D26880/79

D 26880/79
ROSSALL S.
PP 247

Attention is drawn to the fact that the copyright of this thesis rests with its author.

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior written consent.

THE RESISTANCE OF *VICIA FABA* L. TO INFECTION BY *BOTRYTIS*

BY

STEPHEN ROSSALL, B.A. (STIRLING)

Thesis submitted for examination for the degree of

Doctor of Philosophy

University of Stirling

October, 1978

ACKNOWLEDGEMENTS

It is a pleasure to express my gratitude to Dr J. W. Mansfield for his advice and encouragement, particularly in the preparation of the manuscript.

I am most grateful to Mrs. M. Abrahamson for typing this thesis and to my wife for her patience throughout the duration of this research.

ABSTRACT

The mechanisms of resistance of Vicia faba L. to infection by Botrytis have been examined.

Inoculation of leaves detached from growth room grown plants with the avirulent species B. allii, B. cinerea and B. elliptica caused lesion development to occur at very few sites. At lesion free sites spore germination and germ tube growth were inhibited on the leaf surface. The cause of the inhibition of B. cinerea was shown to be a combination of nutritional competition between epiphytic bacteria and fungal spores and also the release into inoculum droplets of a water soluble inhibitor, probably arising from the interaction between conidia and epicuticular wax. The virulent pathogen, B. fabae was not inhibited on the leaf surface and caused lesion development at all inoculation sites. The mechanism by which this species may overcome inhibitors acting on the leaf surface is discussed.

Inhibition of B. cinerea germination was much less frequent on leaves from field grown plants indicating that post-penetration resistance mechanisms may be of more significance to the restriction of avirulent Botrytis spp. in the field.

Whereas B. cinerea typically produced limited lesions in field grown leaves, B. fabae produced lesions which ultimately spread throughout the leaf. Phytoalexin production by V. faba has been strongly implicated in the differential pathogenicity of Botrytis towards the broad bean plant. The antifungal activity of five phytoalexins from V. faba, the furanoacetylenes wyerol, wyerone,

wyerone acid and wyerone epoxide and the isoflavanoid medicarpin has been examined. All the phytoalexins were more active against germ tubes of B. cinerea than B. fabae but differential activity was more marked with wyerone derivatives than with medicarpin. The inhibitors were ranked in the following order of activity; wyerone epoxide, wyerone acid, wyerone, medicarpin and wyerol. The activity of wyerone acid was influenced by pH. B. fabae was found to be less sensitive to wyerone acid than six other species of Botrytis and studies with four isolates of both B. cinerea and B. fabae showed that tolerance to wyerone acid correlated with virulence. At concentrations slightly above those causing complete inhibition of fungal growth, wyerone, wyerone acid and wyerone epoxide were also shown to be toxic to B. cinerea and B. fabae. Results suggested that the phytoalexins probably acted by binding to a specific receptor site within hyphae, but their precise mode of action was unclear.

Studies on the relationships between fungal growth and metabolism of wyerone, wyerone acid and wyerone epoxide showed that the differing abilities of B. cinerea and B. fabae to grow in the presence of the inhibitors was due primarily to differences in sensitivity rather than ability to metabolize the phytoalexins.

The time course of the growth and death of infection hyphae and accumulation of phytoalexins in epidermal tissue has been examined. Results suggested that antifungal concentrations of phytoalexins (particularly wyerone acid) accumulated sufficiently early, and in the correct location to account for the observed cessation in growth of B. cinerea. Within lesions caused by equally large numbers of conidia of B. fabae, however, phytoalexins failed to reach fungistatic concentrations. The biochemical mechanisms underlying the pathogenicity of B. fabae to V. faba are discussed.

CONTENTS

	Page
ACKNOWLEDGEMENTS	(i)
INTRODUCTION	1
(a) Inhibition of fungi on the leaf surface	2
(b) Involvement of phytoalexins in disease resistance	7
(c) Objectives of research	11
(i) Inhibition of conidial germination on the leaf surface	11
(ii) Role of phytoalexins in the differential pathogenicities of B. cinerea and B. fabae	11
MATERIALS AND METHODS	
1. Plant Material	13
(a) Source of seeds	13
(b) Growth of plants in growth rooms	13
(c) Growth of plants under field conditions for studies on leaves and pods	13
(d) Growth of French bean and tomato plants	14
2. Fungi	14
(a) Origin and maintenance of stock cultures	14
(b) Production of conidia	14
(c) Preparation of conidial suspensions	15
3. Culture media	16
(a) Medium X	16
(b) Acid V8 Juice Agar	16
(c) Synthetic pod nutrients (SPN)	17
(d) Galacturonate buffered SPN	17
4. Chemicals	18
5. Measurement of pH	19
6. Determination of pKa of wyerone acid	20
7. Spectral analysis	20
8. Extraction and redeposition of leaf surface waxes	20
(a) Watch glass redeposition	20
(b) Sprayed glass slide redeposition	21
(c) TLC plate redeposition	21
(d) Glass fibre disc redeposition	21

Page

MATERIALS AND METHODS (cont.)

9. Inoculation techniques 22
   (a) Growth room grown leaves 22
   (b) Field grown leaves 23
   (c) Glassware and fibreglass discs 23
   (d) Cotyledons 23
   (e) Pods 23

10. Measurement of infection on leaves 24

11. Preparation of material for light microscopy 24
   (a) Examination of growth on the leaf surface 24
   (b) Examination of growth with epidermal cells 24

12. Preparation of diffusates 25
   (a) Leaf diffusates 25
   (b) Diffusates from glass 25
   (c) Pod diffusates 25

13. Analysis of nutritional status of diffusates 26
   (a) Total carbohydrate 26
   (b) Total amino acids 26

14. Preparation of tissue for extraction 27
   (a) Leaf epidermal strips 27
   (b) Pod endocarp 27
   (c) Cotyledons 27

15. Extraction procedures 27
   (a) Diffusate 27
   (b) Tissue 28
      (i) Leaf tissue 28
      (ii) Cotyledon and pod tissue 28

16. Chromatography 29
   (a) Thin layer chromatography (TLC) 29
   (b) Preparative layer chromatography (PLC) 29
   (c) Gel filtration 30

Page

MATERIALS AND METHODS (cont.)

17. Summary of purification procedures for phytoalexins and phytoalexin metabolites 31
(a) Phytoalexins 31
(i) Wyerone 31
(ii) Wyerone epoxide and wyerol 31
(iii) Wyerone acid 32
(iv) Medicarpin 32
(b) Phytoalexin metabolites 33
(i) Reduced wyerone acid 33
(ii) Wyerol epoxide and dihydrodihydroxy wyerol 33

18. Use of UV absorption spectra to estimate concentration of phytoalexins and phytoalexin metabolites 34

19. Bioassays 35
(a) TLC plate bioassay 35
(b) Slide bioassay 35
(i) Against conidia 35
(ii) Against germ tubes (sporelings) 36

20. Assessment of death of sporelings and conidia 37
(a) By solution replacement 37
(b) By vital staining 38

21. Relationship between growth of germ tubes and metabolism of phytoalexins 38

EXPERIMENTAL WORK AND RESULTS

Section I The inhibition of Botrytis on leaves of Vicia faba 40

Chapter 1 Quantitative studies on the development of Botrytis on the adaxial surface of growth room grown plants 40

Page

Chapter 2 Studies on the causes of inhibition of germination of conidia on leaf surfaces 42

(a) The antifungal activity of diffusates from broad bean leaves 42

(b) Comparative studies on the inhibition of germination of Botrytis on leaves of broad bean, French bean and tomato plants 43

(c) Time course studies on the accumulation of inhibitory activity in inoculum droplets incubated on broad bean leaves 45

Chapter 3 Characterisation of possible source of inhibition of germination of B. cinerea conidia on leaves of V. faba 48

(a) Studies on the inhibitory activity of bacteria within inoculum droplets 48

(b) Studies on the water soluble inhibitor remaining in inoculum droplets after membrane filtration 49

(i) Source of the water soluble inhibitor 49

(ii) Organic extraction of diffusate 50

(iii) UV absorption spectrum of filtered diffusates 51

(iv) Detection of antifungal activity on TLC plate bioassay 52

(c) Studies on the role of epicuticular wax in leaf surface inhibition 52

(i) The growth of B. cinerea conidia on redeposited wax 53

(ii) The antifungal activity of diffusates from wax 53

(iii) Extraction of diffusate from wax with diethylether 54

(iv) UV absorption spectrum of diffusate obtained from redeposited wax 55

(v) Detection of antifungal activity on TLC plate bioassay 55

(vi) Analysis of bean wax by thin layer chromatography 55

Page

Chapter 3 (cont.)

(d) Effect of glucose on leaf surface inhibition of *B. cinerea* 57

(i) Effect of glucose solutions on the development of lesions on broad bean leaves following inoculation with *B. cinerea* 57

(ii) Effect of glucose on the antifungal activity of diffusates from inoculation sites on broad bean leaves 57

(iii) Effect of glucose on the inhibition associated with redeposited wax 58

Chapter 4 Studies on the inhibition of *B. cinerea* conidia on leaves from field grown *V. faba* plants 60

(a) Lesion development on field grown leaves following inoculation with *B. cinerea* 60

(b) Time course studies on the accumulation of inhibitory activity in inoculum droplets incubated on field grown leaves 61

(c) Studies on the involvement of nutrients in the formation of lesions by *B. cinerea* 62

(i) A comparison between lesion development on adaxial and abaxial surfaces of field and growth room grown leaves following incubation with *B. cinerea* 62

(ii) The release of carbohydrates and amino acids into droplets incubated on leaves 62

(iii) The effect of the addition of nutrients to droplets of conidial suspension incubated on growth room grown leaves 64

Page

Section II The inhibition of *Botrytis* by phytoalexins from *V. faba*

Chapter 1 Antifungal activity of phytoalexins 66

(a) Antifungal activity of phytoalexins against conidia of *B. cinerea* and *B. fabae* 67

(b) Antifungal activity of phytoalexins against sporelings of *B. cinerea* and *B. fabae* 67

(c) Effect of pH on antifungal activity of wyerone acid 68

(i) In relation to the pKa of the acid 68

(ii) Effect of pH on the activity of wyerone acid in natural pod nutrients 69

(d) Antifungal activity of wyerone acid towards a range of species of *Botrytis* 70

(e) Antifungal activity of wyerone acid towards a range of isolates of *B. cinerea* and *B. fabae* 70

(f) Effect of wyerone derivatives on rates of growth of *B. cinerea* and *B. fabae* 71

(g) Relationship between metabolism of phytoalexins and growth patterns of germ tubes 72

Chapter 2 Fungitoxicity of phytoalexins 74

(a) Assessment of cell death 74

(i) Visual appearance of fungal structure 74

(ii) Use of vital stains 74

(b) Fungitoxicity of wyerone, wyerone acid and wyerone epoxide 76

(c) Effect of time of exposure on the toxicity of wyerone acid to *Botrytis* 76

(d) Effect of stage of fungal growth on toxicity of wyerone acid to *Botrytis* 77

(i) Comparison between sporelings and ungerminated conidia 77

(ii) Comparison between sporelings of differing germ tube lengths 77

(e) Tolerance of 'secondary' germ tubes to wyerone acid 78

Page

Chapter 3 Studies on the mode of action of phytoalexins 79

(a) Effect of reduced wyerone acid on the antifungal activity of wyerone acid 79

(b) Effect of a range of structurally related compounds on the antifungal activity of wyerone, wyerone acid and wyerone epoxide towards *B. fabae* 80

(c) Studies on the mechanism of inhibition of the activity of wyerone acid by reduced wyerone acid 81

(d) Effect of wyerone acid on leakage of cations from sporelings of *Botrytis* 84

Chapter 4 Fungal development and phytoalexin accumulation in bean leaves 86

(a) The growth and death of infection hyphae of *B. cinerea* and *B. fabae* within epidermis of bean leaves 86

(b) Comparative studies on the growth of infection hyphae of *B. cinerea* and *B. fabae* and the accumulation of phytoalexins within epidermal tissues and inoculum droplets 88

(i) Growth of infection hyphae in the epidermis in relation to phytoalexin accumulation 88

(ii) Accumulation of phytoalexins and phytoalexin metabolites in epidermal tissue and inoculum droplets of bean leaves inoculated with *B. cinerea* and *B. fabae* 90

(iii) Antifungal activity of inoculum droplets removed from leaves 92

(c) Estimation of pH within infected plant cells 93

DISCUSSION

I Inhibition of *Botrytis* on the leaf surface of *V. faba*

(a) Germination of *Botrytis* conidia on the leaf surface in relation to symptom development 95

(b) The accumulation of inhibitory activity in inoculum droplets collected from lesion free sites 96

(c) The effect of nutrients on leaf surface inhibition 104

(d) Conclusions on the inhibition of *Botrytis* on leaves of *V. faba* 106

Page

II Role of phytoalexins in the resistance of *V. faba* to *Botrytis* 107

(a) Antifungal activity of phytoalexins from *V. faba* 107

(i) Bioassay procedures 107

(ii) Relative activity of phytoalexins 108

(iii) Effect of pH on antifungal activity of phytoalexins 110

(iv) Mode of action of phytoalexins 111

(v) Differential sensitivity of fungi to wyerone acid 113

(vi) Relationships between growth of fungal germ tubes and metabolism of phytoalexins 114

(vii) Fungitoxicity of phytoalexins 115

(viii) Effect of fungal growth stage on tolerance to phytoalexins 117

(b) Phytoalexin accumulation and activity *in vivo* 118

(i) Localization and timing of phytoalexin accumulation and cessation of fungal growth 118

(ii) Activity of wyerone acid *in vivo* 121

(iii) Differential pathogenicities of *B. cinerea* and *B. fabae* 122

III General Conclusions 123

BIBLIOGRAPHY

APPENDIX Studies on the role of leaf surface bacteria in inhibiting germination of conidia of *B. cinerea in situ*

(a) Elimination of bacteria from infection droplets using antibiotics

INTRODUCTION

This thesis reports further studies on the resistance of leaves of Vicia faba L. to infection and colonization by Botrytis cinerea Pers. and B. fabae Sard. Both species of Botrytis have been described as the causes of chocolate spot disease of broad and field beans (Wilson, 1937; Ogilvie & Munro, 1947; Leach, 1955). They both cause localized lesions to develop on leaves and pods but under normal field conditions only B. fabae is able to spread from initial infection sites to develop aggressive infection and to sporulate on rotted tissues (Leach, 1955).

The differential pathogenicities of B. cinerea and B. fabae towards V. faba have been demonstrated in the laboratory by inoculating detached leaves (Deverall & Wood, 1961; Purkayastha & Deverall, 1965a; Mansfield & Deverall, 1974a; Hargreaves, Mansfield & Rossall, 1977). For example, Mansfield & Deverall (1974a) found that conidia of B. fabae produced spreading lesions at nearly all inoculation sites but infection development was much more variable following inoculation with B. cinerea. The latter species produced no symptoms, limited lesions and spreading lesions at 26, 73.4 and 0.6% of 516 inoculation sites after 6 days. Germination and germ tube growth by B. cinerea were inhibited on the leaf surface at sites which failed to develop lesions, whereas germ tubes often terminating with appresoria were produced from conidia at lesion sites where restriction of fungal growth occurred after short infection hyphae had been produced.

Preliminary studies on the mechanisms of the inhibition of B. cinerea on the leaf surface of V. faba were reported by Rossall

(1974). In the research reported here this resistance mechanism has been further examined. The involvement of phytoalexins in the restriction of fungal growth at sites where lesions develop has also been assessed.

(a) Inhibition of fungi on the leaf surface

Once deposited on the leaf surface initiation of infection processes depends on germination of the fungal spore. With the exception of powdery mildews, spores of most fungal pathogens require free water in order to germinate (Manners & Hossain, 1963; Schnathorst 1965). The environment existing within a water droplet containing spores of a fungal pathogen supported on the surface of a leaf is relatively complex (Blakeman, 1973). It reflects the interaction between pathogen, plant and leaf surface saprophytic microflora, each responding to changes in physical conditions. The existence of such a three-membered biological system has long been accepted for the zone around plant roots, termed the rhizosphere by Hiltner (1904), but such a system has only recently been accepted as responsible for events on the leaf surface, in the phylloplane. Biological and biochemical changes within such droplets, henceforth termed inoculum droplets, may prevent or induce germination of fungal spores.

Inhibitors and stimulants accumulating within inoculum droplets may be products of the host plant, saprophytic microflora, the fungal pathogen itself or be produced as a result of the interaction between any or all three members of the phylloplane system. As early as 1922 the investigations of Brown suggested that leaves and petals may act as sources of both stimulants and inhibitors of fungal development in overlying inoculum droplets.

The accumulation of nutrients in inoculum droplets has been reviewed by Tukey (1971). A large number of substances have been recorded in leachates derived from plant leaves, including simple sugars, all known amino-acids, growth regulators, organic acids, vitamins, all essential minerals and many trace elements. These nutrients may be utilized by both parasitic and saprophytic micro-organisms in the inoculum droplet.

Fraser (1971) has shown that saprophytic bacteria remove nutrients from inoculum droplets on leaves of chrysanthemum (Chrysanthemum morifolium). The increase in numbers of bacteria after two or three days caused bacterial uptake of nutrients to equal the rate of their release from the leaf. Consequently sugars and amino-acids were almost undetectable in inoculum droplets at these times after inoculation (Blakeman & Fraser, 1971).

Leakage of nutrients from spores of pathogenic fungi on leaf surfaces can also make a significant contribution to enrichment of droplets. Botrytis cinerea has been shown to enrich droplets on chrysanthemum leaves with amino acids and sugars, resulting in an increase in the numbers of epiphytic bacteria (Fraser, 1971).

