungal data for this compound against these fungi has been published.

It is not possible to explain the metabolism of wyerone acid by _B. fabae_ by a process of inhibition induced conversion because wyerol acid appears to be as inactive as reduced wyerone acid in preventing growth of the fungi.

Wyerol epoxide is the only wyerone derivative examined so far, which is more active against germ tube growth of _B. fabae_ than _B. cinerea_. This suggests that the epoxide moiety itself confers some degree of antifungal activity to the molecule independent of the Keto-acetylenic function. Thus it would appear that wyerone epoxide has at least two antifungal groups, which may account for its greater activity than wyerone against _Botrytis_. It is possible that wyerone epoxide acts at two separate sites within the fungus. However as discussed previously, the effect of mixtures of wyerone acid and wyerone epoxide against growth of _Botrytis_ appears to be additive rather than synergistic suggesting that both phytoalexins act on the same site.

It is possible that the inclusion of an oxygen atom between the 4-C and 5-C carbon atoms of wyerone, enhances antifungal properties already conferred by the _cis_ double bond (since wyerol is more antifungal than dihydrodihydroxy

wyerol). It would be interesting to compare the antifungal activities of pure samples of wyerone and wyerol with that of their corresponding dihydro-derivatives to determine the relative degree of antifungal activity conferred by the cis double bond. Inactivation of these phytoalexins, in general is associated with either the reduction of the keto group to the alcohol derivative or saturation of the C≡C suggesting that these moieties are the main factors conferring antifungal activity. It is possible that it is the configuration of the molecule, determined by the position of the C≡C CO groups, rather than the direct action of these groups, that confer the antifungal activity to these molecules and that the affinity of the phytoalexins and their metabolites to the sites of action in the fungus determine their antifungal activity.

(iii) Localization and characteristics of fungal enzymes which metabolize the phytoalexins.

As previously reported for wyerone acid (Mansfield and Widdowson, 1973) no extracellular enzymes capable of degrading this phytoalexin or wyerone could be detected. Similar results have been obtained for phaseollin detoxification by *Fusarium solani* f.sp. *phaseoli* (Van den Heuvel and Van Etten, 1973) and for maackiain conversion by *Stemphylium botryosum* (Higgins 1975). Since the metabolism of the wyerone derivatives involves a reduction of the molecule, the enzymes will require a form of reducing power, probably $NADH_2$ or $NADPH_2$. However no reduced cofactors have, so far, been shown to be produced outside plant or animal cells. This supports the above findings and suggests that phytoalexin conversion occurs intracellularly or on the fungal plasmalemma. In this context it would be interesting to determine if the phytoalexins are metabolized before they came into contact with the site of action or whether the site of action is also the site of phytoalexin conversion.

It remains to be determined if the wyerone phytoalexins are detoxified by an inducible enzyme system in Botrytis, as has been shown for phaseollin detoxification to 1a-hydroxyphaseollone (Vanden Heuvel et al., 1974) by F. solani f. sp. phaseoli (Van den Heuvel and Van Etten, 1973) and maackiain conversion to dihydromaackiain by S. botryosum (Higgins, 1975). However, the possibility that these phytoalexins are metabolized by constitutive enzymes cannot be overlooked, Higgins (1972) provides evidence for the metabolism of medicarpin by non-induced enzymes in Leptosphaerulina briosiana.

It is possible, however, to comment on the metabolism of wyerone acid and wyerone epoxide by B. fabae. Since the intermediate metabolites accumulate in the bathing solutions around the fungal hyphae it is probable that the enzymes converting these phytoalexins are separated either spatially or temporally from each other.

The detection of a blue fluorescent substance at low levels in B. fabae cultures incubated with wyerone epoxide raises an interesting problem concerning the production of the degradative enzymes. Assuming that the enzymes are induced by the substrate, the hypothetical situation illustrated in Fig. 67A can be envisaged. Initially the enzyme converting wyerone epoxide to wyerol epoxide (enzyme A) is induced by wyerone epoxide. As the levels of wyerol epoxide increase, in the bathing solutions, above a threshold value it induces the production of the enzyme converting wyerol epoxide to dihydrod-hydroxy wyerol (enzyme B). However, when enzyme B is induced there is still some unmetabolised wyerone epoxide present which enzyme B may convert to dihydroxydihydro wyerone, and which may be eventually metabolized to dihydro-dihydroxy wyerol by enzyme A Fig. 67B. Dihydrodihydroxy wyerone would be expected to fluoresce blue under UV light (366nm) and have a similar UV absorption spectrum to wyerone epoxide, due to the presence of the Keto group. It would also be expected to have a lower RF value than wyerone epoxide due to

Figure 67

A possible mechanism for wyerone epoxide metabolism by *B. fabae in vitro*

(1) wyerone epoxide

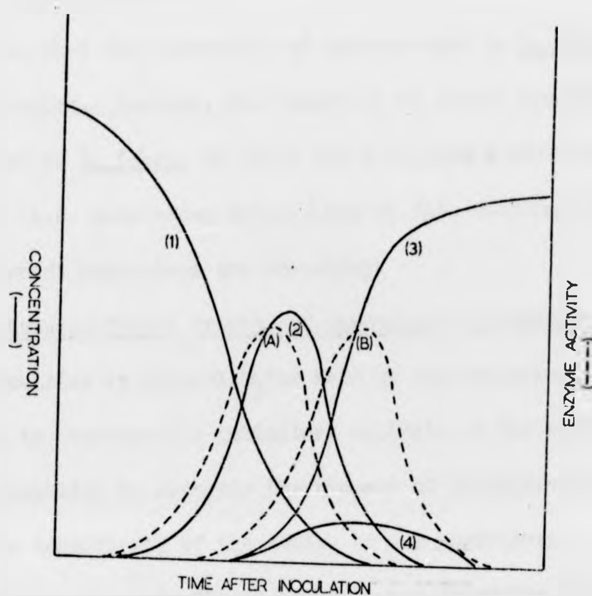
(2) wyerol epoxide

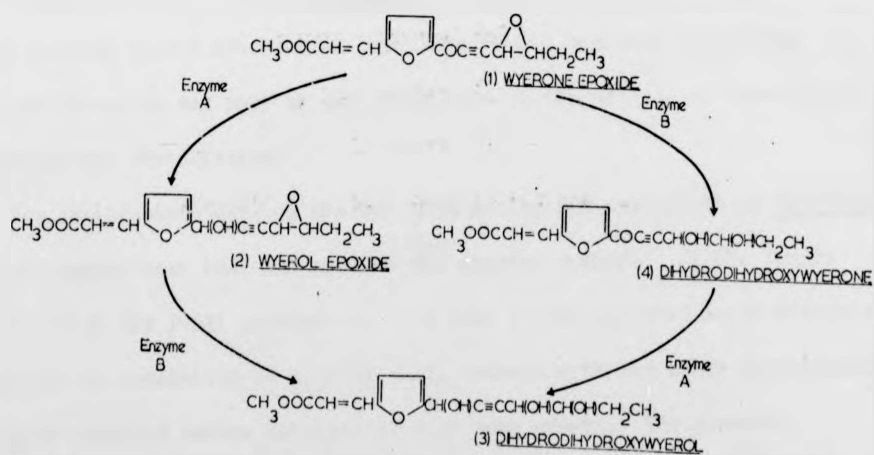
(3) dihydrodihydroxy wyerol

(4) dihydrodihydroxy wyerone

(a) and (B) enzymes (see text for explanation)

Fig.67





the greater polarity of this molecule. The substance WE2 detected as a metabolite of wyerone epoxide formed by B. fabae behaved in the manner predicted for dihydrodihydroxy wyerone.

It is also possible that the metabolism of wyerone acid by B. fabae occurs by a similar mechanism. However, the inability to detect wyerone acid metabolites produced by B. fabae, in which the keto group is retained but the C≡C bond saturated (i.e. substances having λmax at 318, shoulder 330nm), would suggest that different mechanisms are operating.

(iv) The relationship between fungal growth and phytoalexin metabolism.

One of the difficulties in determining the role of phytoalexin metabolism in the ability of fungi to overcome the inhibitory activity of the phytoalexins *in vitro* has been the inability to separate the process of detoxification of the phytoalexin from the sensitivity of the fungus to the phytoalexin. Differences in the ability to degrade the phytoalexin may determine the ability of the fungus to grow, or alternatively may just be an expression of sensitivity.

In my experiments both B. cinerea and B. fabae metabolized wyerone and wyerone epoxide before substantial germ tube growth occurred suggesting that detoxification may play an important role in the ability of these fungi to overcome the phytoalexins.

The relationship between wyerone acid metabolism and growth of Botrytis was more complex than that for wyerone and wyerone epoxide. In the treatments in which the fungi germinated, germ tube growth appeared to be directly related to the metabolism of wyerone acid, whereas with the other phytoalexins metabolism occurred before substantial germ tube growth. The observed pattern of wyerone acid metabolism may be interpreted in two ways. The ability of the fungus to overcome the inhibitory action of wyerone acid may depend on both sensitivity to, and metabolism of, wyerone acid.