The presence of saprophytic micro-organisms in the inoculum droplet may directly inhibit the development of pathogens. The first observation of such an inhibitory process occurring in vivo was made by Crosse (1965). He concluded that saprophytic bacteria, which were a regular constituent of natural inoculum, were able to reduce the severity of leaf scar infection of cherry caused by the pathogen Pseudomonas mors-prunorum. Fraser (1971) suggested that the reason Mycosphaerella ligulicola germinated well on chrysanthemum leaves and

B. cinerea was inhibited was that the latter released greater amounts of nutrients into the inoculum droplet, stimulating increased numbers of saprophytic bacteria which inhibited spore germination. The implication that the activity of bacterial saprophytes is directly inhibitory to B. cinerea spore germination has also been made by Blakeman & Fraser (1971) and Blakeman (1972) for chrysanthemum and beetroot leaves respectively, and by Clark & Lorbeer (1976 & 1977) for spore germination of B. cinerea and B. squamosa on onion leaves.

Fraser (1971), Blakeman & Fraser (1971) and Blakeman (1972) demonstrated that leaf surface bacteria did not produce stable antifungal compounds. Evidence has been obtained by Sztejnberg & Blakeman (1973a) to indicate that the mechanism of bacterial inhibition on leaf surfaces is one of nutrient competition, whereby bacteria set up a steep concentration gradient from within to the outside of the spore, resulting in an increased rate of loss of nutrients from its endogenous reserves. Competition for nutrients derived from the leaf would add to this effect, resulting in spore starvation.

Studies using ^{14}C labelled compounds by Brodie & Blakeman (1975 & 1976) and Blakeman & Brodie (1977) confirmed that bacteria on beetroot leaf surfaces adversely affected germination of B. cinerea conidia by utilizing nutrients, especially amino acids, derived from both conidia and from exogenous sources. Similar conclusions were reached by Ko & Lockwood (1967) in a study of the mechanism of soil fungistasis. However, Leben (1964) and Leben & Daft (1965) showed that Colletotrichum lagenarium, which causes anthracnose of cucumber, could be inhibited by cell-free diffusates obtained from a bacterium from cucumber leaves. Diffusates were later shown to contain antifungal antibiotics.

Fokkema (1973), investigating the infection of rye by Drechslera sorokiniana (Helminthosporium sativum) in the presence of rye pollen, has shown that saprophytic fungi can also inhibit the development of a fungal pathogen. The growth of a superficial mycelium of the pathogen on the leaf surface and the subsequent necrosis of the leaf were reduced in the presence of fungal saprophytes, including Aureobasidium pullulans, Sporobolomyces spp. Crytococcus spp. and Cladosporium spp. which were dominant in the phyllosphere of rye. Fokkema concluded that nutrient competition was the cause of the antagonism between fungal saprophyte and parasite. Fokkema & Lorbeer (1974) have also shown saprophytic fungal flora of onion leaves to be weakly antagonistic towards B. cinerea and Alternaria porri, but not to the onion pathogen B. squamosa.

Within an inoculum droplet germination of spores of a fungal pathogen can also be influenced by interactions with the cuticle, more particularly the epicuticular waxes of the leaf. The role of chemical substances associated with the cuticle in defence against plant disease has been reviewed by Martin (1964). Subsequent research has brought about conflicting evidence concerning the precise role of cuticles in resistance, and Martin and Juniper (1970) were reluctant to conclude that cuticles have much direct inhibitory or stimulatory effect on potential parasites. They considered that resistance might be conferred indirectly by physical effects on inoculum droplets. Heather (1967) suggested that resistance of Eucalyptus bicostata to Phaseoseptoria eucapypti was associated with both the antifungal nature of the surface wax and the limitation of deposition of inoculum resulting from the strongly hydrophobic nature of the leaf surface.

There are, however, several reports that cuticles may influence the

development of fungal pathogens if inoculum droplets are able to adhere to the leaf surface. Certain factions of surface waxes of leaves of apple (Malus domestica) have been shown to inhibit powder mildew (Podosphaera leuchotricha), (Martin, Batt & Burchill, 1957), and surface wax is thought to contribute to the antifungal properties of Ginkgo biloba (Johnston & Spronston, 1965). Venkata Ram (1962) showed that the wax of the tea leaf (Thea sinensis) contains some fractions which stimulate the growth of germ tubes of Pestalotia theae and others that completely inhibit spore germination. Blakeman and Sztejnberg (1973) have shown that germination of B. cinerea was inhibited on waxes extracted from leaves of beetroot and that germination in vivo was improved on leaves where the wax deposit had been reduced by spraying with water or by treatment with chemicals such as trichloracetic acid or ethyl N, N-dipropylthiocambamate (E.D.T.C.). Lampard & Carter (1973) found components of a chloroform extract of wax of berries and leaves of coffee (Coffea arabica) toxic to a range of fungi, including Colletotrichum coffeanum, the causal organism of coffee berry disease.

Cruickshank, Perrin & Mandryk (1977), however suggested that fungitoxic diterpenediols associated with tobacco leaf wax influenced epidermiology of blue mould (Peronspora hyoscyami) but were not involved in the resistance of tobacco to the disease. Schütt (1971) found that total wax and several wax fractions from various Pinus spp. stimulated germination and growth of spores of B. cinerea and Lophodermium pinastri. The less resistant the species from which the wax was extracted, the greater the stimulatory effect of the wax.

It is also possible that microorganisms within the inoculum droplet may modify citucular wax, causing the production of fungal stimulants or inhibitors (Rossall, 1974).

It is clear, therefore that the biological and biochemical processes occurring within inoculum droplets can lead to the inhibition of spore germination. These processes need not involve the metabolism of underlying host cells following inoculation. Reports of metabolic changes being induced in cells before penetration and affecting fungal development on the leaf surface are less common. However, Sinha & Trivedi (1969) demonstrated that fungitoxic substances accumulate in droplets of cell-free diffusates of Helminthosporium oryzae incubated on whole rice leaves and suggested that the inhibitors arose from changes induced within epidermal cells.

(b) Involvement of phytoalexins in disease resistance

The original concept of phytoalexins as antifungal principles produced by plants in response to infection was postulated by Müller & Borger (1941), who examined the production of antifungal compounds by cells of potato tubers as they underwent hypersensitivity to infection by hyphae of incompatible races of Phytophthora infestans. However, the first compound to be isolated, characterized and termed a phytoalexin was pisatin from pea (Cruickshank & Perrin, 1960; Perrin & Bottomley, 1962).

Phytoalexins were defined by Müller (1956) as "antibiotics which are the result of an interaction of two different metabolic systems, the host and the parasite, and which inhibit the growth of micro-organisms pathogenic to plants". Although several authors have proposed revisions of this interpretation (Wood, 1967; Deverall, 1972 & Ingham, 1973) a generally acceptable explicit definition of a phytoalexin is lacking. Van Etten & Pueppke (1976) applied the term phytoalexin to "antimicrobial plant metabolites which undergo enhanced

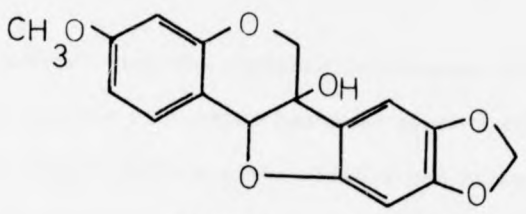
or de novo synthesis, i.e. induction, in response to microbial infection". This may be considered a commonly accepted working definition.

The involvement of phytoalexins in disease resistance has been the subject of many reviews, including those by Cruickshank (1963); Cruickshank, Biggs & Perrin (1971); Deverall (1972 & 1977); Ingham (1972) and Kuc (1972 & 1976).

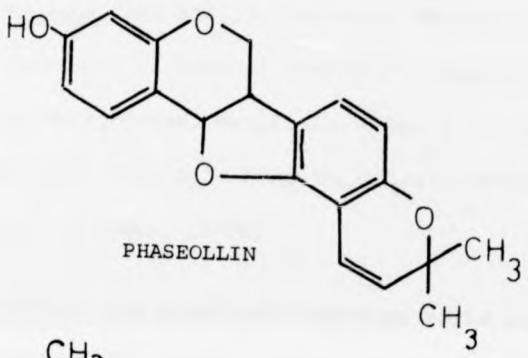
Although phytoalexins have been discovered in several plant families (Kuc, 1976; Deverall, 1977) the majority of published works concern the production of phytoalexins by the Leguminosae and the Solanaceae. Phytoalexins of the Solanaceae characterised to date are all chemically related terpenoids. The involvement of terpenoid phytoalexins in resistance has recently been considered by Kuc, Currier & Shih (1976).

Only phytoalexins of the Leguminosae will be considered here. A number of leguminous plants produce phytoalexins which are closely related to each other, having a pterocarpanoid structure (Fig.1); for example pisatin from pea (Perrin & Bottomley, 1962), phaseollin from French bean (Perrin, 1964; Cruickshank & Perrin, 1963a), glyceollin (formerly hydroxyphaseollin) from soy bean (Burden & Bailey, 1975) and medicarpin from alfalfa (Smith, McInnes, Higgins & Millar, 1971).

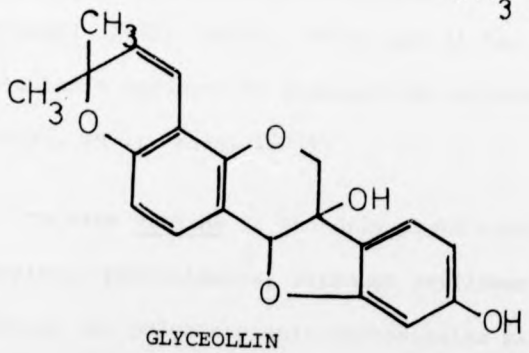
The early investigations of phytoalexin production by infected plants identified only a single compound from each plant, and attempts were made to explain resistance of a host to fungal invasion in terms of the accumulation of a single inhibitor (Bailey & Deverall, 1971; Cruickshank & Perrin, 1963b & 1971; Higgins, 1972 and Mansfield & Deverall, 1974b). Latterly, however, it has been shown that in a number of host-parasite interactions more than one phytoalexin is



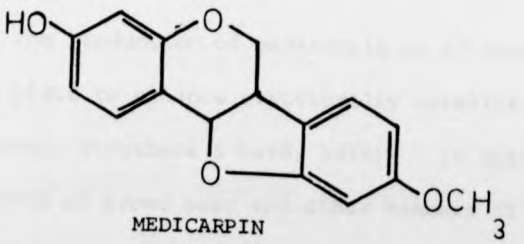
PISATIN



PHASEOLLIN



GLYCEOLLIN



MEDICARPIN

Fig.1 Structures of some pterocarpanoid phytoalexins of the Leguminosae.

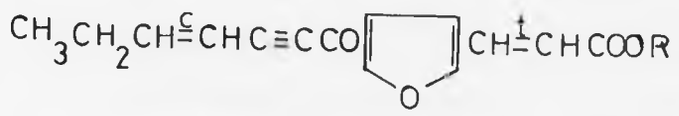
produced, and the possible involvement of a multi-phytoalexin response in disease resistance has been considered (Bailey, 1974; Pueppke & Van Etten, 1975 & 1976; Smith, Van Etten & Bateman 1975; Hargreaves & Mansfield, 1975 and Hargreaves, Mansfield & Rossall, 1977). For example, the following compounds have now been described as phytoalexins from V. faba (see Fig.2); wyerone (Fawcett et al, 1968a, 1969) wyerone acid (Letcher, Widdowson, Deverall & Mansfield, 1970); wyerone epoxide (Hargreaves, Mansfield, Coxon & Price, 1976); wyerol (Hargreaves, Mansfield & Coxon, 1976a) and medicarpin (Hargreaves, Mansfield & Coxon, 1976b).

Wyerone was originally believed to be a preformed inhibitor (Deverall, 1972; Smith, 1973), but it has now been shown to be a phytoalexin produced as a result of infection (Fawcett, Forn & Spencer, 1971; Keen, 1972).

To date V. faba is the only plant examined which produces furano-acetylenic phytoalexins, although safflower (Carthamus tinctorus) produces the polyacetylenic phytoalexins safynol and dihydrosafynol (Allen & Thomas, 1971a & b).

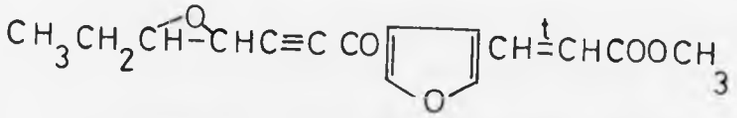
The production of medicarpin is of interest as broad bean is the only plant to produce structurally unrelated phytoalexins (Kuc, 1972; Stoessel, Strothers & Ward, 1976). It also demonstrates a common property of broad bean and other members of the Leguminosae - that is the formation of a pterocarpanoid phytoalexins (Ingham & Harborne, 1976).

The basis of the phytoalexin theory is that during a resistant reaction the inhibitors accumulate to antifungal concentrations around invading fungal hyphae, preventing their growth. During a susceptible

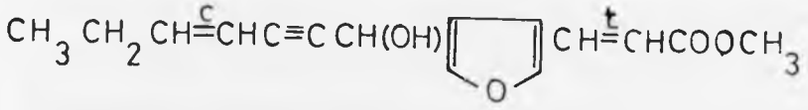


R = CH_3 — wyerone

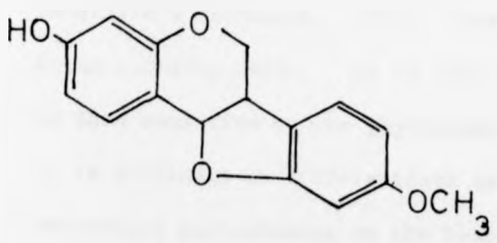
R = H — wyerone acid



wyerone epoxide



wyerol



medicarpin

Fig.2 Structures of known phytoalexins from V. faba.

response inhibitory concentrations are not achieved and consequently the virulent fungus is able to colonize the tissue.

The cause of a susceptible response may be the result of one or more of several possible host parasite interactions. Lower levels of inhibitors are often recovered from tissues undergoing such a response than from tissues exhibiting resistance (Bailey & Deverall, 1971; Higgins & Millar, 1968; Keen 1971; and Mansfield & Deverall, 1974b). Differential accumulation of phytoalexins has been explained in terms of differential induction (Keen, 1971; Varns & Kuć, 1971 and Bailey & Deverall, 1971) and differential abilities to metabolize phytoalexins which are produced to non-antifungal compounds (Higgins, 1972; Mansfield & Widdowson, 1973; Stoessel, Unwin & Ward, 1973; and Van Etten & Smith, 1975). It is also possible that a virulent parasite is less sensitive to the phytoalexins than a non-pathogen. However, it is difficult to differentiate between tolerance and ability to metabolize phytoalexins on the basis of published data, as discussed by Van Etten & Pueppke (1976). Possible differences between virulent and avirulent parasites in terms of phytoalexin production are summarized in Fig. 3.

The accumulation of phytoalexins in different tissues of V. faba following inoculation with B. cinerea and B. fabae was reported by Hargreaves (1976) and Hargreaves et al (1977). Within leaf tissue, wyerone acid was the predominant phytoalexin, achieving a level some 4-5 times that of wyerone during resistant reactions. In susceptible reactions caused by B. fabae, however, phytoalexins accumulated much more slowly over the first 2d after inoculation, then their levels decreased to zero by 3d, suggesting the virulent pathogen was metabolizing the inhibitors.

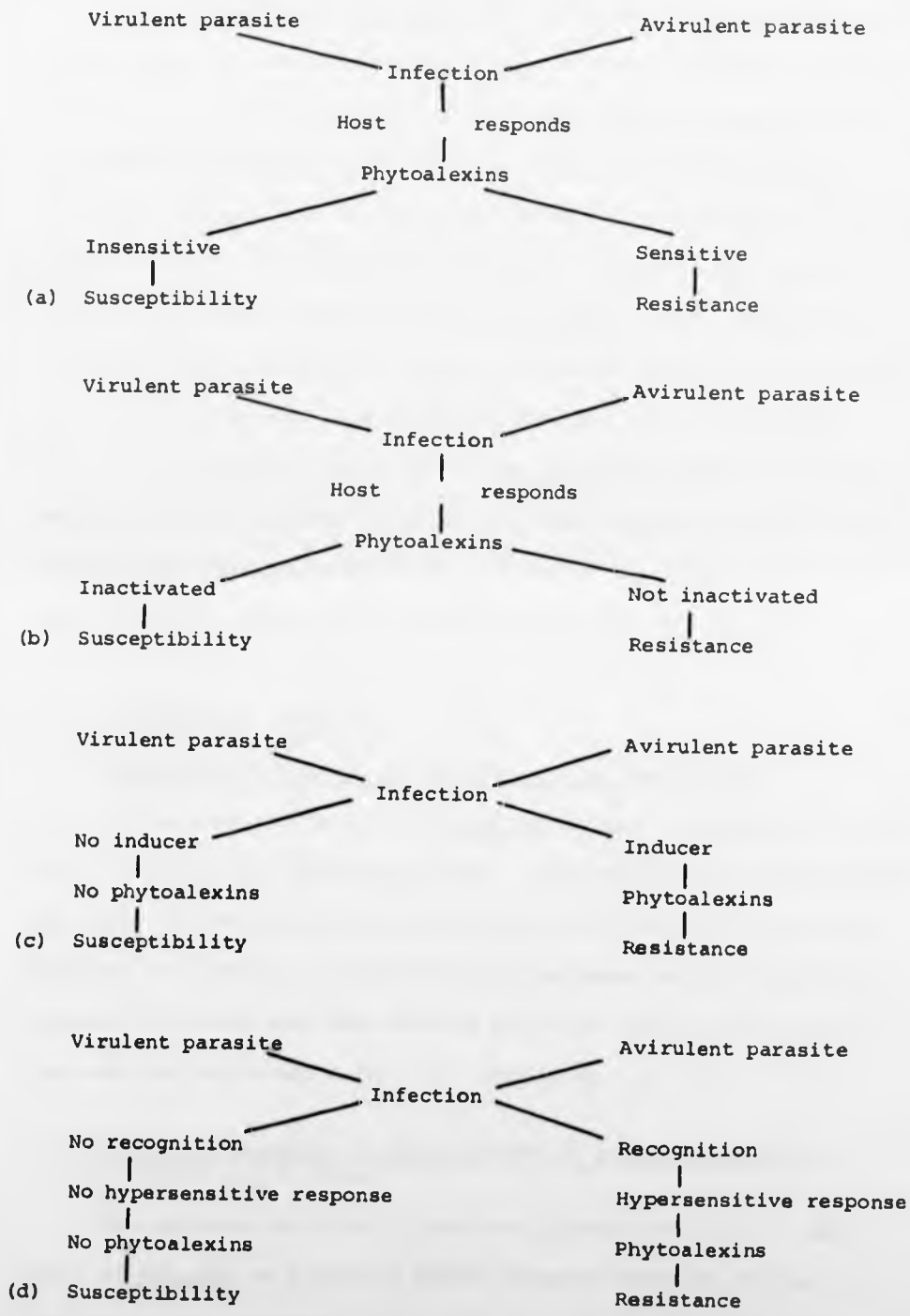


FIG.3 Possible schemes for the involvement of phytoalexins in disease resistance and susceptibility (From Wood, 1974)

Both B. cinerea and B. fabae have been shown to metabolize wyerone, wyerone acid and wyerone epoxide to less antifungal compounds in vitro (Hargreaves, Mansfield & Coxon, 1976a; Mansfield & Widdowson, 1973; Hargreaves, Mansfield, Coxon & Price, 1976). A summary of the structural changes involved is given in Fig.4. The metabolites of wyerone acid and the epoxide were shown to accumulate in spreading lesions in pod seed cavities caused by B. fabae (Hargreaves, 1976), and it was suggested that wyerone may have been detoxified after prior conversion to the acid (Mansfield & Widdowson, 1973; Hargreaves, 1976). In limited lesions caused by B. cinerea no metabolites were detected and phytoalexins accumulated to much greater concentrations. These findings suggested that the virulence of B. fabae was in some way related to its ability to metabolize phytoalexins in vivo.