Alternatively, sensitivity of the fungi to wyerone acid may be the only factor involved and metabolism just a reflection of growth. At the highest wyerone acid concentration tested (17.2 μg/ml) few B. fabae conidia germinated but they produced relatively long germ tubes although little loss of wyerone acid from culture solution was detected. This result would favour the latter explanation of growth of B. fabae in the presence of wyerone acid.

In order to obtain more comparative data, metabolism of the phytoalexins must be measured in terms of loss of phytoalexin/increase in fungal mass, in order to eliminate the growth factor. Perhaps a more useful line of research would be to reduce or remove either the sensitivity of the fungi to the phytoalexins or increase their ability to degrade the phytoalexins, by selecting mutants which are less sensitive or capable of metabolizing the phytoalexin at greater rates.

(v) Metabolism of phytoalexins in relation to the pathogenicity of Botrytis.

Both B. fabae and B. cinerea are able to inactivate the wyerone phytoalexins in vitro and it appears that the only difference which occur in rates of metabolism may be related to differences in the sensitivity of the fungi to the inhibitors. It is likely that the differential pathogenicity of Botrytis towards the broad bean cannot be explained in terms of phytoalexin inactivation alone.

The metabolites of both wyerone acid and wyerone epoxide produced by B. fabae in vitro occurred in spreading lesions caused by B. fabae whereas none of the metabolites produced by B. cinerea in vitro were detected in lesions caused by B. cinerea, suggesting that in infected tissue B. fabae must be predisposed in someway to metabolize the phytoalexins at a much greater rate than B. cinerea. Other factors such as sensitivity of the fungi to the phytoalexins and also perhaps rate of accumulation of the phytoalexin are

probably also involved.

A differential rates of phytoalexin degradation / differential pathogenicity hypothesis, has been suggested for a number of host/parasite interactions (Higgins and Millar, 1969 a and b; Nonaka, 1967; de Wit-Elshove, 1969; Christenson, 1969; Mansfield and Widdowson, 1973). However, it has been shown that a number of non-pathogenic fungi can metabolize phytoalexins from plants which they do not colonize (Heath and Higgins, 1973; Sakuma and Millar, 1972; Stoessl et al 1973; Van den Heuvel and Glazner, 1975). Thus, the results obtained in this work and elsewhere show that it is not possible to extrapolate the in vitro ability of a fungus to metabolize a phytoalexin to the in vivo situation. Any hypothesis of pathogenicity involving phytoalexin metabolism must be based on analysis of infected tissue and the recognition of the inactive compounds as metabolites in tissues invaded by the pathogen.

Both reduced wyerone acid and dihydrodihydroxy wyerol accumulate within lesions caused by B. fabae in pods. However, only traces of wyerol and no reduced wyerone or wyerol epoxide could be detected. In lesions caused by B. fabae changes in wyerol concentrations followed a similar pattern to that observed for other phytoalexins, that is an increase followed by a decrease. None of the fungal metabolites of the phytoalexins, except wyerol, were detected in limited lesions caused by B. cinerea or B. fabae (in cotyledons). The much higher concentrations of wyerol recovered from tissues undergoing a resistant rather than a susceptible response to Botrytis indicate that wyerol is more likely to be part of the host's response rather than a product of fungal metabolism and that in vivo wyerone acid and wyerone epoxide but not wyerone are inactivated directly by B. fabae.

It is possible, however, that in vivo B. fabae is able to further metabolize wyerol formed from wyerone by pathways not detected in vitro.

However, since no reduced wyerone was detected in B. fabae lesions, any metabolic pathway if operating would be expected to be different from that observed for wyerone acid. A more plausible explanation of the observed changes in wyerone concentrations in pods inoculated with Botrytis and the absence of wyerol accumulation in B. fabae lesions is as follows. Wyerol detected in infected tissue is mainly of plant origin perhaps as a precursor of wyerone. B. fabae however, does not come into contact with wyerone due to its deposition on cell walls around cells which are not in physical contact with the fungal hyphae. It has been suggested that wyerone acid synthesis occurs in healthy cells adjacent to dead cells, which may be affected but not yet killed by the fungus (Deverall et al.,1968; Deverall and Vessey, 1969; Mansfield et al., 1974). If this is the case reduced levels of wyerone and wyerol in B. fabae lesions may be due to either a suppression of their biosynthesis, resulting in the channeling of their precursors towards the synthesis of the other phytoalexins or alternatively wyerone may act as a precursor, being converted to the phytoalexins wyerone acid and also perhaps wyerone epoxide by either plant or fungal enzymes. Thus, it is these phytoalexins which come into contact with and are metabolized to inactive forms by B. fabae.

Initially in the phytoalexin metabolism studies the carbon source for fungal growth was supplied as a simple sugar,sucrose. However, it has been shown that the antifungal activity of wyerone acid (Deverall and Rodgers, 1972) and of pisatin and phaseollin (Van Etten, 1973) depends on the nutrient source for fungal growth. De Wit-Elshove and Fuchs (1971) have also shown that the breakdown of pisatin by Fusarium oxysporium f.sp. pisi and F. solani f.sp. pisi is influenced by catabolite repression of the carbohydrate source. It was thought that in vivo the availability of nutrients would depend on the

the activity of fungal enzymes which may not be induced in the synthetic medium used. If induction of these enzymes and utilization of their products (such as cell wall residues) results in a reorganisation of fungal metabolism this may account for an alternative mechanism of phytoalexin metabolism. Using isolated cell walls from broad bean stems as the carbon source, wyerol was still the predominant metabolite of wyerone. However, a small percentage of wyerone was converted to wyerone acid in B. cinerea but not B. fabae cultures. Although no wyerone acid was detected in B. fabae cultures, it is possible that any wyerone acid formed was rapidly inactivated to reduced wyerone acid. However, it was not possible to determine if this occurred. It was proposed that the conversion of wyerone to wyerone acid occurred outside the fungus where as the metabolism of wyerone to wyerol was associated with the fungal hyphae. However, no exoenzymes capable of converting wyerone to wyerone acid were detected. These experiments suggest that the conversion of wyerone to wyerone acid may be due at least in part to fungal enzymes. However, the observed conversion of wyerone to wyerone acid in lesions produced by B. cinerea six days after inoculation when the fungus appeared to be dead, suggests that plant enzymes are also involved in this conversion.

Thus, it can be concluded that, as for B. cinerea, wyerone acid and wyerone epoxide may be the only phytoalexins which come into contact with B. fabae hyphae and it would appear that wyerone may be metabolized by B. fabae via wyerone acid to reduced wyerone acid.

Care must be taken in determining if metabolites are of host or fungal origin. Since reduced wyerone acid and dihydrodihydroxy wyerol do not appear on the first day after inoculation of pod seed cavities with B. fabae and since they accumulate after the phytoalexins have reached maximum levels, it is not unreasonable to suggest that they are fungal metabolites. However, more conclusive evidence would be obtained by showing that these substances are not

induced by culture filtrates, abiotic agents or viruses. Burden et al (1972) used the latter to show that phaseollinisoflavan, a metabolite of phaseollin produced by S. botryosum (Higgins et al., 1974) is formed together with phaseollin in bean tissue. Another difficulty in interpreting the in vivo situation arises from the possibility that the fungal metabolite of one phytoalexin may be the same as that of another phytoalexin. For example, if the structure of PA3b is correct its fungal metabolite would be expected to be dihydrodihydroxy wyerol. Also if wyerone is converted to wyerone acid, it will also contribute to the accumulation of reduced wyerone acid.

It still remains to be determined if B. fabae and B. cinerea are able to metabolize medicarpin and PA3b. It has been shown that B. cinerea is able to metabolize medicarpin obtained from Melilotus alba to the 6a-hydroxyderivative, which is less antifungal than medicarpin (J. Ingham Pers. comm.)

F. An hypothesis to explain the differential pathogenicities of *B. cinerea* and *B. fabae* towards the broad bean.

The broad bean is characterized by a multi-component phytoalexin response to infection by *Botrytis*. Although wyerone acid appears to be the major component of this response contributing to an antifungal environment in restricted lesions caused by *B. cinerea* in pods and leaves, evidence presented here shows that wyerone may also have an important role in the resistance of cotyledons to fungal infection and may accumulate in cell walls around fungal hyphae in pod tissue.

B. fabae was able to invade and colonize pod and leaf tissues. The metabolites of wyerone acid and wyerone epoxide, but not of wyerone accumulated in spreading lesions caused by *B. fabae* in pods. This suggests that wyerone acid, wyerone epoxide and perhaps PA3b, but not wyerone come into contact with, and are taken up by the fungus.

The possible pathways of synthesis and location of the phytoalexins and of their metabolism by *B. fabae* and *B. cinerea* *in vivo* are illustrated in Fig.68.

Both *B. fabae* and *B. cinerea* metabolize the phytoalexins *in vitro*. Differences in their rates of metabolism have been attributed to an expression of the differential tolerance of the fungi to the phytoalexins rather than a real difference in abilities to metabolize the phytoalexins. However, in pod tissue *B. fabae* appears to be able to overcome the phytoalexins response by inactivating the phytoalexins, whereas *B. cinerea* is unable to prevent their accumulation. This suggests that *B. fabae* must be, in some way, predisposed to metabolize the inhibitors.

The differences in the sensitivity of these two fungi to the phytoalexins is an obvious candidate for this predisposition. Growth of *B. cinerea* may

Figure 68

Possible pathways of synthesis and location of the phytoalexins in the broad bean, and of their metabolism by *B. fabae* and *B. cinerea* in the plant.

Figure 68

Possible pathways of synthesis and location of the phytoalexins in the broad bean, and of their metabolism by *B. fabae* and *B. cinerea* in the plant.

Fig.68

inside cell

WYEROL

?

WYEROL
EPOXIDE

cell wall/
plasmalemma

? ?

WYERONE

?

plant or fungal
enzyme?

outside cell

WYERONE
EPOXIDE
(PA3b?)

WYERONE
ACID

?

inside fungus

DIHYDRODI
HYDROXY
WYEROL

REDUCED
WYERONE
ACID

WYEROL

-·-·- metabolism by B.cinerea

- - - - metabolism by B fabae

be inhibited or indeed the fungus killed by low levels of the inhibitors whereas B. fabae being less sensitive may continue to grow and initiate phytoalexin metabolism. However, if phytoalexins accumulate initially at the same rate in lesions caused by both B. cinerea and B. fabae the observed differences in tolerance to the inhibitors may not be sufficent to account *in toto* for their differential abilities to metabolize the phytoalexins. An additional factor giving rise to the ability of B. fabae to metabolize the phytoalexins may be that it is able to reduce the levels of phytoalexins which come into contact with the fungal hyphae, by affecting the rate of production and accumulation of phytoalexins in the infected tissue. Van den Heuvel *et al.* (1973) have demonstrated that an inhibitory concentration of phaseollin was rapidly metabolized by F. solani f. sp. phaseoli provided the fungus was first allowed to adapt through exposure to a non-inhibitory dosage. A similar situation may occur in lesions caused by B. fabae, since although no metabolites were detected on the first day after inoculation of pod seed cavities with B. fabae, lower concentrations of the inhibitors were recovered from these lesions than from similar limited lesions caused by B. cinerea.

An hypothesis incorporating lower rates of phytoalexin synthesis in the response to B. fabae than to B. cinerea, supports one of the main tenets of the phytoalexin theory proposed by Cruickshank (1963) which states that "The basic response that occurs in resistant and susceptible hosts is similar. The basis of differentiation between resistant and susceptible hosts is the speed of formation of the phytoalexin". As illustrated in Fig.69 B. fabae may be exposed to low initial concentrations of the phytoalexins which enable it to metabolize higher inhibitory concentrations of the phytoalexins, whereas in lesions produced by B. cinerea the phytoalexins accumulate at a greater rate and this coupled with its sensitivity to the inhibitors, may account for the inability of B. cinerea to metabolize the phytoalexins *in vivo*.

be inhibited or indeed the fungus killed by low levels of the inhibitors whereas *B. fabae* being less sensitive may continue to grow and initiate phytoalexin metabolism. However, if phytoalexins accumulate initially at the same rate in lesions caused by both *B. cinerea* and *B. fabae* the observed differences in tolerance to the inhibitors may not be sufficent to account *in toto* for their differential abilities to metabolize the phytoalexins. An additional factor giving rise to the ability of *B. fabae* to metabolize the phytoalexins may be that it is able to reduce the levels of phytoalexins which come into contact with the fungal hyphae, by affecting the rate of production and accumulation of phytoalexins in the infected tissue. Van den Heuvel *et al.* (1973) have demonstrated that an inhibitory concentration of phaseollin was rapidly metabolized by *F. solani* f. sp. *phaseoli* provided the fungus was first allowed to adapt through exposure to a non-inhibitory dosage. A similar situation may occur in lesions caused by *B. fabae*, since although no metabolites were detected on the first day after inoculation of pod seed cavities with *B. fabae*, lower concentrations of the inhibitors were recovered from these lesions than from similar limited lesions caused by *B. cinerea*.

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Fig.69

Phytoalexin metabolism by the fungus

Phytoalexin production by the plant

B CINEREA

B FABAE

B. fabae may be able to reduce the rate of phytoalexin accumulation at inoculation sites by killing the host cells responding to fungal invasion at a greater rate than B. cinerea. If phytoalexin production is induced in living cells surrounding necrotic cells initially invaded by the fungus B. fabae may be able to kill these responding cells at a greater rate than B. cinerea, reducing the amount of phytoalexin produced by these cells. Thus, advancing hyphae of B. fabae will be subjected to lower phytoalexin levels than those of B. cinerea. At the same time as this occurs B. fabae will be inducing phytoalexin synthesis in cells adjacent to those undergoing necrobiosis in a similar manner to that described above and this may account for the conclusions reached by Deverall et al.,(1968) and Deverall and Vessey (1969) that both fungi induce similar levels of phytoalexin. The reduced accumulation of wyerone acid in peripheries surrounding sites inoculated with B. fabae in leaves (Mansfield and Deverall 1974 b) supports the suppression hypothesis.

The ability of B. fabae to kill cells at a greater rate than B. cinerea may be a consequence of its ability to produce greater amounts of toxic metabolites. Purkayastha (1968) has shown that culture filtrates produced by B. fabae were more phytotoxic than those produced by B. cinerea.

An explanation of differential pathogenicity based on the production of phytoalexins, the sensitivity of the fungi to the phytoalexin, and the metabolism of the phytoalexins by the fungi may also explain the resistance of cotyledons to both B. cinerea and B. fabae. In cotyledons the cells are closely packed together. It is possible that there are relatively more cells responding to fungal invasion in this tissue than in pod and leaf tissue and that B. fabae is not able to kill the responding cells sufficiently rapidly to prevent the accumulation of the phytoalexins to levels to which it is sensitive and also unable to metabolize. Tomiyama et al.(1958) showed that there was a correlation between the thickness of potato discs and its resistance to Phytophora infestans.

They calculated that there was a minimum number of neighbouring cells to resist the fungus.

One of the problems in assessing the physiological nature of a disease situation is that it is a dynamic interaction of two metabolic systems and any explanation of susceptibility or resistance must take into account a number of factors which are continually changing as the disease progresses.

The appraisal of the role of the multi-component phytoalexin response of the broad bean in the disease reactions of Botrytis in bean tissue afforded by this study is consistent with the involvement of phytoalexins in the resistance of plants to fungal invasion. However, it also illustrates the need for a more critical evaluation of the use of the term phytoalexin with regard to its role in the disease resistance of plants, since compounds such as wyerol, which can be considered as a phytoalexin may not come into contact with the fungus and inhibit growth directly.

A useful tool for future research into the mechanisms involved in the pathogenicity of B. fabae towards the broad bean may be the use of mutants of Botrytis. If mutants could be isolated which are; insensitive to the phytoalexins; able to rapidly metabolize the phytoalexins; or able to produce large quantities of toxic metabolites, and if these characters can be tested either singly or in combination, the relative importance of each factor in the pathogenicity of B. fabae could be critically assessed.

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APPENDIX I

1 Separation of phytoalexins by gel filtration

Preparative separation of the phytoalexins by gel filtration with sephadex LH20 was investigated as an alternative to repeated PLC which involved considerable loss of phytoalexins. Preliminary experiments using 10cm columns (0.8cm diameter) of sephadex LH20 were carried out on Et_2O extracts of either B. fabae infected cotyledons or B. cinerea infected pod tissue. Sephadex powder was allowed to swell in MeOH : 5% benzene overnight and a glass column (0.8cm internal diameter x 15cm long) was packed with a thin slurry of gel to a height of 10cm. Before the start of an experiment the column was flushed out with the eluting solvents for 2 hours, and the flow rate adjusted to 0.2ml/min by restriction of the outlet tube. Samples were applied to the column in 0.5ml MeOH (equivalent to 5 g.f.w. tissue) through a disc of whatman seed test filter paper, which rested on the surface of the sephadex column. Fractions were collected at 5 min. intervals, 0.1ml aliquots of each fraction were applied to 3cm origins on TLC plates and developed in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). The chromatograms were then bioassayed with C. herbarum. The fractions in which inhibitory activity was detected are shown in Table 25. All the phytoalexins, except medicarpin, were detected in the same fractions, however these fractions from infected pod tissue extracts also contained chlorophylls. It was subsequently found that the phytoalexins could be separated from chlorophylls using an alkylated form of sephadex LH20 (obtained from [illegible].Dorn Chemistry dept. Stirling). Unfortunately only a small amount of this type of sephadex was available and preparative scale separations of Et_2O extracts not possible. Separation of the phytoalexins by PLC1 (Fig.9) before fractionating on alkylated sephadex LH20 allowed the use of greater concentrations of phytoalexins with low loading rates.

TABLE 25 Detection of inhibitors in fractions of Et_2O extracts of *B. fabae* infected cotyledon and *B. cinerea* infected pod tissue after gel filtration using sephadex LH-20.