(c) Objectives of research

(i) Inhibition of conidial germination on the leaf surface

The cause of the failure of Botrytis conidia to germinate on broad bean leaves has been further examined. The involvement of phylloplane epiphytic bacteria and epicuticular wax in the inhibition have been assessed, and studies on the effects of nutrients on the inhibitory processes involved have been related to the possible significance of leaf surface inhibition under field conditions.

(ii) Role of phytoalexins in the differential pathogenicities of B. cinerea and B. fabae

The antifungal activity of purified phytoalexins against germ tubes of Botrytis at a similar growth stage to infection hyphae inhibited within epidermal tissue was examined, and the relationships between tolerance and ability to metabolize the phytoalexins has been determined. More critical studies have been made of the timing of the

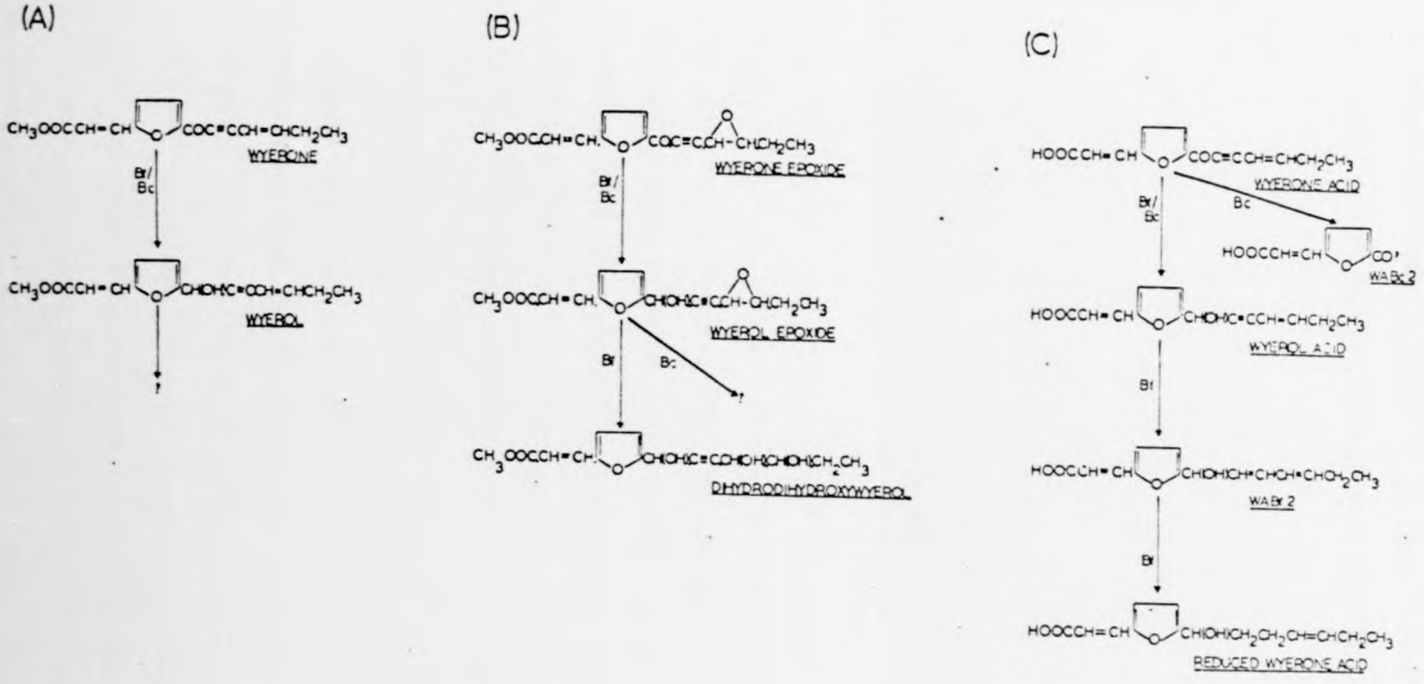


Fig.4 In vitro metabolism of wyerone (A), wyerone epoxide (B) and wyerone acid (C) by B. cinerea and B. fabae. (From Hargreaves, 1976).

cessation in growth of infection hyphae and phytoalexin accumulation in epidermal tissue and attempts were made to assess the fungitoxicity of phytoalexins both *in vitro* and *in vivo*. Preliminary studies on the mode of action of wyerone acid were also carried out.

MATERIALS AND METHODS

1. Plant Material

(a) Source of seeds

Broad bean (*Vicia faba* L.) cv. "Aquadulce claudia" was used throughout the project. Seeds were supplied by "Sutton and Sons Ltd.", Reading.

(b) Growth of plants in growth rooms

Seeds were germinated between layers of moist tissue paper at 20°C in the dark. After 3-4d seeds with an emergent radical were sown in "John Innes No.2" potting compost, in 6" diameter pots, three seeds per pot. Pots were kept on trolleys in a growth room at 20°C with a 16h photoperiod, illumination being provided by a bank of "Phillips Reflectalite" fluorescent tubes supplemented with tungsten filament lamps to give a light intensity of c. 120W/m^2 in the region of plant material. Plants were watered daily from the base of the pots to avoid damaging leaves. Four fully expanded bifoliate leaves were produced 3-4 weeks after planting.

(c) Growth of plants under field conditions for studies on leaves and pods

Seeds were sown in Stirling University research gardens from December until March to give plants for use in the following Spring and Summer. Healthy, undamaged bifoliate leaves were detached from plants by cutting the petiole near to the stem with a razor blade. Cut petioles were immediately wrapped in moist tissue paper and leaves were rapidly transported to the laboratory in plastic boxes lined with wetted paper towels. Pods were harvested from

plants as soon as the seeds had swollen. All field grown material was used as soon as possible after collection.

(d) Growth of French bean and tomato plants

Tomato (Lycopersicum esculentum) cv. "Money Maker" and French beans (Phaseolus vulgaris) cv. "Canadian Wonder" seeds were sown individually in "John Innes No.2" potting compost in 3" diameter pots. Plants were grown under conditions identical to those described for the growth of broad beans in growth rooms. Leaves were removed from plants when four fully expanded leaves developed.

2. Fungi

(a) Origin and maintenance of stock cultures

Cultures of species of Botrytis and Cladosporium herbarum were originally obtained from Dr. J. W. Mansfield. Stocks were initially maintained on slants of acidic V8 juice agar at 4°C and under sterile liquid paraffin. However, as it proved difficult to maintain pathogenicity of Botrytis cultures using these techniques, stocks were subsequently stored under liquid nitrogen. Aliquots of c. 1 ml of suspension of mycelial fragments and conidia in 10% (v/v) glycerol were injected into glass ampoules and sealed. Ampoules were stored in a "Vivostat" (British Oxygen Company) and a fresh ampoule from the stocks was used for each subsequent subculture of strains onto agar.

(b) Production of conidia

Conical flasks (250 ml) containing c. 40 ml of the appropriate medium were inoculated with the fungus after the surface of the agar had been wetted with sterile distilled water (SDW). Fungus was introduced as a small piece of agar containing sporulating

myceli um or as a suspension from a thawed liquid nitrogen storage ampoule. The fungus was spread uniformly across the surface of the medium by gentle shaking. Cultures were grown at 20°C under "Phillips, Black Light" fluorescent tubes with an emission spectrum of 310-410 nm and a maximum at 360 nm, with a 16h photoperiod.

(c) Preparation of conidial suspensions

Suspensions of Botrytis conidia were prepared from 10-14d old cultures. About 20 ml SDW was added to flasks containing the sporulating culture and conidia were released into suspension by scraping with a glass rod. Mycelial debris was removed by filtering through four layers of sterile muslin. The suspension was collected and washed twice with SDW following centrifugation at 1000g for two minutes. The concentration of conidia was estimated by haemocytometer counts and a suspension of required concentration made by appropriate dilution. Unless stated otherwise for specific experiments, concentrations of 5×10^5 and 10^5 conidia/ml were prepared for experiments on leaf surface inhibition and studies involving phytoalexins respectively. Where conidia were to be suspended in a medium other than water, volumes of standard suspension were centrifuged prior to resuspension in the appropriate media. Isolates of B. cinerea and B. fabae routinely used were identified as Bc 39 and BF PBI6, and Bc Vn 2 and BF VF 2 for leaf surface and phytoalexin studies respectively.

3. Culture media

(a) Medium X (Last and Hamley, 1956)

Content:

(i)	Glucose	10g
	Mycological peptone	2g
	Casein hydrolysate (acid)	3g
	$KH_2 PO_4$	1.5g
	$Na NO_3$	6g
	KCl	0.5g
	Yeast nucleic acids	0.5g
	Distilled Water	200 ml
(ii)	$Mg SO_4 . 7H_2 0$	0.5g
	Distilled water	100 ml
(iii)	Oxoid agar No.3	30g
	Distilled water	700 ml

Preparation: The solutions (i) and (ii) were prepared whilst (iii) was placed in a boiling water bath. When the agar had dissolved the three solutions were mixed. Aliquots (40 ml) were disposed into 250 ml conical flasks which were sealed with cotton wool bungs before autoclaving for 20 min. at 1 Kg cm^{-2}.

(b) Acid V8 Juice Agar

Content:

V8 Juice (Campbell's Soups Ltd.)	200 ml
Distilled water	800 ml
Oxoid agar No. 3	20g

Preparation: The agar and distilled water were placed in a boiling water bath until the agar had dissolved, the V8 Juice was

added and the pH of the mixture was adjusted to 5.0 with 1M NaOH solution. Ten ml aliquots were dispensed into McCartney bottles and autoclaved as described above. The bottles were allowed to cool at an angle to give slants for maintenance of stock cultures.

(c) Synthetic pod nutrients (SPN)

Content:

Sucrose	5g
Casein hydrolysate (acid)	380mg
KH_2PO_4	100mg
$MgSO_4.7H_2O$	50mg
Distilled water	1 litre

Preparation: The pH of the mixture was adjusted to the desired value either by addition of galacturonic acid or by mixing with galacturonate buffer as described below. After being dispensed into appropriate bottles the mixture was autoclaved in the usual way.

(d) Galacturonate buffered SPN

A 20mM solution of galacturonic acid was titrated against 20mM NaOH to give a 10mM solution of sodium galacturonate. This salt was mixed with 10mM galacturonic acid (pK c. 3.3) to give a 10mM galacturonate buffer of desired pH. The resulting buffer was then added to double strength SPN to give SPN buffered with 5mM galacturonate buffer. The pH to which the buffer had to be adjusted to give the required final SPN pH was determined experimentally. For example galacturonate buffered SPN at pH 4.0 required mixing of equal volumes of double strength SPN and 10mM buffer at pH 3.8.

4. Chemicals

All chemicals used were of analytical grade whenever possible. Organic solvents (supplied by British Drug Houses Ltd. (BDH) and Fisons Ltd.) used in tissue extraction were redistilled before use as some batches of diethyl ether (Et_2O) were found to contain antifungal substances.

Spray reagents for thin layer chromatography (TLC) were prepared according to the methods described in 'Dyeing Reagents for Thin Layer and Paper Chromatography' produced by E. Merck Ltd., Darmstadt, W. Germany. Sprays used are summarized below.

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

Spray solution : 0.4% (v/v) 2,4-DNP in 2M HCl

Treatment: Above solution sprayed consecutively with 0.2% (v/v) solution of potassium hexacyano perate III in 2M HCl. After spraying saturated ketones show blue colour immediately and saturated aldehyde derivatives show an olive green colour more slowly. Unsaturated carbonyl derivatives change only slowly or not at all.

(ii) Lead (IV) acetate-rosaniline

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

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

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

(iii) Picric acid

Spray solution : 0.05M ethanolic picric acid solution.

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

(iv) Rhodamine 6G (Halloway & Challen, 1966)

Spray solution : 1% aqueous rhodamine 6G.

Treatment: Above solution sprayed onto plates. Compounds appeared as deep pink spots on pale pink background. Used to locate constitutents of extracted wax.

(v) Universal indicator (BDH)

Spray solution : Undiluted indicator.

Treatment: Sprayed directly onto chromatograms, acidic groups appeared pink on green background.

(vi) Vanillin - sulphuric acid

Spray solution : vanillin (3g) was dissolved in 100ml EtOH containing 0.5ml concentrated sulphuric acid.

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

5. Measurement of pH

Measurements were made with a Pye Unicam PW of 418 pH meter fitted with a Radiometer GK 2321C semi-micro electrode.

6. Determination of pKa of wyerone acid

The pKa of the phytoalexin wyerone acid was determined by Dr B. Cox, Chemistry Department, Stirling University. Measurements were made by recording the shift in λ maximum of the UV absorption spectrum of the dissociated and undissociated forms of the acid at different pH values.

7. Spectral analysis

Ultraviolet (UV) spectra were obtained on a Pye Unicam SP1800 spectrophotometer. Analysis of potassium, magnesium and sodium concentrations in solutions was carried out using a Perkin Elmer 103 atomic absorption spectrophotometer. Mass spectral analysis was carried out by Dr. D. Dance, Chemistry Department, Stirling University, using a Jeol JMS-D100 mass spectrophotometer.

8. Extraction and redeposition of leaf surface waxes

Chloroform has been widely used as a solvent for wax extraction since it was shown to remove wax effectively from the surface of leaves (Fernandes, Baker & Martin, 1964). Fully expanded bifoliate leaves were detached from growth room grown plants and dipped in chloroform ($CHCl_3$) for one second only to minimise the possibility of co-extraction of cellular components of the leaf together with epicuticular wax. For each leaf extracted 1 ml of solvent was used. Wax solution was used immediately or stored at -20°C in the dark.

Several methods of wax redeposition were developed for studies on its antifungal properties.

(a) Watch glass redeposition

Wax solution (5 ml) was pipetted onto a clean watch glass and

the solvent allowed to evaporate slowly in a fume cupboard. Dishes were inoculated only when the characteristic smell of $CHCl_3$ was no longer detectable.

(b) Sprayed glass slide redeposition

Aliquots (2 ml) of wax solution were sprayed onto glass slides with an atomiser spray powered by oxygen free nitrogen (OFN) at 1 Kg/cm^2 from a distance of 6-8". The solvent rapidly evaporated on contact with the slide, leaving a uniform layer of redeposited wax. Slides were placed in a vacuum desiccator for 2h before use to ensure complete removal of $CHCl_3$.

(c) TLC plate redeposition

Precoated cellulose TLC plates (Merck, 5781) were cut to the size of the microscope slides with a diamond glass cutter and sprayed with wax solution as described above.

(d) Glass fibre disc redeposition

A modified version of the technique described by Jeffree, Baker & Holloway (1975) was used. The apparatus required was constructed as illustrated in Fig.6. Wax solution was slowly poured into the sealed apparatus through the glass fibre disc (Whatman, GF/C) to ensure the wick was fully saturated. The apparatus was then placed in a fume cupboard, where the draught aided the evaporation of the solvent. After the evaporations of 5 ml of solution from the graduated glass jar, the disc was removed and placed in a vacuum desiccator for at least 12h to ensure complete removal of solvent.

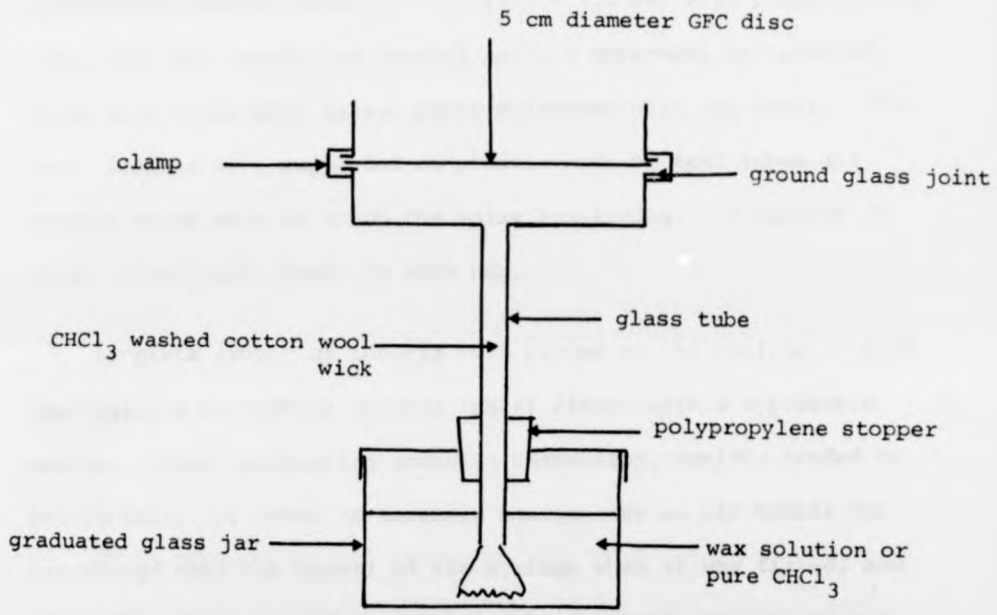


Fig.6 Apparatus for wax redeposition.