	FRACTION NUMBER(X2 = MINUTES)	
	B. FABAE INFECTED COTYLEDON	*B. CINEREA* INFECTED POD
WYERONE	12-24	12-16
WYERONE EPOXIDE	12-20	12-16
WYEROL	12-17	12-14
MEDICARPIN	-	38-40
PA3b	12-16	12-14
WYERONE ACID	12-17	12-20

2 Separation of wyerone epoxide by gel filtration

Samples of wyerone epoxide were obtained by PLC1 Fig.70 A and B shows the UV absorption spectra of the epoxide recovered after this separation from B. fabae infected cotyledon and B. cinerea infected pod tissue. These crude samples of wyerone epoxide were applied to a column of alkylated sephadex LH20 in 0.5ml MeOH and eluted as described above. Fractions were collected every 2 minutes and their UV absorption spectra recorded in MeOH. The fractionation of the epoxide from both sources is shown in Fig.71. The major contaminant of wyerone epoxide was a 312nm absorbing substance (possible wyerol), since this substance has little absorbance at 347nm, the amount of each substance could be determined in mixtures by calculating the absorbance of wyerone epoxide at 312nm from a known absorbance at 347nm (the ratio of 347 : 312nm absorbance of wyerone epoxide was calculated for pure wyerone epoxide as 1.8). Subtracting this calculated absorbance from that obtained at 312nm gave the absorbance due to wyerol.

The isolation of pure wyerone epoxide by this method was not very successful, only two fractions collected contained little or no contaminating substances. Further purification required an additional PLC step, which reduced the efficency of this method. Fig.70 C and D show the UV absorption spectra of wyerone epoxide obtained after PLC of the combined sephadex fraction (15-22) in $CHCl_3$ (2% EtOH) followed by $CHCl_3$: petrol (2:1) Yields of 2.8 and 1.3mg were obtained from B. fabae infected cotyledons (2 kg.f.w.) and B. cinerea infected pods (0.5Kg.f.w.) respectively, six days after inoculation.

3 Isolation of medicarpin by gel filtration.

During preliminary studies on the development of a gel filtration method for isolating wyerone epoxide, it was observed that medicarpin was

Figure 70

UV absorption spectra of wyerone epoxide after PLC1 in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) and after gel filtration and PLC of these extracts.

(A) Wyerone epoxide from B. fabae infected cotyledon tissue after PLC1.

(B) " " " B. cinerea " pod " " " .

(C) " " " B. fabae " cotyledon " " gel filtration and PLC.

(D) " " " B. cinerea " pod tissue after gel filtration and PLC.

Fig 70

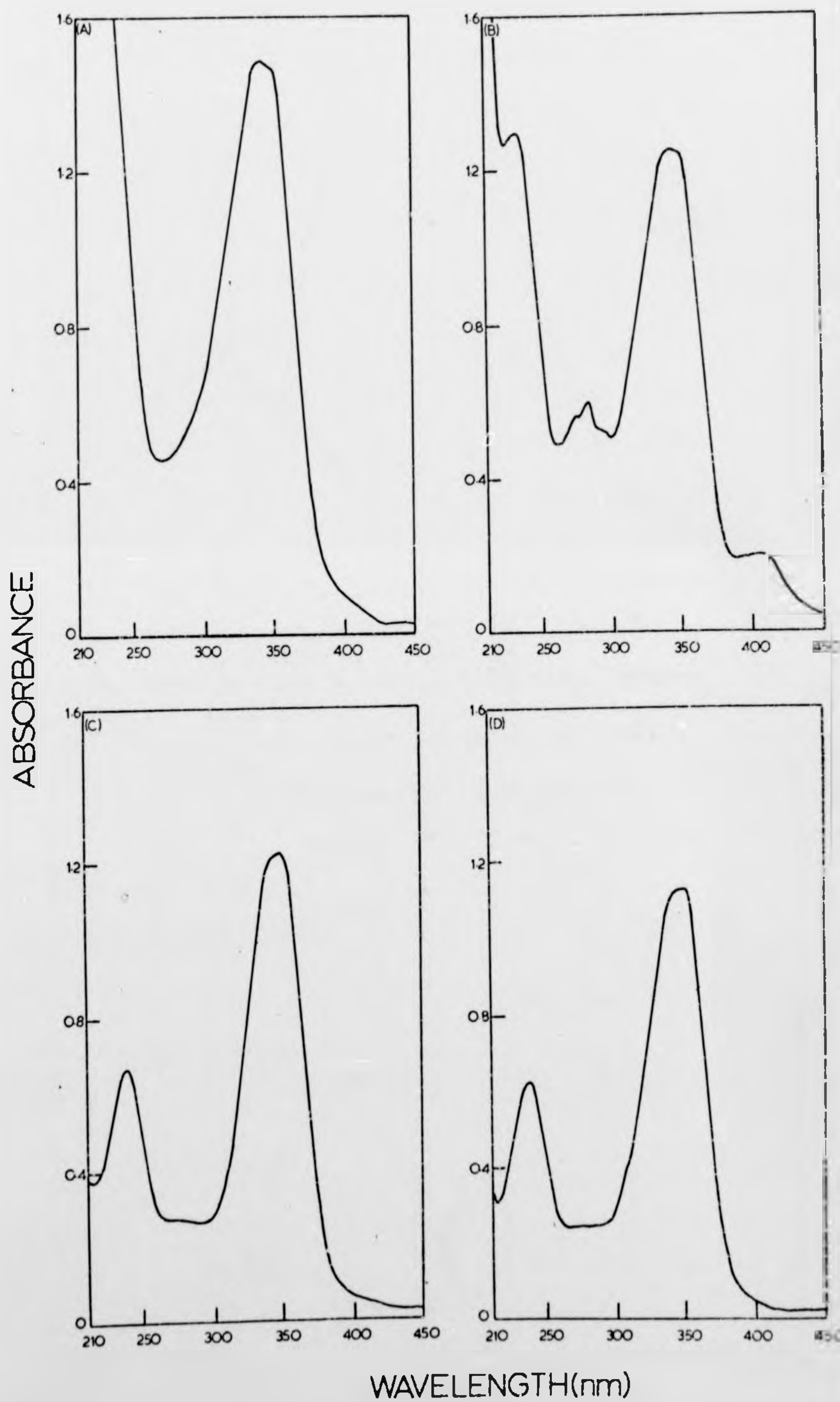


Figure 71

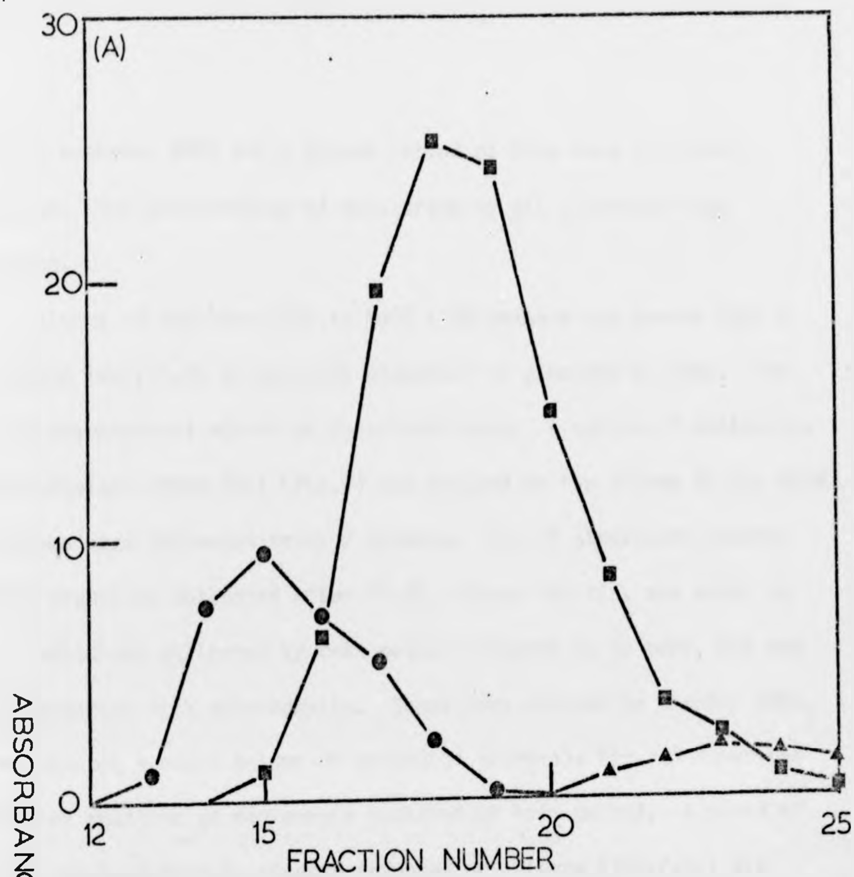
Fractionation of wyerone epoxide using alkylated LH20 sephadex from B. fabae infected cotyledon tissue (A) and B. cinerea infected pod tissue (B). Fractions were collected every two minutes.

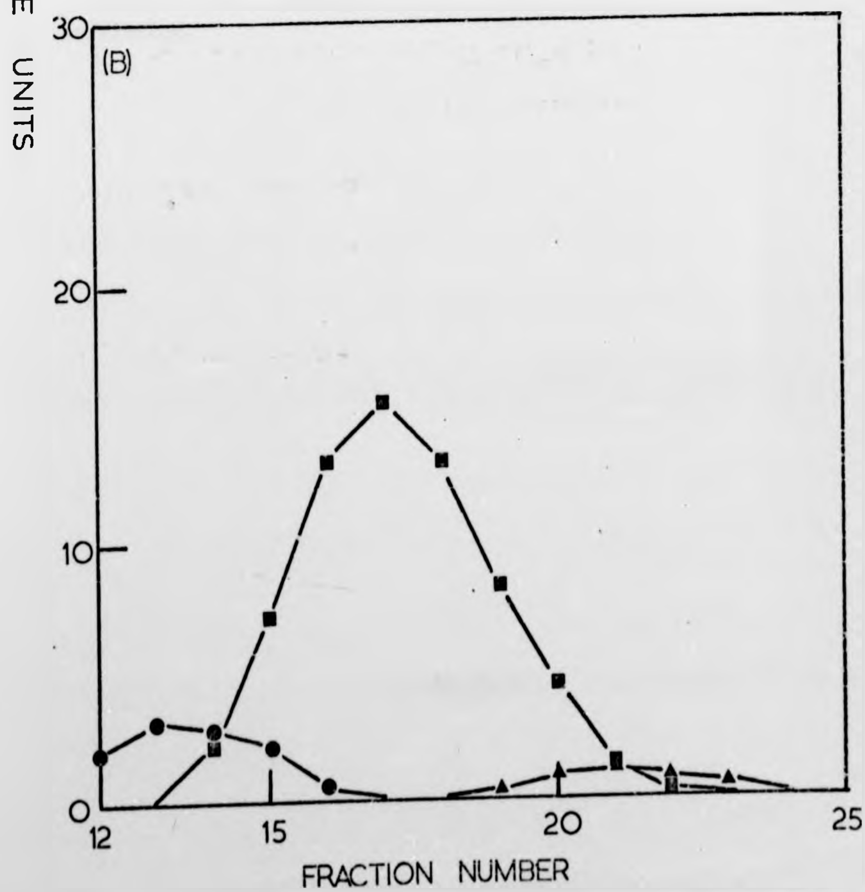
■ wyerone epoxide (λmax 347nm).

● unidentified substance (wyerol λmax 312nm).

▲ unknown substance (λmax 282nm).

Fig.71





retained on sephadex LH20 for a longer period of time than the other phytoalexins. The purification of medicarpin by gel filtration was investigated.

A slurry of sephadex LH20 in MeOH : 5% benzene was poured into a column (4.5cm long, 2.54 cm internal diameter) to a height of 25cm. The column was prepared and eluted as described above. A sample of medicarpin (Fig.72A) obtained after PLC1 (Fig.9) was applied to the column in 2ml MeOH and fractions were collected every 2 minutes. The UV absorption spectra in MeOH of fractions collected after 70,80, 90 and 100 min. are shown in Fig.73. Medicarpin collected by this method appeared to be pure, but was still contaminated with chlorophylls. These were removed by passing $CHCl_3$ solutions through a small column of activated charcoal. Fig.72B shows the UV absorption spectrum of medicarpin isolated by this method. A yield of c 3 mg was obtained from B. cinerea infected pod tissue (1Kg.f.w.) Six days after inoculation.

Figure 72

UV absorption spectra of medicarpin before (A) and after (B) gel filtration using LH20 sephadex.

Fig.72

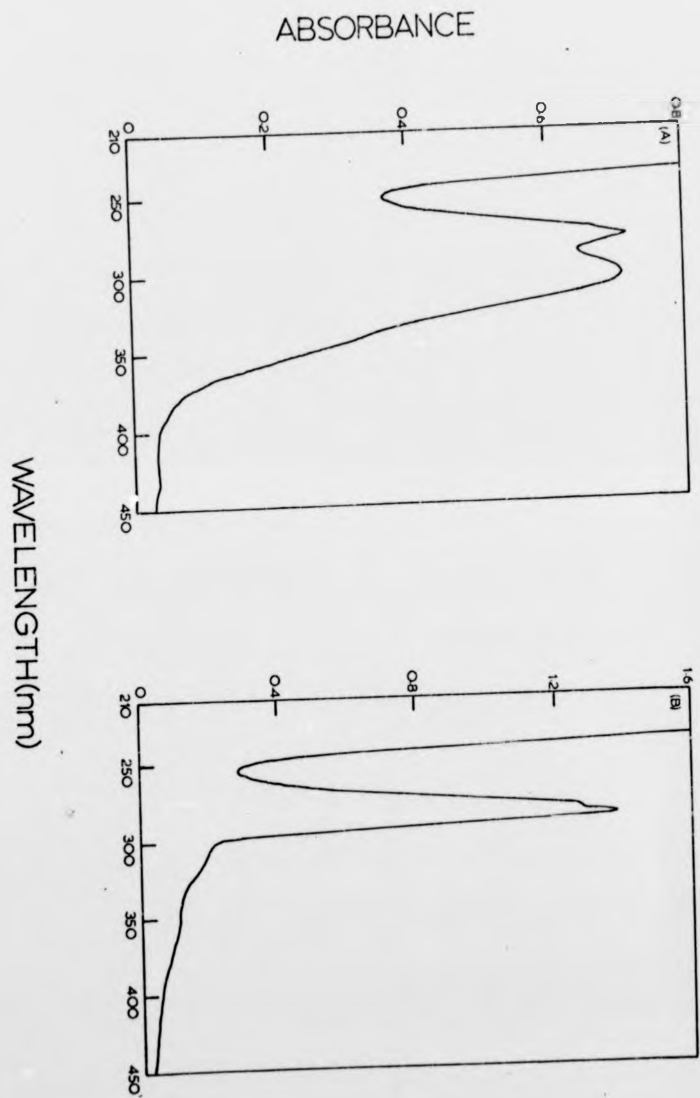
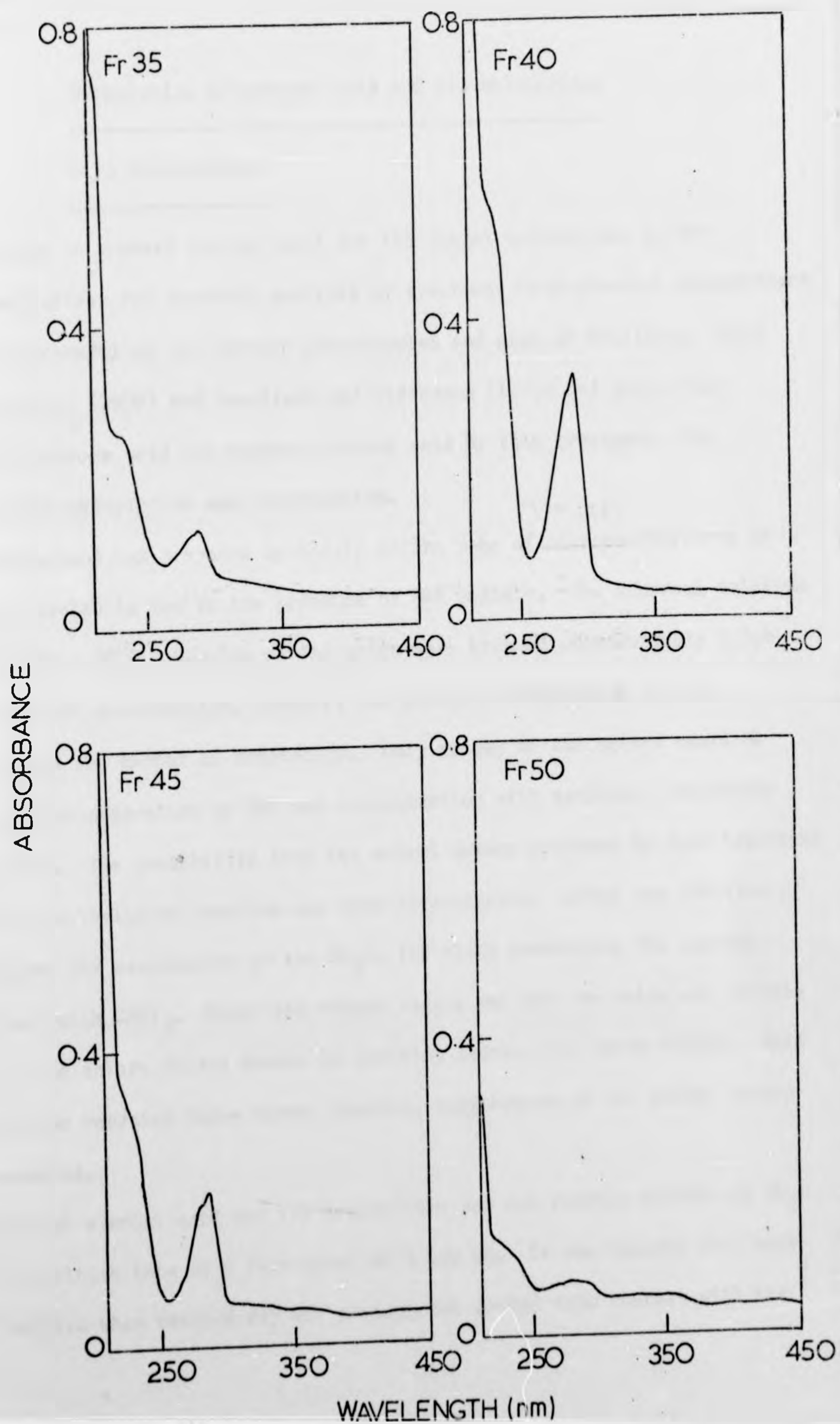


Figure 73

UV absorption spectra of the fractions 35, 40, 45 and 50 of medicarpin collected after gel filtration using LH20 sephadex.