9. Inoculation techniques

(a) Growth room grown leaves

The first to fourth fully expanded bifoliate leaves were cut from plants with a sharp razor blade. Cut petioles were immediately wrapped in moist tissue paper. Detached leaves were placed inside transparent plastic boxes (17.5 × 11.5 × 5.0 cm) with tight fitting lids, with the abaxial or adaxial surface uppermost as required. Boxes were lined with tissue paper moistened with tap water. The leaf laminae were supported on plastic mesh or test tubes and petiole wicks made to touch the moist box lining. A maximum of three leaves were placed in each box.

Droplets (20µl) of inocula were placed on the surface of each leaf using a micrometer syrings (Agla) fitted with a hypodermic needle. When inoculating conidial suspension, conidia tended to settle out. In order to maintain homogeneity an air bubble was introduced into the barrel of the syringe when it was filled, and after five droplets had been applied to the leaf the syringe was inverted so that the air bubble stirred the suspension. The number of droplets per leaf varied in individual experiments.

When large volumes of diffusate were required, leaves were inoculated using a sterile pasteur pipette, the use of a micrometer syringe being too time consuming. As many droplets as possible were placed on each leaf, the limiting factor being that if too many droplets were placed on the leaf they coalesced and tended to run off. Leaves were incubated in a growth cabinet at 18 ± 1°C and illuminated for 16h each day by a bank of fluorescent tubes.

(b) Field grown leaves

Leaves, transported to the laboratory as described earlier, were carefully examined for signs of damage and soil contamination. Damaged leaves were discarded and contaminated leaves gently washed in a stream of distilled water and blotted dry with tissue paper. Arrangements in plastic boxes and subsequent inoculation and incubation was as described for growth room grown material, except that, being larger only one or two leaves were placed in each box.

(c) Glassware and fibreglass discs

Glass slides, watch glasses and fibre glass discs were supported as necessary on test tubes and plastic mesh in plastic boxes lined with moist tissue paper. Inoculation and incubation was as described for leaves.

(d) Cotyledons

Broad bean seeds were germinated for 36-48h 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 (abaxial) side downwards on plastic mesh over moist tissue paper in large plastic boxes. Cotyledons bearing signs of bacterial rot and discolouration were discarded. Cotyledons were then inoculated by flooding the upper surface with inocula with the aid of a pasteur pipette or a "Shandon" laboratory spray gun. Inoculated tissues were incubated at 18°C in the dark.

(e) 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 with a sharp razor blade, exposing the seed cavities.

Seeds were discarded and the half pods cut into pieces bearing two or three seed cavities. Larger segments tended to curl up on incubation. Before inoculation seed cavities which showed signs of natural infection or which had little endocarp tissue were discarded. The half pod pieces were placed on moist tissue paper in large plastic boxes and inoculated using a sterile 10 ml pipette. Approximately 0.3 ml of inocula was placed in each cavity, and the tissues were incubated in sealed boxes at 18°C in the dark.

10. Measurement of infection on leaves

A pictorial key to the system used is given in Figure 7 (from Mansfield, 1972). Allocation to grades was made on the basis of the percentage browning/blackening of the leaf tissue under the inoculum droplet. Lesions spreading beyond the area of the droplet were graded on the extent of spread beyond the infection site.

11. Preparation of leaf material for light microscopy

(a) Examination of growth on the leaf surface

Leaf discs bearing inoculum droplets were cut from detached leaves and bleached with chlorine gas generated by the oxidation of concentrated HCl by $KMnO_4$ crystals. Ammonia vapour was blown over the bleached discs to neutralise excess chlorine (Janes, 1962). A drop of cotton blue lactophenol stain was added to each inoculum droplet and a cover slip put in place. This method rendered conidia clearly visible for microscopical examination without dislodging them from the leaf surface.

(b) Examination of growth within epidermal cells

Shallow cuts in the form of a square immediately around an

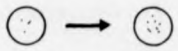
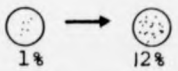
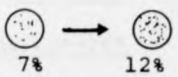
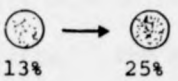
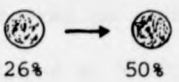
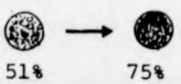
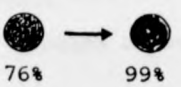
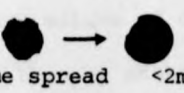
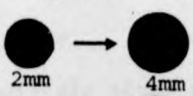
Grade boundaries	Grade mean
0%	0
 1% 6%	3.5
 1% 12%	6.5
 7% 12%	9.5
 13% 25%	19
 26% 50%	38
 51% 75%	63
 76% 99%	87.5
100%	100
 some spread <2mm ring	S
 2mm 4mm ring	S_1
4mm 6mm ring	S_2
>6mm wide ring of browning beyond the infection site	S_3

Fig.7 Grading system for measurement of infection on leaves.

inoculum droplet on a leaf were made with a sharp razor blade. With care it was then possible to peel off the epidermis underlying the droplet using fine curved forceps. Abaxial epidermis was more easily removed. Detached tissue was mounted on a glass slide in water for microscopical observation of growth of infection hyphae within the epidermis and visible cellular responses by the plant.

12. Preparation of diffusates

(a) Leaf diffusates

Droplets were collected from the leaf surface using a sterile pasteur pipette. The bulked droplets, henceforth termed diffusate, were transferred to a centrifuge tube surrounded by crushed ice, and centrifuged at 1000g for two minutes to remove any conidia and leaf surface debris which were present. Bacteria (including Sporobolomyces spp.) were removed from diffusates when required by filtration through a membrane filter (13 mm diameter, pore size 0.45 µm) held in a "Swinny adaptor" (Millipore Corporation). Bacteria were recovered from the filter by passing a volume of SDW, equal to the original volume of diffusate, through the filter in the reverse direction, then by shaking the membrane in the resulting suspension for one minute. All diffusates and bacterial suspensions were used immediately after collection.

(b) Diffusates from glass

Droplets were collected from glassware and fibreglass discs and treated as described for diffusates from leaves.

(c) Pod diffusates

With small volumes of diffusates, inoculum droplets were collected from pod seed cavities using a pasteur pipette; however, large volumes

were collected under suction into a Buchner flask surrounded by crushed ice. After collection the diffusate was either used immediately or stored at -20°C in the dark.

Throughout all collections, and subsequent extraction procedures described, illumination was kept to a minimum.

13. Analysis of nutritional status of diffusates

(a) Total carbohydrate

Determination of total carbohydrate in diffusates was made by measuring the colour reaction given with anthrone reagent (Hewitt, 1958). Anthrone reagent was made up, containing 0.2g anthrone, 8 ml EtOH, 30 ml distilled water and 100 ml concentrated H_2SO_4. Aliquots (1 ml) of diffusates were mixed with 10 ml aliquots of anthrone reagent and heated for seven minutes in a boiling water bath. After cooling absorbance was measured at 620 nm. Concentrations of total carbohydrate were expressed as µg glucose/ml of solution following comparison with a glucose calibration curve.

(b) Total amino acid

The level of total amino acid in diffusates was measured using the colour reaction given with ninhydrin (Rosen, 1957). Samples (1 ml) of diffusate were added to 0.5 ml cyanide-acetate buffer (pH5.0-5.2) and 0.5 ml 3% (w/v) ninhydrin solution in methyl cellosolve. After heating for 15 minutes in a boiling water bath absorbance was measured at 570 nm following suitable dilution with isopropyl alcohol : water (1:1, v/v). Concentration of total amino acid in diffusate was expressed as µg valine/ml following comparison with a valine calibration curve.

14. Preparation of tissue for extraction

(a) Leaf epidermal strips

Strips of epidermis were peeled from the leaf underlying inoculum droplets as described earlier. Excised strips were collected in a beaker of known weight cooled on crushed ice. The total weight of tissue was calculated before extraction.

(b) Pod endocarp

Pod endocarp tissue was removed from previously inoculated pod segments by scraping it out with a small spatula. It was collected in a beaker of known weight, cooled on ice. Tissue collected was extracted immediately or stored at -20°C in the dark.

(c) Cotyledons

Infection sites were sliced from inoculated cotyledons with a razor blade and stored as described for pod endocarp tissue.

As described for diffusates, throughout all tissue collections and extraction illumination was kept to a minimum.

15. Extraction procedures

(a) Diffusate

All diffusates were extracted by partitioning with diethyl ether (unless stated otherwise) by shaking three times with twice the volume of solvent. Large volumes (> 10 ml) were extracted in a separating funnel whereas small volumes were partitioned in a test tube after agitation with a pasteur pipette. If the residual water phase was required for antifungal activity determinations, excess solvent was removed by bubbling through a stream of OFN until the ethereal smell was no longer detectable.

(b) Tissue

(i) Leaf tissue

The small quantities of leaf tissue collected for phytoalexin estimation were extracted by grinding four times in diethyl ether (Et_2O) (1:50, w/v) with a glass homogeniser (MSE). A maximum of 0.2g of tissue was extracted on each occasion and care was taken to keep the extracts as cool as possible.

(ii) Cotyledon and pod tissue

Cotyledon and pod tissue were extracted for bulk collection of phytoalexins. Excised infected cotyledon tissue was initially coarsely ground for 5 seconds in an omnimixer (Sorval) at half speed. Conical flasks (500 ml) were then approximately one third filled with the ground tissue, which was covered with Et_2O. Flasks were placed on an orbital incubator at 4°C and 60 r.p.m. for a minimum of 12h. The supernatant ether was carefully decanted off and replaced with a similar volume of fresh solvent. After shaking for a further one hour, flasks were placed in a freezer at -20°C. When the tissue became completely frozen, the liquid ether phase was bulked with the original extract. Extraction was carried out with c. 10 ml Et_2O/g of tissue.

Infected pod tissue was extracted after first grinding in Et_2O (1:1, w/v) in an omnimixer for three 15 second bursts at half speed. The resultant slurry was shaken three times with twice its volume of ether in a separating funnel and the ether collected. After the third extraction the residual water phase was frozen to remove remaining ether to add to the extract.

All collected ether extracts were dried in vacuo at 25°C in a

rotary evaporator (Büchi, Rotavap R). Traces of water present in extracts were evaporated following the addition of a little EtOH and dried extracts were redissolved in a suitable solvent for bioassays or further purification.

16. Chromatography

(a) Thin Layer Chromatography (TLC)

All analytical TLC was carried out using precoated plates (Merck, silica gel 60 F_{254}, 0.25mm thick). Extracts were applied to chromatograms in MeOH or $CHCl_3$ as necessary using drawn out pasteur pipettes. Plates were developed in solvent systems described in individual experiments, after which they were dried and bands located by spray reagents or examination under UV light at 254 or 366nm (Camag Universal Lamp). Bands detected were marked with pencil. Located phytoalexins were eluted with MeOH or $CHCl_3$ after being carefully removed from the plate with a spatula. Recovered silica gel was suspended in solvent shaken with a "Whirlimixer" when centrifuged at 1000g for 5 minutes. Concentrations of phytolexins in the resultant supernatant could be estimated by UV absorption spectrophotometry by reference to published molecular extinction coefficients for the compounds as described later.

(b) Preparative layer chromatography (PLC)

Preparative chromatography was carried out on plates coated in the laboratory. Glass plates (20 × 20 cm) were thoroughly washed in hot detergent solution (Teepol) then rinsed with tap water and dried. The plates were loaded onto a "Shandon" Unoplan leveler model P. Silica gel ("Merck", GF_{254} type 60) was weighed into a conical flask and mixed with distilled water (1:2, w/v). The

mixture was shaken vigorously for 90 seconds and the slurry spread onto the plates as a layer 1-1.5 mm thick within 15 seconds. After 5 minutes the plates were transferred to a drying rack and the rack placed horizontally in an oven at 110°C for 10 minutes. The plates were finally activated by storage in the oven for a further 50 minutes in the vertical plane. The plates were used the same day or stored in a desiccator.

Methanolic extracts were applied to the plates with a pasteur pipette fitted with a small cotton wool wick to avoid damage to the delicate surface of the absorbent layer. After development in a suitable solvent system bands were detected using UV light as described for TLC, and marked with a sharp pointer. Elution was carried out using MeOH or $CHCl_3$ depending on the polarity of the compound. Silica gel was removed from the plates by filtering through cotton wool.

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

(c) Gel filtration

Gel filtration was carried out on a 2.5 × 75 cm column packed with "Sephadex LH20" in MeOH. Extracts (from c.100g FW tissue) were applied to the top of the column in 2 ml MeOH, and the flow rate adjusted to 2 ml/minute. Separation of components of the extract was monitored using an "LKB uvicord 4700" uv absorptiometer at 254 nm and a flat bed recorder (Servoscribe 1s). Fractions were collected at 10 minute intervals with an "LKB 7000" fraction collector. Further separation of constituents of individual fractions was carried out using TLC or PLC.

17. Summary of purification procedures for phytoalexins and phytoalexin metabolites

(a) Phytoalexins

Milligram quantities of phytoalexins were purified from cotyledon and pod tissue extracts for the preparation of stock solutions.

(i) Wyerone

Cotyledon tissue (c. 100g) collected 6d after inoculation with B. fabae, was extracted with Et_2O and applied to the top of a column of "Sephadex LH20" for gel filtration in MeOH. Fractions containing wyerone were collected from 2 hours 50 minutes until 3 hours 20 minutes after application, bulked and dried down in vacuo at 25°C. This semi-purified extract was loaded onto PLC plates in MeOH (1g FW tissue/cm plate) and plates were devloped in Hexane:Acetone (2:1, v/v) and $CHCl_3$: Petroleum spirit (60-80° BR)(2:1, v/v). Wyerone was identified as a large band, fluorescing blue (366 nm), at R_F c. 0.6. Precise measurement of R_F values was especially difficult on preparative plates as compounds tended not to run as fine bands and some variation between batches of plates was unavoidable. The centre of the wyerone band only was collected and eluted with $CHCl_3$ and redissolved in a known volume of MeOH.

(ii) Wyerone epoxide and wyerol

Procedures for the purification of wyerone epoxide and wyerol were identical to wyerone. Wyerone epoxide was recognized on chromatograms as a small blue fluorescent (366 nm) band immediately below wyerone at R_F c. 0.5. Following elution of this band contaminating wyerone was removed by repeated development (up to three times) of fresh PLC chromatograms in $CHCl_3$: Petroleum spirit (2:1, v/v). Pure wyerone epoxide was detected below contaminating

wyerone and eluted with $CHCl_3$ before redissolution in MeOH.

Wyerol was identified on the original chromatograms as a non-fluorescent band which quenched background fluorescence (254 nm) at R_F c. 0.4. Elution was carried out with $CHCl_3$.

(iii) Wyerone acid

Wyerone acid was purified from an extract of pod endocarp tissue collected 5d after inoculation with B. cinerea. The extract was applied to the "Sephadex" column for gel filtration in MeOH. Fractions containing wyerone acid were collected from 3h until 3h 30 minutes after application, then applied to PLC plates and developed as described for the purification of wyerone. The acid was identified on chromatograms as a blue fluorescent band (366 nm) as R_F c. 0.05. After elution in MeOH wyerone acid was reapplied to PLC plates (2g FW tissue/cm plate) for further purification by development in Et_2O:MeOH (6:1, v/v). The phytoalexin was eluted from plates in MeOH as the centre of a large blue fluorsecent band (366 nm) at R_F c. 0.3.

(iv) Medicarpin

Medicarpin was obtained from the same extract as wyerone acid. Fractions containing medicarpin were collected from the "Sephadex" column from 6h until 6h 30 minutes after application and bulked fractions were applied to PLC plates in MeOH. The phytoalexin was recovered as a dark quenching band (254 nm) at R_F c. 0.3 after two repeated developments in $CHCl_3$. Elution was carried out using MeOH.

A phytoalexin was considered pure if it ran as a single spot when developed an analytical TLC plates and if its UV absorption spectrum corresponded with the known spectrum of the compound

(Hargreaves, 1976). Spectra of wyerone, wyerone acid, wyerone epoxide, wyerol and medicarpin are given in Figs. 8-12 respectively.

(b) Phytoalexin metabolites

(i) Reduced wyerone acid

Reduced wyerone acid was purified from an ether extract of pod endocarp tissue (c. 200g) 5d after inoculation with B. fabae. The extract was applied to the "Sephadex LH20" column in MeOH. Fractions containing reduced wyerone acid were collected from 5h until 5h 30 minutes after application and subjected to PLC. Plates were developed in Et_2O MeOH (8:1, v/v) and reduced wyerone acid was recognized as a dark quenching band (254 nm) at R_F c. 0.4. The band was eluted with MeOH.

(ii) Wyerol epoxide and dihydrodihydroxy wyerol

Wyerol epoxide and dihydrodihydroxy wyerol were prepared from wyerone epoxide by in vitro metabolism by mycelium of B. cinerea and B. fabae respectively. Aliquots (10 ml) of wyerone epoxide (25 µg/ml) in SPN were added to 40 conical flasks (50 ml). Twenty flasks each were inoculated with a 0.5 cm disc of agar bearing speculating mycelium of B. cinerea or B. fabae. Flasks were incubated in the dark at 18°C on an orbital incubator at 120 rpm. Loss of the phytoalexin was monitored by UV spectrophotometry against an SPN blank, reduction in absorption at 347 cm corresponding to disappearance of the inhibitor. Reduction in absorption at 347 nm closely paralleled an increase at 310 nm, and when no further reduction was observed (24h with B. fabae and 48h with B. cinerea) the contents of the flasks were extracted with Et_2O. Extracts were redissolved in MeOH and subjected to PLC. Plates were developed in Hexane:Acetone (2:1, v/v). Wyerol epoxide was recognised in the B. cinerea

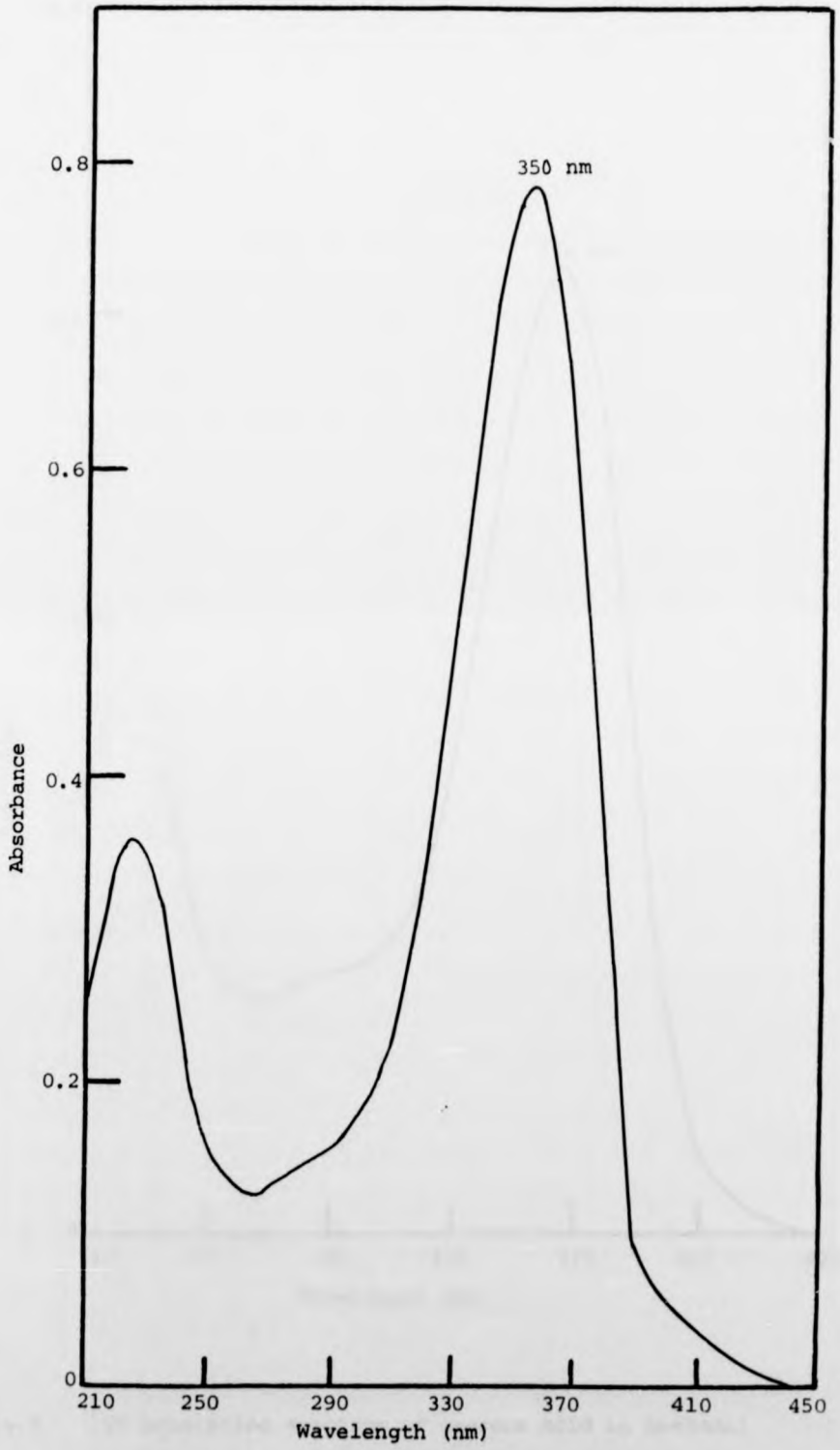


Fig.8 UV absorption spectrum of wyerone in methanol

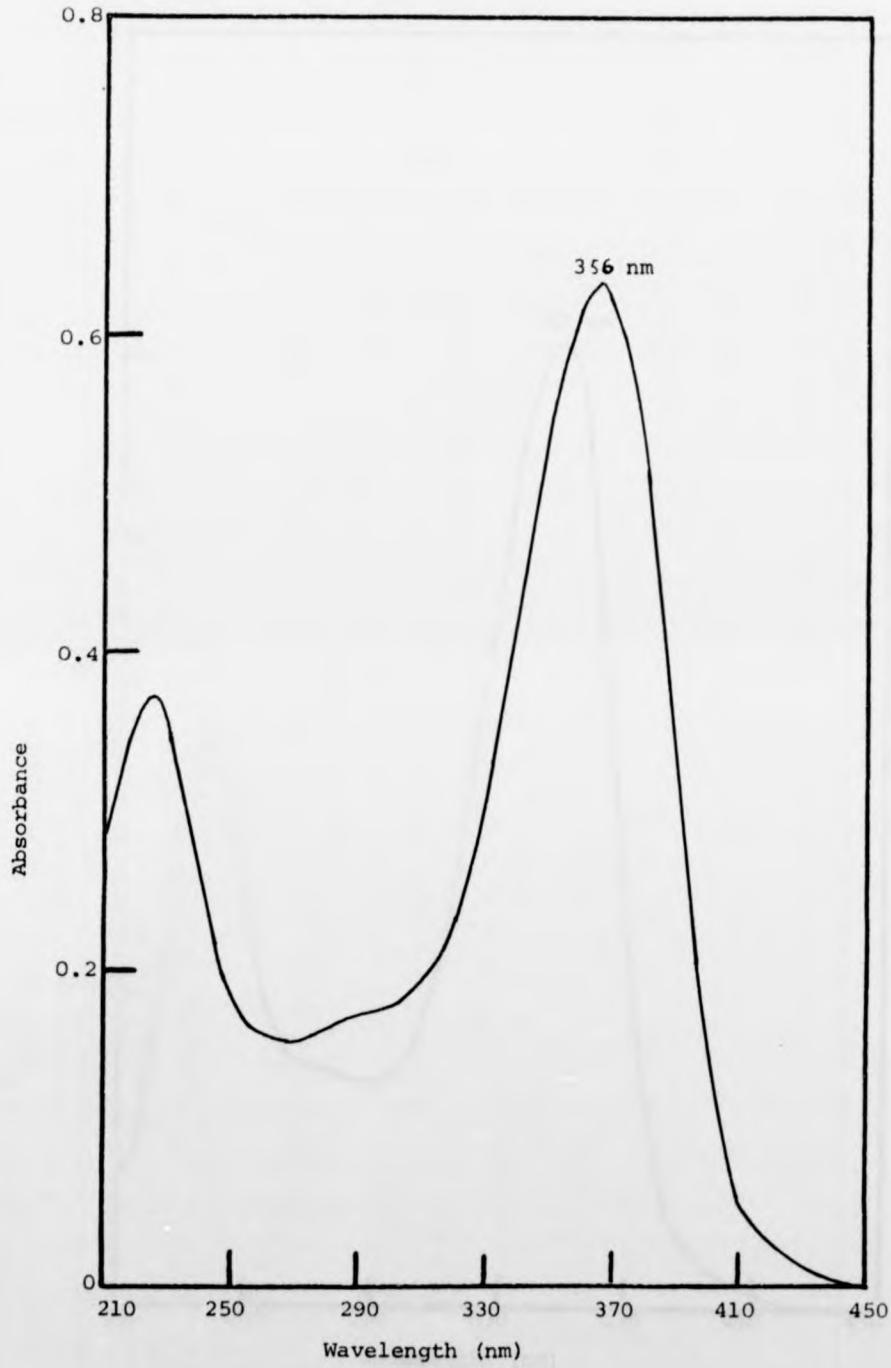


Fig.9 UV absorption spectrum of wyerone acid in methanol

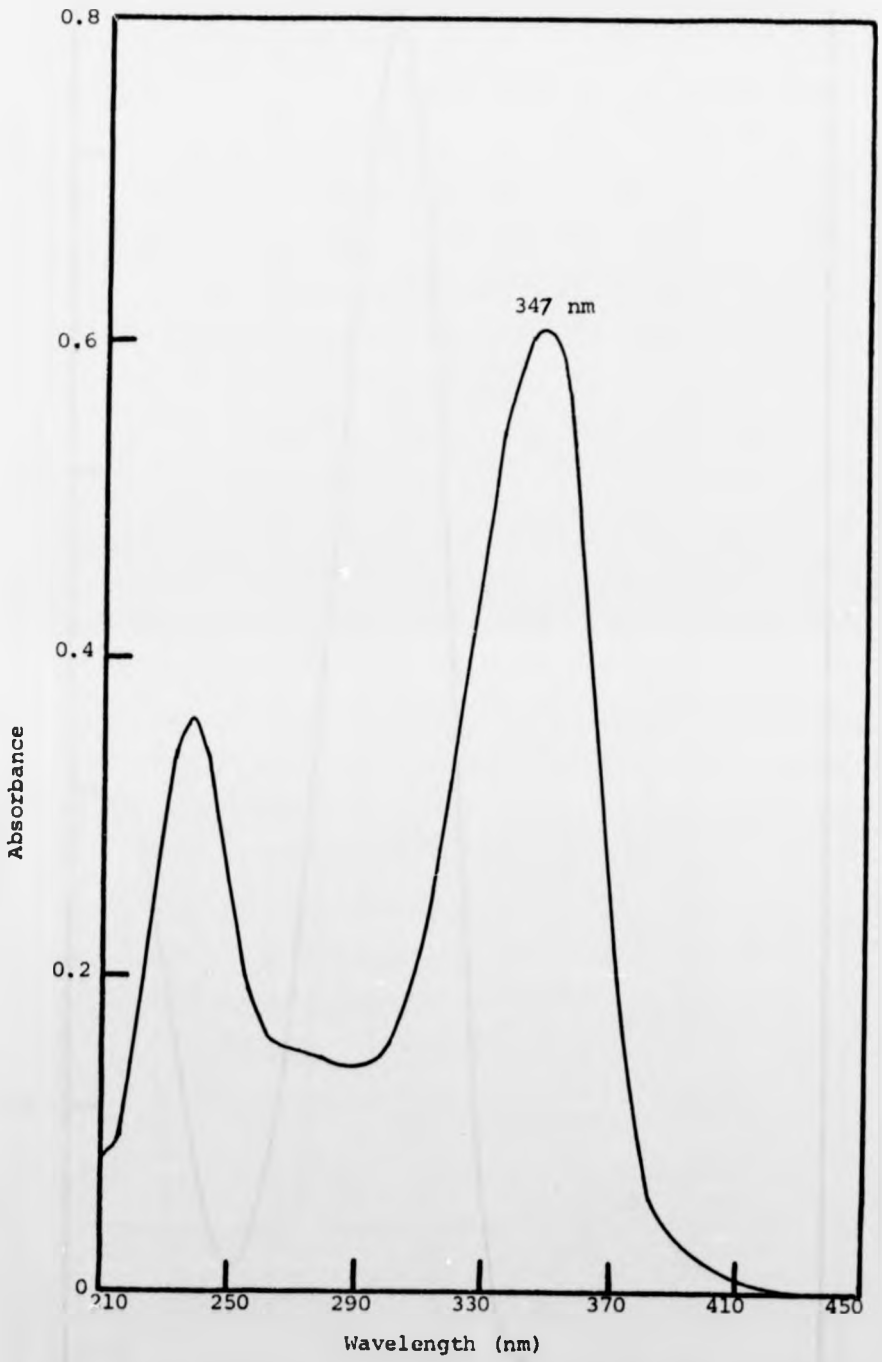


Fig.10 UV absorption spectrum of wyerone epoxide in methanol

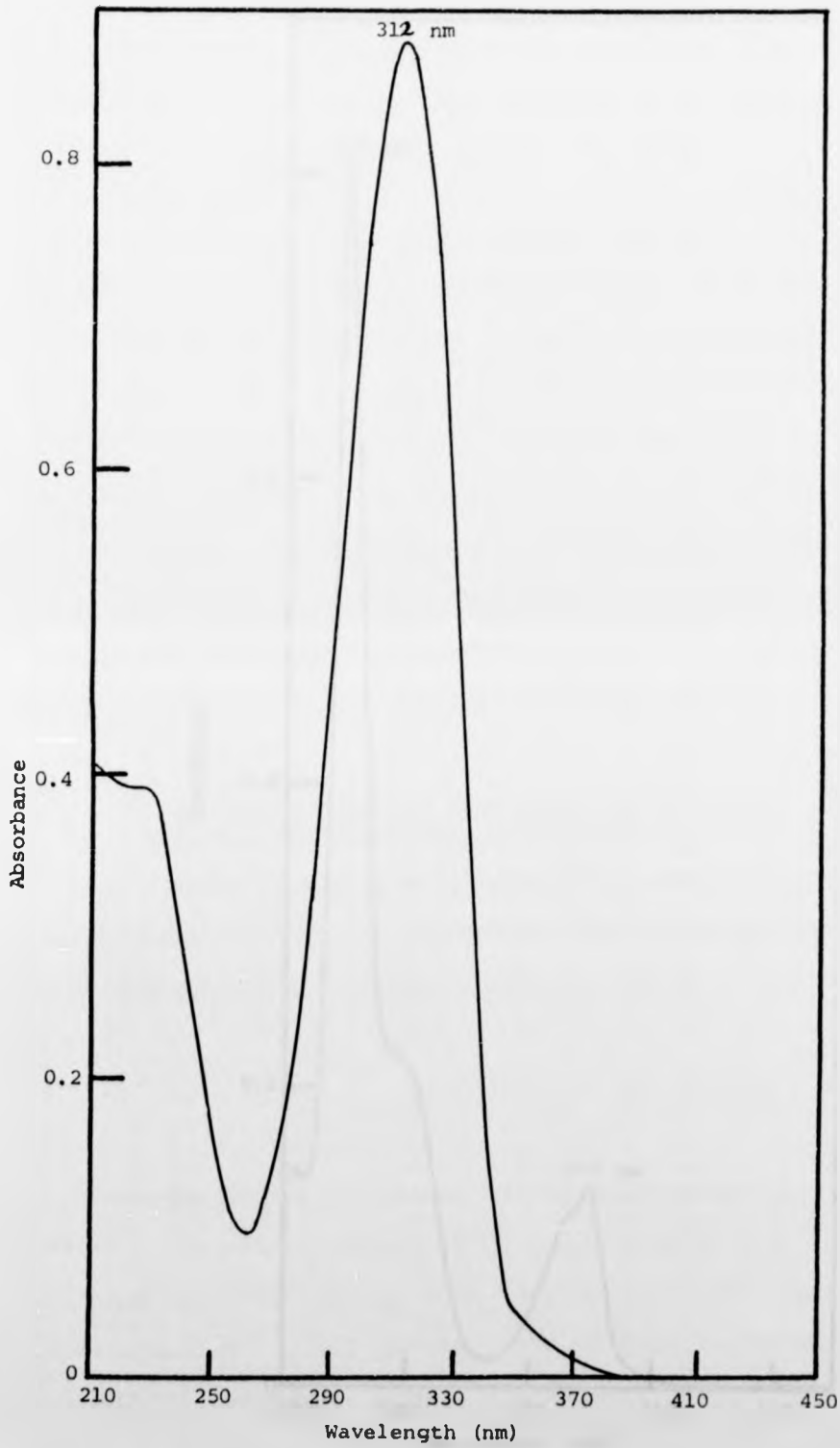


Fig.11 UV absorption spectrum of wyerol in methanol

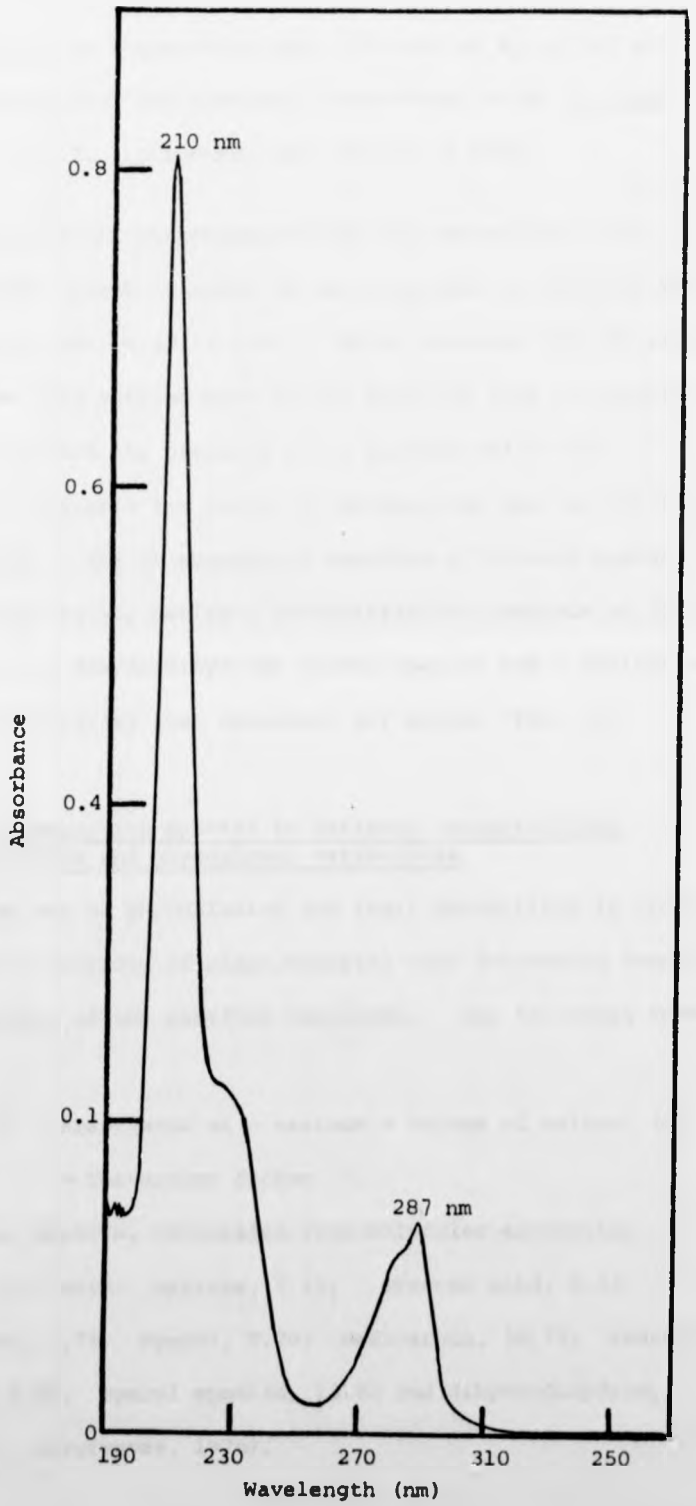


Fig.12 UV absorption spectrum of medicarpin in methanol

metabolism extract as a quenching band (254 nm) at R_F c. 0.5 and dihydrodihydroxy wyerol was similarly identified in the B. fabae extract at R_F c. 0.2. Compounds were eluted in MeOH.