Fig. 73



APPENDIX II

Methylation of wyerone acid and its metabolites with diazomethane

Attempts to convert wyerone acid and its fungal metabolites to the methyl derivatives for spectral analysis by treatment with ethereal diazomethane proved unsuccessful at the Norwich laboratories and also at Stirling. Since Letcher *et al.* (1970) and Mansfield and Widdowson (1973) had previously methylated wyerone acid and reduced wyerone acid by this treatment, the procedure for methylation was investigated.

Diazomethane was prepared by slowly adding 5-6g of nitrosomethylurea to 250ml Et_2O cooled in ice in the presence of KOH pellets. The ethereal solution was stored at - 20°C. Samples of the acids were treated consecutively 2,3,4 or 5 times with diazomethane, however, the amount of conversion did not increase with the number of treatments. Only traces of the esters could be detected after separation by TLC and visualization with vanillin - sulphuric acid reagent. The possibility that the methyl esters produced by this treatment inhibit the methylation reaction was then investigated. After the addition of diazomethane and evaporation of the Et_2O, the vials containing the samples were rinsed with $CHCl_3$. Since the methyl esters but not the acids are soluble in $CHCl_3$, the esters formed should be removed, leaving the acids behind. This procedure was repeated three times, however, only traces of the methyl esters were recovered.

Although wyerone acid and its derivatives are not readily soluble in Et_2O they do partition into Et_2O from water at a low pH. It was thought that most of the samples when treated dry was perhaps not coming into contact with the

diazomethane because of their insolubility in Et_2O. Three approaches were followed to investigate this possibility.

1. Diazomethane was added dropwise to samples in 0.1ml MeOH, then diazomethane was added in excess.

2. Et_2O solutions of the acids were prepared by partitioning the acids between water and Et_2O, the resulting Et_2O containing the acids was treated with diazomethane.

3. The surface area of the samples in contact with the diazomethane was increased by depositing the acids as a thin layer in a 100ml evaporating flask, 20ml of diazomethane were added and the flask shaken for 5 minutes before removing the Et_2O solution.

None of these methods gave an efficient conversion of the acids to their methyl esters. It is probable that the failure of these methods is due to the fact that the acids are not soluble in Et_2O under alkaline conditions. Thus, they do not come into contact with the ethereal diazomethane solutions, which are stored under alkaline conditions for reasons of safety.

APPENDIX III

Phytoalexin production by detached leaves from greenhouse grown plants

In chapter 3 it was shown that the phytoalexin response of detached leaves from garden grown plants was essentially similar to that of pod tissue. However, preliminary experiments suggested that leaves from greenhouse grown plants showed a different phytoalexin response. In the following experiments on greenhouse grown leaves only TLC plate bioassays were carried out and thus, the phytoalexins detected could therefore only be tentatively identified on the basis of RF data.

1. Accumulation of phytoalexins in greenhouse grown leaves following infection by *B. cinerea* and *B. fabae*.

Detached leaves were prepared from plants grown during February, 1975, and inoculated with conidial suspensions of *B. cinerea*, *B. fabae* or sterile distilled water. The symptoms caused by *B. cinerea* were extremely variable, at some inoculation sites no sign of infection were visible whereas at others, the degree of infection which developed ranged from very slight flecking (grade 6.5) to spreading lesions (grade S_2 - S_3). Tissue was collected for analysis from sites where infection occurred but not from sites where the lesion had spread into uninoculated tissue. In contrast to *B. cinerea*, *B. fabae* invariably invaded leaf tissue causing lesions between grades 38 and 63 within 12 hours after inoculation. During the first and second days after inoculation *B. fabae* had rotted and blackened the tissue beneath the inoculum droplet and spread into uninoculated tissue. Four days after inoculation *B. fabae* had completely rotted and blackened the leaf tissue and had sporulated on the rotted tissue by the sixth day after inoculation. The progress of *B. fabae*

through greenhouse grown leaves was therefore far more rapid than through garden grown leaves. No visible symptoms were detected on leaves at any time after inoculation with water alone.

At intervals after inoculation, the inoculum droplets and underlying tissue were collected (0.5 - 1.0 g.f.w.) and combined for extraction with MeOH. Et_2O extracts (equivalent to 0.25 g.f.w.) prepared from MeOH extracts as described previously were separated by TLC in hexane : acetone (3:1) followed by $CHCl_3$: petrol (2:1). The inhibitory bands which developed after bioassaying the developed chromatograms with *C. herbarum* are illustrated in Plate 14.

In extracts of *B. cinerea* infected tissue one inhibitory band was detected at R*F* 0.5 - 0.6 within 12 hours after inoculation. On the third day this band was resolved into two separate zones of inhibition which corresponded to wyerone epoxide (R*F* 0.56) and wyerol (R*F* 0.5). Wyerone (R*F* 0.65) and wyerone acid (R*F* 0.03) both accumulated in infected tissue and were first detected two days after inoculation.

An inhibitory band (R*F* 0.5) identical to that detected in extracts of *B. cinerea* infected tissue was present at 12h and 1 day but not 2 days after inoculation with *B. fabae*. Wyerone acid accumulated on the first day but decreased on the second and could not be detected on the third day after inoculation. *B. fabae* was apparently able to prevent the accumulation of phytoalexins and spread through the leaf tissue. No inhibitory bands corresponding to PA3 (Medicarpin and wyerone derivative PA3b) were detected in any of the extracts. No antifungal substances were detected in tissue collected from leaves inoculated with water alone.

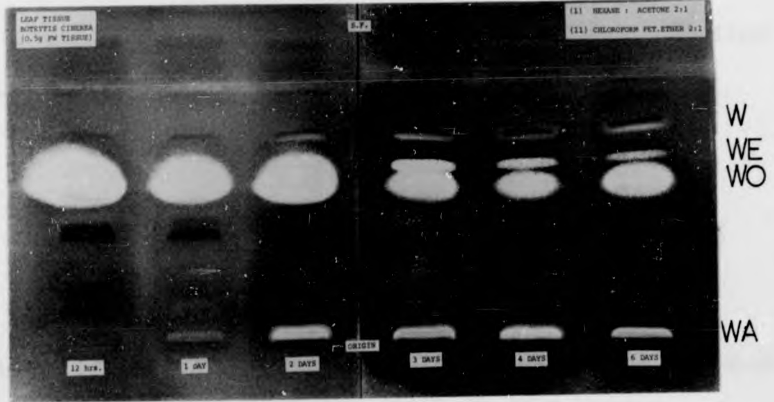
2. Accumulation of phytoalexins in greenhouse grown leaves bearing different grades of lesion caused by *B. cinerea*.

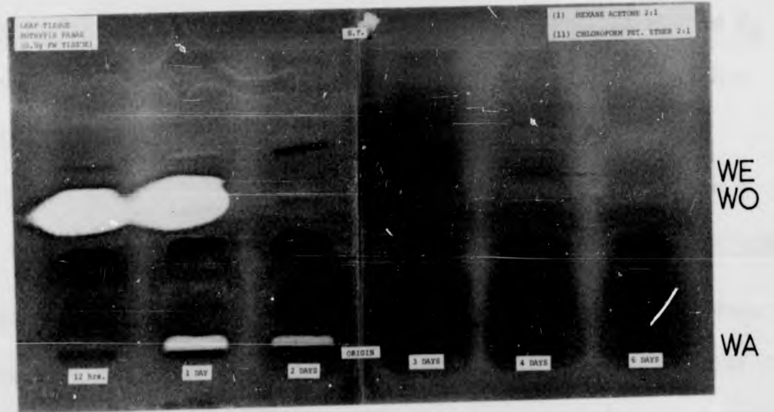
Ninety leaves were inoculated with conidial suspensions of *B. cinerea*.