Following elution, structures of the two metabolites were confirmed by development of spots of the compounds on analytic TLC plates in Hexane:Acetone (2:1, v/v). After location with UV radiation (254 nm) plates were sprayed with picric acid and lead IV acetate-rosaniline to confirm the presence of an epoxide and a diol respectively. Criteria for purity of metabolites were as described for phytoalexins. The UV absorption spectrum of reduced wyerone acid is given in Fig.13, having a characteristic λ maximum at 300 nm. Wyerol epoxide and dihydrodihydroxy wyerol spectra had λ maximum at 310 nm, being similar to that described for wyerol (Fig. 11).

18. Use of UV absorption spectra to estimate concentrations of phytoalexins and phytoalexin metabolites

Concentrations of phytoalexins and their metabolites in stock solutions and in extracts of plant material were determined from UV absorption spectra of the purified compounds. The following formula was used:

Yield (μg) = Absorbance at λ maximum × Volume of solvent (ml) × Conversion factor

Conversion factors, calculated from molecular extinction coefficients (ε), were: wyerone, 9.55; wyerone acid, 9.04; wyerone epoxide, 9.75; wyerol, 9.29; medicarpin, 38.78; reduced wyerone acid, 9.80; wyerol epoxide, 13.80 and dihydrodihydroxy wyerol, 16.30, (Hargreaves, 1976).

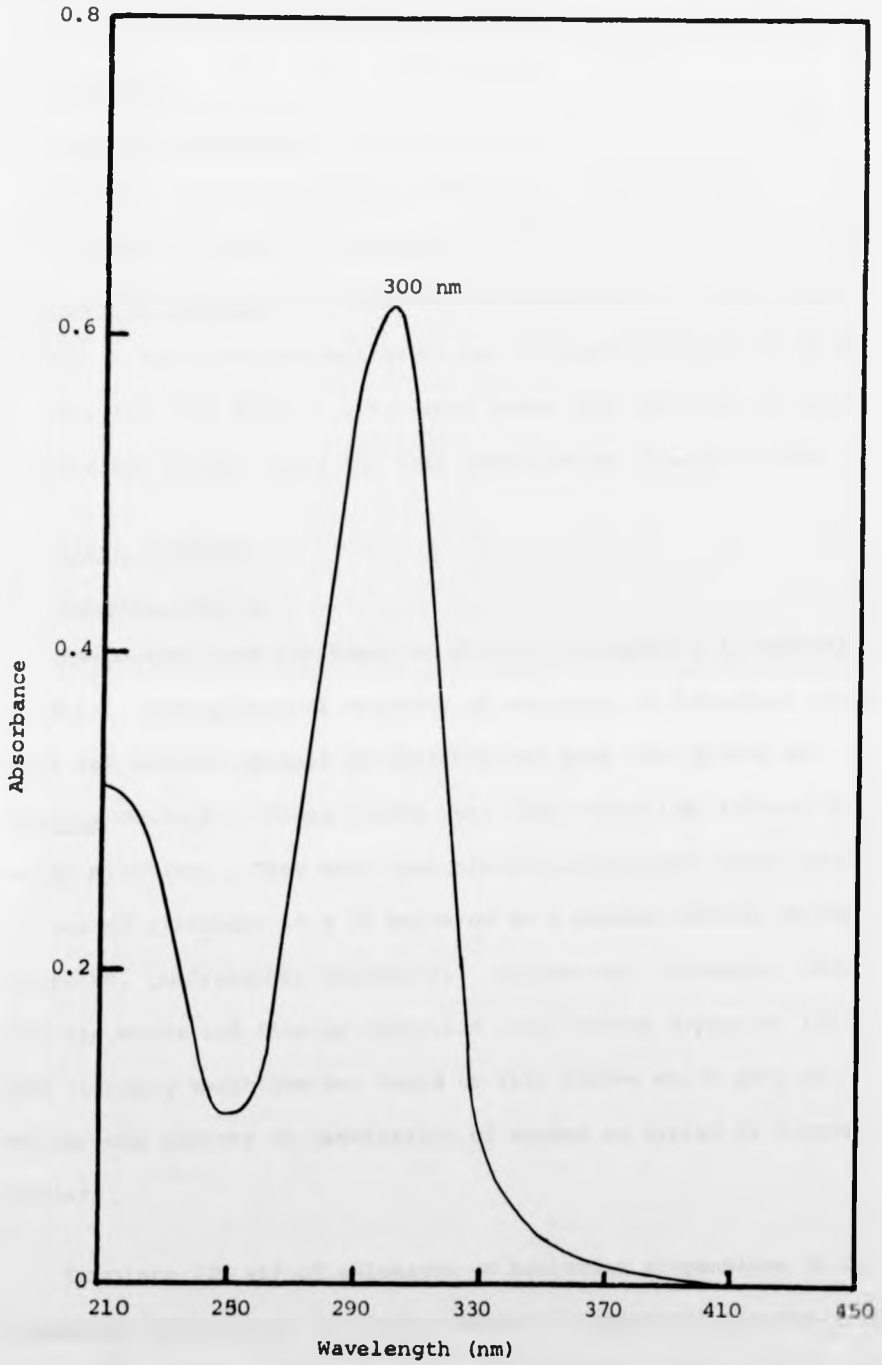


Fig.13 UV absorption spectrum of reduced wyerone acid in methanol

19. Bioassays

(a) TLC plate bioassay

Antifungal substances were detected in chromatograms of extracts by the method devised by Klarmann and Stamford (1968). Spores of Cladosporium herbarum in Czapek-dox liquid medium at pH 5.0 were sprayed on developed chromatograms and incubated at 25°C in moist chambers for four days. Inhibitory zones were detected as areas of white silica gel where the dark green fungus failed to grow.

(b) Slide bioassay

(i) Against conidia

The method used was based on that of Purkayastha & Deverall (1965b). The biological activity of solutions on bacterial suspensions was assayed against germination and germ tube growth of Botrytis conidia. Glass slides were first washed by rubbing in hot teepol solution. They were then placed in stainless steel racks and soaked overnight in a 2% solution of a surface active detergent (Decon 90, Quadralene or Haemosol). Slides were thoroughly rinsed with tap water and finally distilled water before drying at 120°C. This cleaning technique was found to give slides which gave no deleterious effects on germination of spores or spread of bioassay droplets.

Droplets (20 µl) of solutions or bacterial suspensions to be bioassayed were placed on slides using an "Eppendorf" pipette. Two droplets were placed on each slide and a minimum of two slides were prepared for each treatment. Slides were supported on test tubes in plastic boxes lined with moist tissue paper. Droplets (1 µl) of conidial suspension (5×10^5 conidia/ml) in SDW or SPN as required was added to the centre of each droplet with a micrometer

syringe (Agla). Bioassays were incubated at 18°C in the dark, for 24 hours unless stated otherwise.

At the end of the incubation the conidia were fixed and stained by the addition of a small drop of cotton blue lactophenol to each bioassay droplet. Percentage germination was estimated as the mean of five replicate counts of 100 conidia between four replicate droplets. Germination was considered the production of a germ tube of any length. Germ tube length was recorded as a mean of at least 35 randomly selected germ tubes between replicates. Germ tubes with a mean length less than c. 100 µm were measured with a calibrated micrometer eyepiece whereas with larger ones, measurement was made using camera lucida drawings and a map measurer. The total length of all germ tubes produced by each conidium was recorded. Statistical comparison between mean length of germ tubes and control germ tube length was made using a 'D-test' when required.

(ii) Against germ tubes (sporelings)

The antifungal activity of phytoalexins was routinely measured against germ tubes (sporelings) rather than conidia. Glass slides were washed and arranged in plastic boxes as described previously. Each slide was inoculated with two droplets (20 µl) of buffered SPN. Droplets (1 µl) of Botrytis conidial suspensions (5×10^5 conidia/ml) were added to the centre of each droplet and the slides were incubated for 6 hours or longer as required at 18°C. After incubation conidia had germinated and produced germ tubes c. 50 µm in length. Surrounding droplets of SPN were removed by tilting the slide and absorbing displaced liquid with filter paper. Weighing experiments indicated that 90% of the original volume was removed using this method. Sporelings were clearly visible, adhered strongly to the glass.

Droplets (20 µl) of phytoalexin solutions in buffered SPN were then placed over sporelings and the bioassays were incubated in the dark at 18°C for 18 hours unless otherwise stated. Concentrations of phytoalexin solutions were adjusted to compensate for dilution by residual SPN around sporelings. Dissolution of phytoalexins, except polar wyerone acid, in SPN was aided by the addition of 2% MeOH. This had no inhibitory effect on control fungal growth. At the end of incubation bioassays were stained with cotton blue lactophenol and germ tube lengths were estimated by measuring a minimum of 35 randomly selected germ tubes between replicate droplets.

Antifungal activity of phytoalexins against conidia and sporelings were expressed as ED_{50} and minimum inhibitory dose (MID) concentrations. The parameters were measured from graphs of germ tube growth against phytoalexin concentration.

20. Assessment of death of conidia and sporelings

(a) By solution replacement

Assessment of viability of conidia and sporelings after incubation in the presence of phytoalexins was made by replacing the inhibitory solution with fresh SPN and observing subsequent growth. Bioassay droplets were removed with filter paper as described earlier and replaced with droplets (20 µl) of fresh SPN. This was left in place for c. 10 minutes to wash the sporelings then replaced with a second droplet of SPN. Bioassays were incubated at 18°C for a further 12-18 hours then fixed and stained by the addition of a small droplet of cotton blue lactophenol. Comparison of dead and living structures was made by microscopical examination, dead sporelings having a germ tube length of only c. 50 µm whereas living ones having

grown to >200 µm. Four replicate counts of 100 structures were made for each treatment.

(b) By vital staining

Viability was also assessed by vital staining. The following stains were tested: 0.01% aqueous fluorescein diacetate (Rotman and Papermaster, 1966); 0.5% Evans blue, 0.1% neutral red and 0.1% phenosafranine (Widholm, 1972) and 0.5% trypan blue (Hargreaves, personal communication). After incubation in the presence of phytoalexins a droplet (c. 10 µl) of stain was added to the bioassay droplets before microscopical examination. Sporelings were only counted as dead when the entire structure, conidium and germ tube, gave a positive reaction in the presence of a particular stain. Cover slips were not put in place until immediately prior to examination lest anaerobiosis should have a deleterious effect on the fungal structures. Four replicate counts of 100 sporelings or conidia were made for each treatment.

21. Relationship between growth of germ tubes and metabolism of phytoalexins

Studies on the relationship between germ tube growth and phytoalexin metabolism were carried out in plastic petri dishes (5 cm. diameter). Preliminary investigations demonstrated that 4.5 ml of aqueous solution was necessary to overcome surface tension and completely cover the base of the dish. Aliquots (4.5 ml) of conidial suspensions of B. cinerea and B. fabae (2.5×10^4 conidia/ml) in buffered SPN were placed in petri dishes and incubated for six hours at 18°C in humid conditions. The SPN was then carefully decanted off, leaving a uniform layer of sporelings (germ tube length c. 50 µm) adhered to the dish. Portions (4.5 ml) of phytoalexin solutions at

the desired concentration in SPN were added to each dish. Dishes were incubated under standard conditions for varying periods, after which bathing solutions were decanted off. Concentrations of phytoalexins in these solutions was estimated by UV spectroscopy against an SPN blank. Loss of absorbance at λ maximum for each phytoalexin corresponded to loss of the inhibitor from solution. Growth of sporelings was examined in the same dishes after staining with cotton blue lactophenol, as described for slide bioassays.

EXPERIMENTAL WORK AND RESULTS

SECTION I

THE INHIBITION OF *BOTRYTIS* ON LEAVES OF *VICIA FABA*

CHAPTER 1

QUANTITATIVE STUDIES ON THE DEVELOPMENT OF *BOTRYTIS* ON THE ADAXIAL LEAF SURFACE OF GROWTH ROOM GROWN PLANTS

Twelve fully expanded bifoliate leaves were inoculated with six droplets (10 µl) of conidial suspensions of *B. cinerea* (isolates 39 and SR), *B. elliptica* and *B. fabae* (isolate PBI6) on separate half leaflets. The development of symptoms was recorded, after incubation for 24 and 48h, using the grading system illustrated in Fig.7. Results obtained (Table 1) show that each of the avirulent species caused lesions at less than 20% of inoculation sites after 24h incubation. By 48h a small increase in the number of lesions and symptom severity was recorded.

However, *B. fabae* caused the production of lesions of at least grade 19 at all sites 24h after inoculation, and by 48h over 10% had spread from the original site of inoculation.

Microscopical observation showed that the germination and germ tube growth of *B. cinerea* was inhibited at symptomless sites compared to that at sites where visible symptoms had developed (Plate 1, a and b). The growth of *B. cinerea* conidia 24h after inoculation of glass is shown in Plate 1(c). These observations suggested that the development of conidia on leaves was inhibited at symptomless

Plate 1

Growth of *B. cinerea* (39) conidia 24h after inoculation onto:

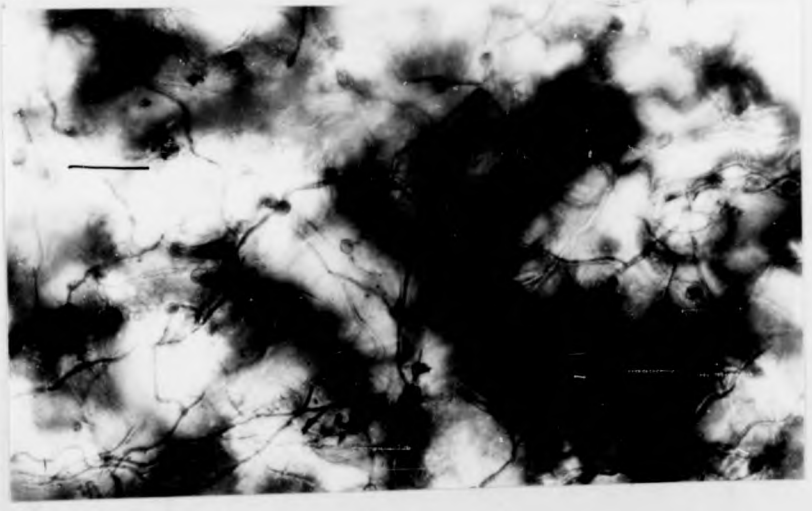
(a) leaves (no lesion site);

(b) leaves (lesion site);

(c) glass slide

Bar = 50 µm

a



b

c

Plate 1

TABLE 1 Lesion formation on the adaxial surface of leaves of V. faba 24h and 48h after inoculation with Botrytis

Infection grade	Percentage of inoculation sites in infection grade following inoculation with							
	B. cinerea (39)		B. cinerea (SR)		B. elliptica		B. fabae	
	24h	48h	24h	48h	24h	48h	24h	48h
0	90	79	95	88	84	76	0	0
3.5	3	5	3	6	4	4	0	0
6.5	3	6	2	4	5	4	0	0
9.5	2	6		2	5	8	0	0
19	2	4			2	6	4	0
38						2	12	8
63							19	16
87.5							49	28
100							16	40
S								14

sites, and stimulated at sites where lesions developed.

The rates of germination of B. cinerea (39 and SR) B. elliptica and B. fabae (PBI6; used in further studies unless stated otherwise) were then compared on glass slides and on leaves over a 24h period. Symptomless sites were selected for examination of the avirulent species. Sites inoculated with B. fabae were selected at random. Symptoms were first observed at 16h and 20h after inoculation of leaves with B. fabae and the other species respectively. Data obtained are given in Fig.14. B. fabae germinated more rapidly than B. cinerea or B. elliptica on both substrates, giving c.90 and 100% germination on glass slides and leaves respectively. Spores of all species germinated more slowly on the leaf surface than on glass, and the avirulent species achieved only c. 10% germination after 24h on leaves compared to c. 85% on glass.