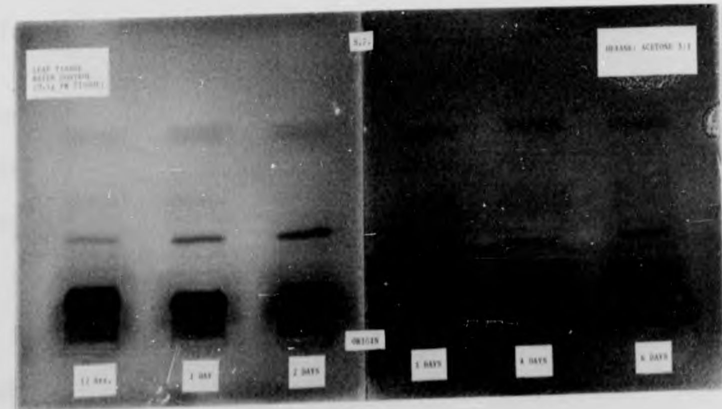
Plate 14

TLC plate bioassays of extracts from 0.5g leaf tissue collected 12h, 1,2,3,4 and 6 days after inoculation with conidial suspensions of either B. cinerea or B. fabae or distilled water alone. Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1). W, wyerone; WE, wyerone epoxide; WO, wyerol; WA wyerone acid.

Plate 14







Four days later the infected tissue and overlying infection droplets were collected and separated into four groups of different lesion grade (Mansfield and Deverall 1974 a)

Group 0 – water droplets retained, no symptoms visible.

Group 1-2 – lesion grade below 19

Group 3-5 – lesion grade between 19 and 63

Group 6 – lesion grade greater than 63 but not spreading.

Inoculation sites where the inoculum droplet had been displaced and where _B. cinerea_ had caused spreading lesions were disregarded. Infection development was extremely variable. Plate 15 shows a leaf from this experiment which illustrates this variability. The lesions formed varied from grades 0 to S_3 where droplets were retained and grades 0-S_1 where droplets had been either displaced or dried up.

Extracts prepared as described above were separated by TLC in hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1) and bioassayed with _C. herbarum_. Plate 16 shows the inhibitory bands detected in the different lesion grades. Results suggest that wyerone epoxide, wyerol and wyerone acid increase with increasing symptom development. Wyerone was detected at highest concentrations in lesion group 3-5. Virtually no phytoalexins were detected in extracts of _B. cinerea_ infected tissue where no symptoms developed.

There were marked differences between the phytoalexin response of greenhouse and field grown leaves. A large inhibitory band was detected 12h after inoculating greenhouse grown leaves but not garden leaves or pods, with either _B. cinerea_ or _B. fabae_. In _B. cinerea_ lesions this inhibitory band was resolved into two zones of inhibition corresponding to wyerol epoxide and wyerol with time, whereas in lesions caused by _B. fabae_ this band disappeared after

Plate 15

Symptoms developed on a broad bean leaf 4 days after inoculation with suspensions of B. cinerea conidia.

Lesion Number		
1	Droplet	not retained, no symptoms
2	"	retained, no symptoms
3	"	" Grade 6.5
4	"	not retained, Grade 38
5	"	" " Grade S
6	"	retained, Grade S_2
7	"	not retained, Grade 6.5
8	"	" " Grade 6.5
9	"	retained, Grade 63
10	"	" Grade 38
11	"	no symptoms
12	"	not retained, Grade 6.5
13	"	retained, Grade 19
14	"	not retained, Grade 6.5

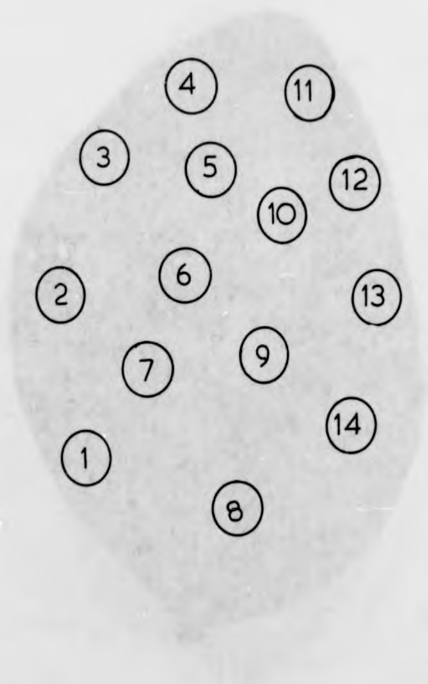

4
11
3
5
12
10
6
2
13
9
7
14
1
8

Plate 15

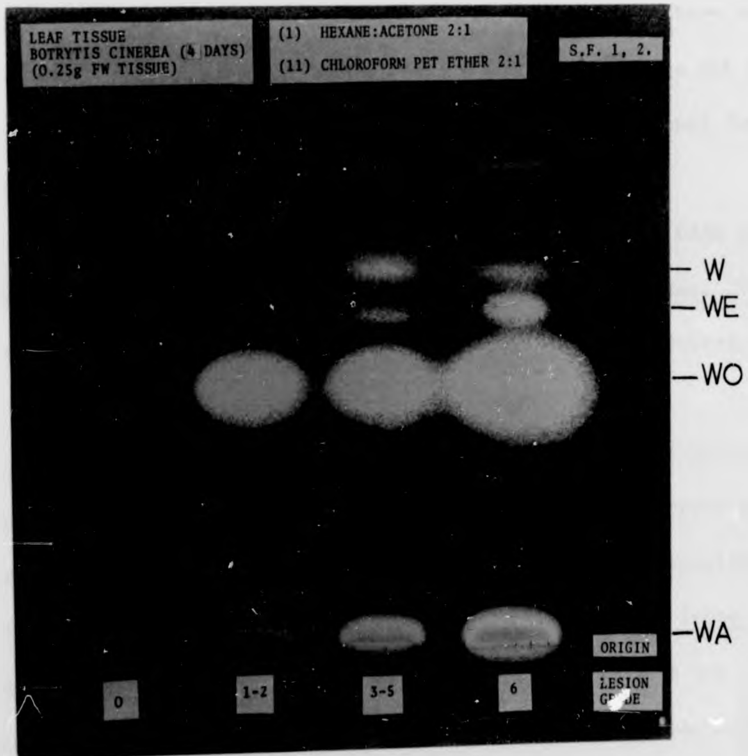
Plate 16

TLC plate bioassays of extracts from 0.25g leaf tissue collected 4 days after inoculation with suspensions of *B. cinerea* conidia. Solvents: hexane : acetone (2:1) followed by $CHCl_3$: petrol (2:1), W, wyerone; WE, wyerone epoxide; WO, wyerol; WA, wyerone acid.

Lesion grade

0 -	water droplet retained but no symptoms visible
1-2 -	lesion grade 19 (25%)
3-5 -	" " 19-63 (25 - 75%)
6 -	" " 63 but not spreading (75-100%)

Plate 16



one day. It was not possible to determine the character of this inhibitor, It may be a consequence of high wyerol and/or wyerone epoxide concentrations, since in B. fabae extracts this inhibitory band appeared to be composed of two components. Alternatively the band may be due to an unknown substance which is only active in the early phases of the disease reaction, if the latter is the case it would be interesting to determine if it is the product of fungal enzymes or arises due to *de novo* synthesis. However, if this inhibitory band is due to wyerol it is possible to speculate that this substance does not come into contact with the fungus and may instead represent a precursor pool for the other phytoalexins.

The lack of an inhibitory band corresponding to medicarpin and PA30 presents another anomaly in the phytoalexin response of greenhouse grown leaves. However, the concentrations of these inhibitors may have been too low to be detected in the TLC plate bioassays.

Greenhouse grown leaves do, however, show patterns of changes in phytoalexin concentrations after infection with both fungi similar to those observed in pods and garden grown leaves. (J.W. Mansfield pers. comm). The phytoalexins accumulate with time at *B. cinerea* inoculation sites, whereas in *B. fabae* lesions the phytoalexins accumulate to lower levels then decrease as the infected tissue becomes rotted and blackened, and eventually disappear as the fungus spreads into uninoculated tissue. Similar patterns of wyerone acid accumulation in leaves after infection with either *B. cinerea* or *B. fabae* have been reported (Mansfield and Deverall 1974 b). Phytoalexin production by leaves appears to be correlated to the degrees of necrosis as previously discussed.

These observations raise serious questions concerning the use of plants grown under artificial conditions in studies designed to elucidate naturally

occurring mechanisms of disease resistance in plants.

The variability of *B. cinerea* infection on leaves encountered in this study has been investigated in detail by Mansfield and Deverall (1974 a). They showed that the loss of the water droplet above inoculation sites during the first day after inoculation inhibited lesion formation by *B. cinerea*. However, where the droplet was retained they detected two patterns of *B. cinerea* infection, in one few or no lesions were formed and in the other, restricted lesions developed with marked browning of the inoculation site. Variation in lesion development could not be related to droplet position, however, plants differed in their susceptibility to lesion formation by *B. cinerea*.

The lack of germination recorded at some sites which failed to develop symptoms suggest that the growth of *B. cinerea* was inhibited on the leaf surface by some factor other than phytoalexin accumulation. A water soluble inhibitor which can be overcome by nutrients has been detected in infection droplets at such sites (Rossall, 1974). Alternatively, *B. cinerea* germination may be inhibited due to competition with bacteria for nutrients (Blakeman and Frazer, 1971; Blakeman, 1972.) Leaf surface waxes have also been implicated in the inhibition of *B. cinerea* on leaf surfaces (Blakeman and Sztenjnberg, 1973)

Stimulation of growth and germination at some inoculation sites may have resulted from the presence of nutrients randomly distributed before inoculation, either as cuticular consituents or dried exudates from epidermal cells as described by Martin and Juniper (1970). Nutrients may also accumulate after inoculation by exosmosis into the inoculum droplet as suggested by Brown (1922). Variation in the distribution of nutrients in the leaf surface may be due to differences in the cuticle and/or underlying cells.

In conclusion it would appear that the resistance of bean leaves to *B. cinerea* involves other factors as well as phytoalexin. This makes an appraisal of the role of the phytoalexin response in preventing fungal colonization of this tissue difficult at present.

APPENDIX IV

The activity of wyerone, wyerone epoxide, wyerol and wyerone acid in TLC plate bioassays using *C. herbarum*

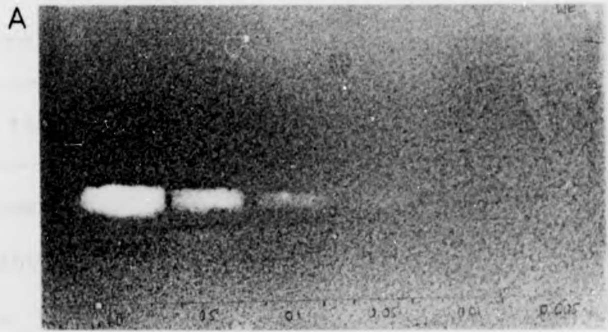
The TLC plate bioassay (Karlman and Stanford, 1968) proved to be invaluable for screening plant extracts for antifungal compounds. However, the extent of inhibition of *C. herbarum* caused by the phytoalexins differed greatly. For example, wyerone epoxide gave rise to an inhibitory band when only very low concentrations were detected by UV spectrophotometry, whereas wyerol could be detected in extracts where no inhibition band was detected. To investigate the differential activity of phytoalexins against *C. herbarum* on TLC plates a range of concentrations (0.005 - 10 μg) of pure samples of wyerone, wyerone epoxide, wyerol and wyerone acid were applied to 3cm origins, separated by TLC, and the developed chromatograms bioassayed with *C. herbarum*. Plate 17 shows the inhibitory bands which developed in each treatment. The relative ability of the phytoalexins to inhibit *C. herbarum* growth followed the order wyerone epoxide > wyerone > wyerone acid > wyerol. Wyerone epoxide was active at much lower concentrations than the other phytoalexins. 0.5 μg wyerone epoxide was able to inhibit fungal growth. Wyerone and wyerone acid had similar activity being detected at 5 - 10 μg level. However, wyerol did not inhbit growth even at the highest concentration tested (10 μg).

Plate 17

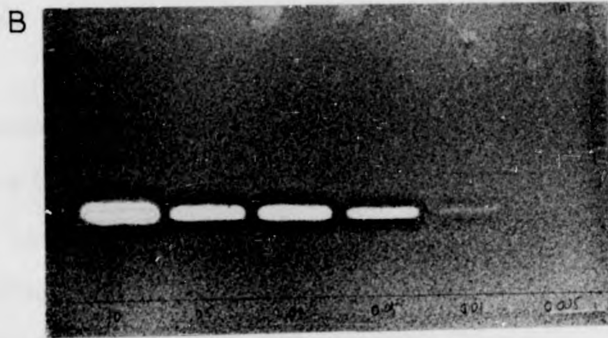
The activities of wyerone (A), wyerone epoxide (B), wyerol (C) and wyerone acid (D) in TLC plate bioassays using *C. herbarum*.

Plate 17

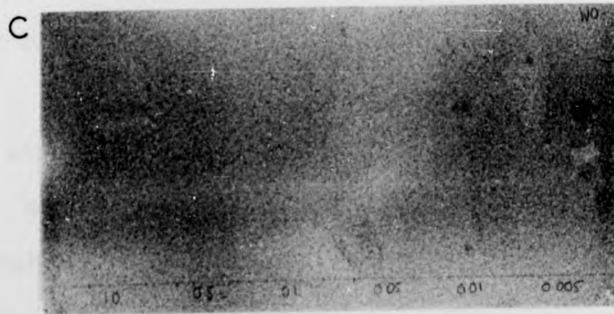
A



B



C



D

10 5 1 0.5 0.1 0.05 (ug)

APPENDIX V

Investigations on the conversion of wyerone to wyerone acid in pod tissue infected with B. cinerea

Results obtained from investigation of the localization of wyerone (p.51) suggested that this inhibitor was converted to wyerone acid in vivo. In order to investigate wyerone - wyerone acid conversion and relate it to the disease situation attempts were made to localize and measure the activity of the esterase enzymes possibly converting wyerone to wyerone acid in B. cinerea infected tissue.

Initially a histochemical stain for esterases was tested, involving the use of α napthyl acetate (Pitt and Coombes, 1968) A 1% stock solution of α napthyl acetate in 50% acetone was prepared and stored at 4°C. For use, 0.5 - 1.0ml of stock solution was pipetted into 50ml distilled water and 5ml 200mM phosphate buffer (pH8) and 20-50mg fast blue B. salt were then added and the mixture stirred then filtered. The tissue was incubated in the clean stain at below 20°C for between 5 and 20 minutes. Esterase activity was detected as a dark brown colouration.

Both 3 and 6 day old B. cinerea lesions gave no particulate localization of esterase activity. However, during the test it was noticed that if the inoculum droplet was left on the inoculation site it rapidly developed the characteristic positive colouration of the stain suggesting that esterase activity was present in the inoculum droplet.

Two biochemical methods of assaying esterase activity were investigated.

(a) Direct measurement of loss of wyerone and the appearance of wyerone acid by extracting the reaction mixture with Et_2O followed by TLC separation and UV spectroscopy.

(b) Indirect measurement of wyerone acid production by monitoring changes in pH of the reaction mixture.

Initially the direct method (a) was used to investigate esterase in inoculum droplets. 150 μg wyerone in 50μl MeOH was added to 5ml of inoculum droplets from 3 day old B. cinerea lesions in pod seed cavities. The reaction mixture was incubated at 25°C in the dark and 1ml aliquots removed at hourly intervals. Wyerone acid already present in the inoculum droplet made detection of increases in wyerone acid difficult but no loss of wyerone occurred over a 4h period . It was considered that there were three possible reasons for this lack of activity.

1) Wyerone acid present in the diffusate may suppress enzyme activity 2) MeOH in which wyerone was added may also inhibit the enzyme 3) The pH of inoculum droplets (4.2) may not be optimal for esterase activity.

Each of these possibilities were examined. It was found possible to remove phytoalexins from inoculum droplets by dialysis (24 x 70 visking tubing) against water at 4°C overnight. Wyerone was added in 50μl MeOH or deposited on the side of the reaction vessel, and the pH of the inoculum droplets adjusted to 8.0 with 0.1 M NaOH. Table 25 shows the recovery of wyerone and wyerone acid from reaction mixtures containing: 5ml dialysed inoculum droplets from 3 day old B. cinerea lesions at pH 4.2 or 8.0 or sterile distilled water, 150 μg wyerone deposited on sides of the reaction vessel or added in 50μl MeOH. After incubating for 4h at 25°C in the dark the recoveries of wyerone and wyerone acid from the reaction mixtures was measured. Conversion of wyerone to wyerone acid was detected in all treatments at pH8. Addition of wyerone in MeOH had no effect on the conversion of wyerone to wyerone acid. Attempts to repeat this experiment using either NaOH to adjust the pH or 100mM phosphate buffer (pH 8.0) failed repeatedly.

The possibility that the enzyme was localized on cell walls and investigated.

TABLE 26 Recovery of wyerone and wyerone acid from reaction mixtures containing 5ml inoculum droplets from pod seed cavities 3 days after inoculation with B. cinerea, at pH8.0 or pH4.7, and 150ug wyerone either deposited or in solution, after incubating for 4h at 20C.

(μg)	WYERONE	WYERONE ACID
Solution of wyerone incubated with sterile distilled water at pH8.0.	139.6	-
Solution of wyerone incubated with inoculum droplets at pH4.7.	119	4.3
Solution of wyerone incubated with inoculum droplet at pH8.0.	42.7	54.5
Deposited wyerone incubated with inoculum droplets at pH4.7.	124.1	5.3
Deposited wyerone incubated with inoculum droplets at pH8.0.	31.0	67.8
Inoculum droplet incubated alone at pH4.7.	-	6.3

activity detected in the above experiment may have been due to contamination of the diffusate with tissue fragments, since any enzyme present in the inoculum droplet would not be active because of the low pH. After removal of inoculum droplets, B. cinerea infected pod tissue was collected 3 days after inoculation soaked in 0.5M NaCl (1:1 w/v) and agitated with the aid of a magnetic stirrer for 1 hour. The NaCl extract was dialysed against water and assayed as described above at pH8. No activity was detected. Finally whole tissue was homogenised in 100mM phosphate buffer (pH 8.0), centrifuged at 850g for 10 minutes, and after dialysis, assayed for esterase activity, however, again no activity was detected.

These results suggest that the esterase hydrolysing wyerone to wyerone acid accumulates only in inoculum droplets above infected tissue. However, further critical analysis of this conversion is required.

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