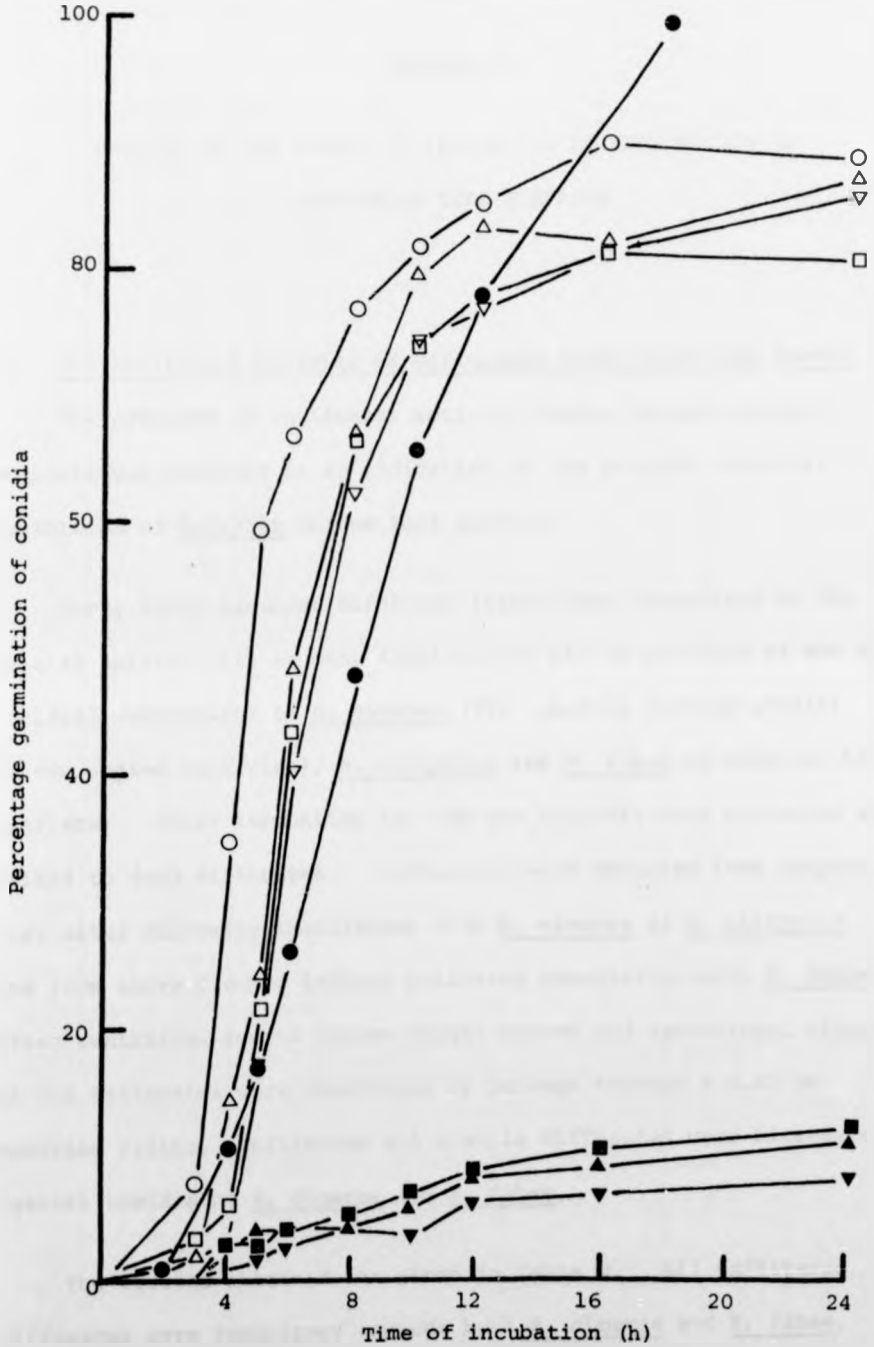


Fig.14 Time course for germination of Botrytis spp. on glass slides (open symbols) and leaves of V. faba (closed sumbols). B. cinerea (39) = ▲ △ B. cinerea (SR) = ▼ ▽ B. elliptica = ■ □ and B. fabae = ● ○

CHAPTER 2

STUDIES ON THE CAUSES OF INHIBITION OF GERMINATION OF CONIDIA ON LEAF SURFACES

(a) The antifungal activity of diffusates from broad bean leaves

The presence of antifungal activity within bulked inoculum droplets was examined as an indication of the possible cause(s) of inhibition of Botrytis on the leaf surface.

Forty fully expanded bifoliate leaves were inoculated on the adaxial surface with as many droplets (10 µl) as possible of SDW and conidial suspensions of B. cinerea (39; used in further studies unless stated otherwise), B. elliptica and B. fabae on separate half leaflets. After incubation for 24h the droplets were collected and bulked to form diffusates. Diffusates were obtained from symptomless sites following inoculation with B. cinerea or B. elliptica and from above flecked lesions following inoculation with B. fabae. After centrifugation to remove fungal spores and sporelings, aliquots of the diffusates were sterilized by passage through a 0.45 µm membrane filter. Unfiltered and sterile diffusates were bioassayed against conidia of B. cinerea and B. fabae.

The results obtained are given in Table 2. All unfiltered diffusates were inhibitory towards both B. cinerea and B. fabae. The most active diffusates were those obtained from sites inoculated with B. cinerea and B. elliptica. Diffusates from sites inoculated with B. fabae or SDW alone exhibited less antifungal activity, and all diffusates were slightly more active against B. cinerea than B. fabae.

TABLE 2 Activity of diffusates from broad bean leaves against B. cinerea and B. fabae

Source of diffusate	Growth of conidia in bioassays: B. cinerea % germination[a] (% G)	B. cinerea Germ tube length[b] (GTL)	B. fabae % G	B. fabae GTL
Control[c]	89.2 (83-94)	35.2	88.4 (82-94	42.3
Water on leaf (Unfiltered	44.6 (36-50)+	23.4*	56.0 (49-62)+	30.6*
Water on leaf (Filtered	70.2 (66-76)+	31.5	76.6 (70-81)+	38.2
B. cinerea on leaf (Unfiltered	24.0 (18-28)+	18.8*	28.4 (21-34)+	23.7*
B. cinerea on leaf (Filtered	34.4 (26-35)+	21.0*	38.8 (34-43)+	28.2*
B. elliptica on leaf (Unfiltered	21.2 (15-24)+	19.1*	29.0 (24-33)+	25.3*
B. elliptica on leaf (Filtered	28.4 (20.33)+	23.7*	40.3 (36.46)+	26.3*
B. fabae on leaf (Unfiltered	63.6 (58-59)+	32.1	75.4 (68-81)+	36.9
B. fabae on leaf (Filtered	>95+	49.8	>95+	61.7*

a Mean of 5 replicates; figures in parentheses, maximum range between replicates

b Mean of 35 germ tubes

c Growth in SDW on glass slides

\+ Range of replicates outside range of control

* Significantly different from control ($p = 0.001$)

Membrane filtration removed some activity in all treatments. Filtered B. cinerea and B. elliptica diffusates retained considerable inhibitory activity, but bulked water droplets were only weakly active. By contrast, B. fabae diffusate became stimulatory to both fungi assayed following filtration.

These results suggested that the presence of epiphytic micro-organisms within diffusates rendered them inhibitory to germination of Botrytis conidia. In addition certain diffusates, particularly those collected from sites inoculated with B. cinerea and B. elliptica contained antifungal principle (or principles) which was not removed by membrane filtration.

(b) Comparative studies on the inhibition of germination of Botrytis on leaves of broad bean, French bean and tomato plants

Before studying the inhibition of germination of B. cinerea conidia on the surface of broad bean leaves in greater depth, it was decided to compare the development of a range of Botrytis species on leaves of broad bean, French bean and tomato plants, grown under identical conditions. The percentage germination of conidia of B. alii, B. cinerea (39 and SR), B. elliptica and B. fabae (PBI6 and SS1) on leaves and glass slides was examined 24h and 48h after inoculation. On tomato and French bean leaves fewer than 10% of inoculation sites had developed visible symptoms by 48h after inoculation with any fungus. Fungal development was therefore examined at symptomless sites on these plants and on broad bean leaves inoculated with avirulent species. B. fabae had produced lesions at all sites on leaves of broad bean 1d after inoculation.

With the exception of B. fabae on broad bean leaves, germination

TABLE 3 The germination of Botrytis conidia on leaf surfaces and glass slides

(a) 24h after inoculation

Fungus	Percentage germination of conidia on surface of[a]			
	Broad bean	French bean	Tomato	Glass slide
B. allii	4.2 (2-5)	8.4 (6-11)	1.8 (4-9)	86.6 (82-89)
B. cinerea (39)	12.2 (9-16)	24.0 (20-30)	19.2 (15-23)	91.2 (84-96)
B. cinerea (SR)	7.6 (5-9)	15.2 (11-18)	14.0 (10-18)	85.4 (79-90)
B. elliptica	12.0 (6-16)	21.0 (17-25)	15.0 (11-19)	84.8 (79-90)
B. fabae (PB16)	100 b	48.2 (40-54)	44.2 (39-49)	94.0 (87-97)
B. fabae (SS1)	100 b	40.6 (37-45)	41.8 (36-47)	87.6 (80-92)

(b) 48h after inoculation

Fungus	Percentage germination of conidia on surface of[a]			
	Broad bean	French bean	Tomato	Glass slide
B. allii	5.6 (3-9)	10.0 (7-13)	8.4 (6-11)	67.8 (84-91)
B. cinerea (39)	16.2 (11-19)	28.8 (25-32)	21.0 (17.24)	95.0 (90-98)
B. cinerea (SR)	10.2 (7-12)	14.6 (11-17)	14.8 (11-19)	87.2 (84-92)
B. elliptica	14.0 (10-17)	22.2 (18-26)	20.0 (16-23)	91.4 (87-93)
B. fabae (PBI6)	100 b	56.2 (50-63)	48.2 (44-53)	94.8 (90-98)
B. fabae (SS1)	100 b	51.4 (46-57)	40.8 (37-45)	86.2 (83-90)

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses, maximum range between replicates

b - Visible symptoms present

and germ tube growth by all species of Botrytis was inhibited on each of the plants examined. Germination of conidia of B. cinerea (39 and SR), B. allii and B. elliptica was lower on broad bean than on the other leaves.

The antifungal activity of bulked inoculum droplets collected 24h and 48h after inoculation on to leaves of broad bean, French bean and tomato, and also onto glass slides were then compared before and after membrane filtration. B. cinerea (39) was used to assay the diffusates, collected from four leaves or slides for each assessment.

Diffusates obtained from glass slides inoculated with water, either before or after filtration, allowed the same growth of B. cinerea (39) conidia as in fresh SDW controls. Conidial suspensions collected from glass slides gave diffusates which stimulated growth both before and after filtration. For example diffusates collected after 24h incubation from B. allii allowed 92.4% germination and growth of germ tubes 44.7 µm in length, and in diffusates from B. fabae 96.8% germination and germ tubes of 52.9 µm were recorded.

The results of bioassays on diffusates from leaves is recorded in Tables 4, 5 and 6. One day after inoculation diffusates from all the interactions examined were antifungal before membrane filtration. Diffusates obtained from broad bean leaves inoculated with B. fabae were the least inhibitory, causing a reduction in germination but not of mean germ tube length. Membrane filtration reduced inhibitory activity of all diffusates collected after 24h, B. fabae diffusates from broad bean becoming stimulatory. However, all the other conidial suspensions retained some inhibitory activity

TABLE 4 The growth of conidia of B. cinerea (39) in diffusates recovered from broad bean leaves inoculated with suspensions of Botrytis in SDW or water alone

Source of diffusate	Growth of B. cinerea conidia in bioassays							
	Diffusates collected 24h after inoculation				Diffusates collected 48h after inoculation			
	Unfiltered		Filtered		Unfiltered		Filtered	
	% G[a]	GTL[b]	% G	GTL	% G	GTL	% G	GTL
Control[c]	86.2 (80-91)	34.7			82.8 (78-89)	35.1		
Water on leaf	40.0 (36-45)+	25.3**	68.2 (62-75)+	32.9	35.2 (30-40)+	23.6**	63.8 (58-60)+	32.7
Bc39 on leaf	20.6 (18-24)+	17.0**	31.0 (26-38)+	18.9**	0+	-	0+	-
BcSR on leaf	22.8 (20-27)+	23.4**	38.6 (32-45)+	26.9**	0+	-	0+	-
BF PBI6 on leaf[d]	67.8 (60-72)+	37.0	100+	65.8**	100+	103**	100+	159**
BF SS1 on leaf[d]	60.4 (55-66)+	35.2	100+	60.8**	100+	107**	100+	150
B. allii on leaf	28.0 (24-36)+	21.5**	39.8 (34-47)+	28.9*	0+	-	14.6 (10-17)+	20.2**
B. elliptica on leaf	20.0 (17-24)+	21.0*	28.8 (22-32)+	26.5**	0+	-	12.2 (9-16)+	17.9**

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - Growth in SDW on glass

d - Diffusate collected from inoculation sites with visible symptoms; all other diffusates from symptomless sites

\+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.005)

** - Significantly different from control (p = 0.001)

TABLE 5 The growth of conidia of B. cinerea (39) in diffusates recovered from French bean leaves inoculated with suspensions of Botrytis in SDW or water alone

Source of diffusate	Growth of *B. cinerea* conidia in bioassays							
	Diffusates collected 24h after inoculation				Diffusates collected 48h after inoculation			
	Unfiltered		Filtered		Unfiltered		Filtered	
	% G[a]	GTL[b]	% G	GTL	% G	GTL	% G	GTL
Control[c]	84.0 (80-90)	36.9			87.0 (81-92)	37.3		
Water on leaf	51.2 (46-55)+	27.8*	76.8 (71-82)	35.6	31.2 (24-36)+	20.1*	68.4 (60-73)+	32.6
Bc39 on leaf	30.4 (26-33)+	22.6*	68.8 (64-75)+	39.2	21.2 (16-25)+	17.0*	31.6 (25-34)+	24.7*
BCSR on leaf	33.2 (30-37)+	23.1*	72.8 (68-77)+	36.2	22.8 (19-25)+	19.7*	35.4 (30-41)+	26.9*
BF PBI6 on leaf	32.4 (27-37)+	20.2*	50.6 (45-54)+	33.9	18.0 (14-21)+	16.0*	24.2 (18-27)+	23.6*
BF SS1 on leaf	30.2 (27-34)+	23.5*	52.8 (47-58)+	34.9	21.2 (17-27)+	21.0*	26.8 (14-34)+	26.2*
B. allii on leaf	35.2 (30-41)+	26.0*	72.4 (67-77)+	36.7	27.6 (22-34)+	23.9*	35.8 (30-43)+	26.8*
B. elliptica on leaf	27.8 (23-35)+	21.0*	48.8 (43-55)+	33.8	19.4 (15-24)+	18.9*	31.2 (28-37)+	26.0*

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to ranges between replicates

b - Mean length of 35 germ tubes (μm)

c - Growth in SDW on glass

\+ - Range of replicates outside range of control

* - Significantly different from control ($p = 0.001$)

TABLE 6 The growth of conidia of B. cinerea (39) in diffusates recovered from tomato leaves inoculated with suspensions of Botrytis in SDW or water alone

Source of diffusate	Growth of B. cinerea conidia in bioassays			
	Diffusates collected 24h after inoculation		Diffusates collected 48h after inoculation	
	Unfiltered		Filtered		Unfiltered		Filtered	
	% G[a]	GTL[b]	% G	GTL	% G	GTL	% G	GTL
Control[c]	87.4 (82-93	33.9			83.0 (81-90)	33.1		
Water on leaf	45.2 (40-49)[+]	25.0*	77.8 (72-83)	30.8	32.8 (25-37)[+]	24.0*	49.0 (43-57)[+]	26.9*
Bc39 on leaf	25.8 (21-29)[+]	22.1*	57.0 (52-65)[+]	29.6	12.2 (8-15)[+]	18.2	25.6 (20-32)[+]	23.7*
BcSR on leaf	29.2 (25-34)[+]	24.5*	59.8 (51-64)[+]	31.2	18.0 (11-22)[+]	21.6*	24.8 (20-30)[+]	26.0*
BF PBI6 on leaf	28.8 (24-34)[+]	22.0*	58.0 (51-65)[+]	33.6	14.6 (11-24)[+]	17.2*	20.0 (17-25)[+]	22.5*
BF SS1 on leaf	27.0 (21-32)[+]	23.2*	52.2 (49-57)[+]	35.6	12.0 (9-17)[+]	18.0*	18.8 (14-25)[+]	23.7*
B. allii on leaf	29.6 (25-33)[+]	24.5*	59.0 (51-66)[+]	31.2	17.8 (13-23)[+]	19.2*	30.4 (27-41)[+]	24.1*
B. elliptica on leaf	26.6 (20-30)[+]	24.7*	55.8 (50-62)[+]	36.5	11.2 (7-14)[+]	15.9*	26.0 (20-31)[+]	19.8*

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - Growth in SDW on glass

+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.001)

after filtration, this residual activity being greatest in diffusates from broad bean leaves. Filtration of water droplets collected after incubation for 24h on tomato and French bean leaves removed inhibitory activity, whereas some residual activity was noted in water diffusates from V. faba at this time.

Two days after inoculation B. fabae diffusates from V. faba were stimulatory. All other diffusates were inhibitory to germination of B. cinerea. Droplets recovered from broad bean leaves were most active. In particular, B. cinerea diffusates completely inhibited conidial germination even after membrane filtration which reduced inhibition in other diffusates collected after 48h.

In conclusion, results suggested that epiphytic microorganisms removed by membrane filtration were the main cause of inhibition of germination of B. cinerea conidia in diffusates. Removal of such microorganisms usually allowed increased fungal growth. In addition, some residual activity was noted in certain filtered diffusates, being most notable in conidial suspensions of B. allii, B. cinerea and B. elliptica incubated on broad bean leaves. On the basis of these findings, the examination of the inhibition of B. cinerea on leaves of V. faba was continued.

(c) Time course studies on the accumulation of inhibitory activity in inoculum droplets incubated on broad bean leaves

Results described in Fig.9 and Table 2 showed that by 6h after inoculution the germination of B. cinerea had reached 40-50% on glass slides but was less than 5% on leaves at this time and increased little over the next 48h. Mansfield and Deverall (1974a) also suggested that at symptomless sites on leaves of V. faba surface growth of B. cinerea ceased by 12h. It is therefore clear that if

the factors causing the antifungal activity detected in diffusates collected from broad bean leaves 24h and 48h after inoculation are also responsible for inhibition of germination *in situ* then they must be present within inoculum droplets by at most 6h after inoculation. The kinetics of the accumulation of inhibitory principles detected in diffusates was therefore examined.

Droplets of water and of *B. cinerea* conidial suspension were incubated for 6, 12, 24 and 48h on broad bean leaves and bioassayed before and after membrane filtration in the usual way. In addition the antifungal activity of membrane filter washings was also examined. Washings were recovered by passing a volume of SDW equivalent to the original volume of diffusate through the filter in the reverse direction to the original filtration in order to dislodge any micro-organisms trapped by the filter. Samples of filter washings were plated out onto nutrient and V8 juice agar, and after incubation it could be seen that the population of microorganisms within filter washings was largely bacterial, with a small proportion of pink yeasts (*Sporobolomyces* spp.). Henceforth microbes removed from inoculum droplets by membrane filtration will be termed bacteria.

Bioassay results obtained are given in Table 7. Prior to membrane filtration the diffusate obtained from leaves inoculated with conidial suspension was inhibitory after 6h incubation, but that obtained from water alone failed to inhibit the development of *B. cinerea* (39) conidia until 24h after inoculation. After 2d *B. cinerea* diffusate was totally inhibitory but water diffusate still supported some germination and germ tube growth. The bacterial suspensions obtained from filter washings after sterilization of *B. cinerea* diffusates were more active than those recovered from water

TABLE 7 Antifungal activity of unfiltered and filtered diffusates and of filter washings collected at different times after inoculation onto broad bean leaves

Source of diffusate	Growth of *B. cinerea* (39) conidia in bioassays of diffusates collected at different times after inoculation							
	6h		12h		24h		48h	
	% G[a]	GTL[b]	% G	GTL	% G	GTL	% G	GTL
Conidia on leaf[c]	43.0 (38-54)+	31.0**	46.4 (42-50)+	29.2**	27.2 (20-32)+	20.2**	0+	
Membrane filtered conidia on leaf	63.8 (55-70)+	35.6	66.2 (62-70)+	33.7*	36.0 (31-39)+	23.1**	0+	
Filter washings	62.8 (54-70)+	38.2	54.4 (50-60)+	40.1	32.8 (30-36)+	25.0**	29.0 (20-41)+	21.1**
Water on leaf	75.0 (68-81)	36.2	80.0 (72-85)	37.0	41.2 (35-46)+	24.1**	33.2 (29-41)+	19.6**
Membrane filtered water on leaf	89.4 (87-93)	39.0	90.8 (88-94)	35.7	75.4 (70-79)	35.0	68.0 (60-75)+	33.6*
Filter washings	76.8 (69-81)	38.5	70.0 (60.74)+	36.5	37.0 (34-40)+	26.1**	33.6 (29-37)+	21.8**

	% G	GTL
Growth in SDW control	80.0 (75-87)	38.6

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - *B. cinerea* (39) in SDW

\+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.05)

** - Significantly different from control (p = 0.001)

TABLE 7 Antifungal activity of unfiltered and filtered diffusates and of filter washings collected at different times after inoculation onto broad bean leaves

Source of diffusate	Growth of *B. cinerea* (39) conidia in bioassays of diffusates collected at different times after inoculation							
	6h		12h		24h		48h	
	% G[a]	GTL[b]	% G	GTL	% G	GTL	% G	GTL
Conidia on leaf[c]	43.0 (38-54)+	31.0**	46.4 (42-50)+	29.2**	27.2 (20-32)+	20.2**	0+	
Membrane filtered conidia on leaf	63.8 (55-70)+	35.6	66.2 (62-70)+	33.7*	36.0 (31-39)+	23.1**	0+	
Filter washings	62.8 (54-70)+	38.2	54.4 (50-60)+	40.1	32.8 (30-36)+	25.0**	29.0 (20-41)+	21.1**
Water on leaf	75.0 (68-81)	36.2	80.0 (72-85)	37.0	41.2 (35-46)+	24.1**	33.2 (29-41)+	19.6**
Membrane filtered water on leaf	89.4 (87-93)	39.0	90.8 (88-94)	35.7	75.4 (70-79)	35.0	68.0 (60-75)+	33.6*
Filter washings	76.8 (69-81)	38.5	70.0 (60.74)+	36.5	37.0 (34-40)+	26.1**	33.6 (29-37)+	21.8**

	% G	GTL
Growth in SDW control	80.0 (75-87)	38.6

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - *B. cinerea* (39) in SDW

\+ - Range of replicates outside range of control

* - Significantly different from control ($p = 0.05$)

** - Significantly different from control ($p = 0.001$)

droplets incubated on the leaf surface. Slight activity was detected in the former after 6h incubation on the leaf, whereas the latter only became marginally antifungal by 12h.

Differences were also observed between the activity of conidial suspension and water droplets following membrane filtration. That collected from B. cinerea inoculation sites was antifungal as early as 6h after inoculation, becoming totally inhibiting by 48h, whereas diffusate from leaves inoculated with SDW had only a very slight activity after 24h (not significant) and was still only moderately antifungal by 48h.

In conclusion, the results suggest that the presence of conidia within inoculum droplets caused an increase in antifungal activity of the total diffusate. Increased activity was also detected in membrane filtered diffusate and in the bacterial suspension obtained from filter washings. As previous results had indicated that a suspension of Botrytis conidia incubated in SDW produced a stimulatory diffusate, the increased antifungal activity probably arose from an interaction(s) between conidia and other components of the inculum droplet.

It would appear that in diffusate collected from sites on the leaf inoculated with conidial suspension, antifungal activity began to accumulate sufficiently early to account for the inhibition of conidia observed in situ. Bacteria and the residual water soluble inhibitor remaining after filtration appeared to contribute equally to total activity of diffusates during the first 24h after inoculation. In further studies some characteristics of both sources of inhibition were examined.

CHAPTER 3

CHARACTERISATION OF POSSIBLE SOURCE OF INHIBITION OF GERMINATION OF B. CINEREA CONIDIA ON LEAVES OF V. FABA

(a) Studies on the inhibitory activity of bacteria within inoculum droplets

There are two possible mechanisms by which bacteria could inhibit germination and germ tube growth of conidia of B. cinerea; the production of an antifungal compound or compounds by the bacteria or inhibition resulting from nutritional competition between bacteria and conidia. The following experiment was carried out to examine the possible production of a water soluble inhibitor by bacteria in vitro.

Bifoliate bean leaves were inoculated with droplets (10 µl) of a suspension of B. cinerea conidia. After incubation for 24h droplets were collected and a 10 ml sample of the resulting diffusate membrane filtered. Bacteria were recovered by washing the filter with 10 ml of SDW, and samples of filter washings were bioassayed against B. cinerea conidia. Other aliquots of the bacterial suspension were inoculated onto glass slides which were also inoculated on the opposite half with control droplets (10 µl) of SDW. After incubation for 24h droplets were collected from the glass slides and membrane filtered. The cell free diffusates obtained were then bioassayed against B. cinerea in the usual way and their effect on the development of conidia compared with that of the original bacterial suspension. The results obtained (Table 8) illustrate that whilst the bacterial suspension obtained from filter washings was inhibitory, membrane filtered diffusates obtained from droplets of bacterial suspension and SDW incubated on glass slides gave similar growth to SDW controls.

TABLE 8 The antifungal activity of bacterial suspensions from broad bean leaves before and after membrane filtration

Source of diffusate	Growth of *B. cinerea* conidia in bioassays		
	Percentage germination[a]		Germ tube length[b]
Water control	86.2	(81-91)	40.1
Membrane filtered water on glass	87.0	(78-93)	39.2
Bacterial suspension	33.6	(29-37)[+]	24.5*
Membrane filtered bacterial suspension	88.4	(83-92)	41.6

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

\+ - Range of replicates outside range of control

* - Significantly different from control ($p = 0.001$)

This demonstrated that bacteria did not produce an inhibitor.

Subsequent studies to attempt to ascertain the role of leaf surface bacteria in inhibiting germination of Botrytis conidia on bean leaves in situ were made by endeavouring to remove them from the biological system described within the injection droplet. This was attempted by incorporation of the broad spectrum antibiotic chloramphenicol with inoculum droplets and by the growth of a sterile broad bean plant. However, both techniques failed to give satisfactory results, as described in Appendix 1.

(b) Studies on the water soluble inhibitor remaining in inoculum droplets after membrane filtration

(i) Source of the water soluble inhibitor

Previous results, given in Tables 2, 4 and 7, suggested that antifungal activity remaining in diffusates after membrane filtration were of leaf origin, more particularly arising through the interaction between conidia of avirulent fungi and the leaf. However, the inhibitory activity could arise from several possible interactions between the three components of the phyllosphere, the leaf, conidia and epiphytic bacteria. All the possible interactions between these components were therefore examined for their abilities to produce inhibitors.

Droplets (10 µl) of SDW, filter washings prepared as previously described and suspensions of B. cinerea condia in SDW or in filter washings were inoculated onto leaves and glass slides. After incubation for 24h droplets were collected from each treatment and the resulting diffusates membrane filtered. The cell free diffusates were then bioassayed in the usual way.

Results of the bioassays are given in Table 9, from which it can be seen that the interaction between the leaf and conidial suspension produced diffusates with the greatest inhibitory activity. No inhibitors were produced by bacteria or conidia, either alone or together when incubated on glass slides, conidial diffusates being slightly stimulatory. With leaf inoculations the bacterial suspension caused the formation of diffusates no more antifungal than those obtained from inoculation with SDW alone, and a combination of bacteria and conidia produced a slightly less active diffusate than spores alone. This reduced activity may have been a result of suppression of conidial metabolism on the leaf surface by bacteria added to the inoculum droplet. Results obtained therefore confirmed that epiphytic bacteria did not produce stable inhibitors and demonstrated that the antifungal activity of filtered diffusates recovered from leaves was produced as a result of the interaction between conidia and the leaf.

(ii) Organic extraction of diffusate

Organic extraction of diffusate obtained from symptomless sites following inoculation of leaves with conidia of B. cinerea (henceforth termed diffusate) was attempted with diethyl ether (Et_2O) and chloroform ($CHCl_3$), the extraction procedures being similar for each solvent. An aliquot of diffusate was shaken three times with twice its volume of solvent, and the resulting organic phases were bulked together. Residual solvent was removed from the water phase by the passage of a stream of oxygen free nitrogen (OFN) until the characteristic smell of the solvent was no longer apparent. The organic phase was then taken to dryness at 25°C in vacuo and redissolved in a volume of SDW equal to the original volume of diffusate. Dissolution

TABLE 9 Production of antifungal activity residual in diffusates after membrane filtration by possible interactions within the phyllosphere

Source of diffusate	Growth of *B. cinerea* (39) conidia in bioassays		
	Percentage germination[a]		Germ tube length[b]
Control[c]	86.8	(81-92)	40.3
Water on leaf	68.0	(61-72)+	36.7
Conidia on leaf	32.6	(30-36)+	19.6**
Filter washings on leaf	69.8	(64-76)+	36.0
Conidia and filter washings on leaf	38.4	(35-43)+	27.9**
Water on glass	84.0	(79.90)	38.1
Conidia on glass	97.2	(94-100)+	51.2**
Filter washings on glass	84.6	(81-90)	38.2
Conidia and filter washings on glass	94.8	(92-98)	45.6*

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - Control growth in SDW

\+ - Range of replicates outside range of control

** - Significantly different from control (p = 0.001)

* - Significantly different from control (p = 0.01)

was aided by the prior addition of ethanol to give a final concentration of 2% (v/v). This concentration was found to have no deleterious effects on germination and germ tube growth of conidia in controls.

The antifungal activities of the total diffusate, ether phase and aqueous phase were then determined. The data shown in Table 10 are the means of three replicate extraction of diffusate with Et_2O. It is clear that Et_2O extraction removed inhibitory activity from the diffusate, the residual aqueous phase becoming stimulatory. However, the redissolved ether soluble fraction did not inhibit germination or germ tube growth by B. cinerea. Partitioning with chloroform ($CHCl_3$) gave similar results, indicating that the inhibitor(s) removed by extraction was probably unstable or volatile.

(iii) UV absorption spectrum of filtered diffusates

The phytoalexins produced by broad bean tissue as listed by Hargreaves, Mansfield and Rossall (1977) all have characteristic UV absorption spectra (Hargreaves, 1976). Wyerone derivatives have a peak of UV absorption ranging from 347-360 nm and medicarpin has characteristic peaks at 282 and 287 nm. To examine the possibility that the water soluble inhibitor was a known phytoalexin or a mixture of them, the UV absorption spectrum of a cell free diffusate collected 24h after inoculation of leaves with conidia of B. cinerea was recorded. The spectrum obtained is illustrated in Fig.15. Above 200 nm no peaks of UV absorption were detected, suggesting the inhibitory activity in the diffusate was not due to the presence of a known phytoalexin or phytoalexins.

TABLE 10 The antifungal activity of filtered diffusate and of its ether and water soluble phases

Source of fraction for bioassay	Growth of *B. cinerea* (39) conidia in bioassays		
	Percentage germination[a]		Germ tube length[b]
Control[c]	86.2	(80-92)	39.1
Total diffusate	32.6	(24-39)[+]	19.7*
Water soluble fraction	96.0	(92-100)	63.2*
Ether soluble fraction	79.8	(74-85)	26.7

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - Control growth in SDW

\+ - Ranges of replicates outside range of control

* - Significantly different from control ($p = 0.001$)

(iv) Detection of antifungal activity on TLC plates bioassay

Thin layer chromatography plate bioassays with Cladosporium herbarum have been extensively used to demonstrate the accumulation of phytoalexins in tissues of V. faba following infection by Botrytis. The detection of an inhibitor(s) in diffusate was therefore attempted using this technique.

Before application to TLC plates, the diffusates were dried and redissolved in ethanol. Water was removed by rotary evaporation in vacuo at 40°C or by freeze drying. Loading rates of plates ranged from 1-5 ml diffusate per cm of origin. Chromatograms were assayed before and after development in a range of solvent systems which allowed separation of the known phytoalexins from V. faba. Solvent systems used were:

1. Hexane: Acetone (2:1), followed by chloroform: Petroleum spirit (60-80°) (2:1)
2. Chloroform
3. Chloroform: Petroleum spirit (60-80°) (2:1)
4. Diethyl ether: Methanol (8:1)

No inhibitory zones were produced when diffusates were assayed on TLC plates before or after development in these systems. These results confirmed that inhibitory activity was not caused by known phytoalexins and once again indicated the volatile or unstable nature of the active principle present in diffusate.

(o) Studies on the role of epicuticular wax in leaf surface inhibition

Several authors have suggested that epicuticular wax may inhibit the development of fungal propagules on the leaves of various plants. Experiments were therefore designed to examine the role of waxes in

the inhibition of germination of B. cinerea conidia on the surface of broad bean leaves.

(i) The growth of B. cinerea conidia on redeposited wax

The growth of B. cinerea conidia on layers of redeposited wax extracted from bean leaves was examined. Two replicate wax layers were prepared by redeposition of wax onto watch glasses, glass slides, cellulose TLC plates and fibre glass discs. These layers were inoculated with droplets (10 µl) of a suspension of B. cinerea conidia and after 24h incubation the growth of conidia was compared to growth on control substrates to which wax free chloroform had been added. Results obtained (Table 11) clearly show that growth of B. cinerea was inhibited on wax redeposited by each of the methods tested.

(ii) The antifungal activity of diffusates from wax

Because of their observed antifungal activity epicuticular waxes were considered as a potential source of the water soluble inhibitor detected in diffusates from leaves. The presence of inhibitory activity in droplets of water on B. cinerea conidial suspension was examined after their incubation for 24h on redeposited wax.

Six glass slides and four fibre glass discs were treated with wax solution or chloroform to give wax layers and solvent controls respectively. The substrates obtained were inoculated with droplets (10 µl) of B. cinerea conidial suspension and SDW on opposite halves. After incubation for 24h the droplets were collected and the resulting diffusates membrane filtered, and bioassayed against conidia of B. cinerea. The results (Table 12) show that conidia incubated on wax gave rise to antifungal diffusates with both methods of redeposition tested. By contrast water incubated on the wax layers gave stimulatory

TABLE 11 Growth of B. cinerea conidia on redeposited broad bean leaf wax

Method of wax redeposition	Growth of B. cinerea in bioassays[a]					
	Solvent control			Wax solution		
	% G[b]		GTL[c]	% G		GTL
Watch glass	87.4	(80-93)	39.6	53.2	(46-67)+	19.2*
Glass slide	84.6	(80-87)	36.0	55.0	(49-62)+	21.2*
TLC plate	90.2	(84-92)	33.7	54.2	(51-69)+	19.7*
Fibre glass	84.0	(80-87)	33.0	50.6	(46-63)+	17.9*

a - Results represent the mean of two repeated experiments

b - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

c - Mean length of 35 germ tubes (µm)

+ - Range of replicates outside range of solvent control

* - Significantly different from solvent control ($p = 0.001$)

TABLE 12 The antifungal activity of diffusates collected from redeposited wax layers

Source of diffusate	Growth of *B. cinerea* conidia in bioassays					
	Chloroform control			Wax redeposition		
	% G[a]		GTL[b]	% G		GTL
Water on glass slides	81.2	(77-85)	33.2	93.8	(90-97)+	51.2**
Conidia on glass slides	87.8	(82-94)	44.0*	54.2	(50-59)+	24.6**
Water on fibre glass disc	-		-	95.0	(91-98)	60.0**
Conidia on fibre glass disc	-		-	57.2	(52-63)+	25.2**

	% G		GTL
Control growth[c]	84.2	(80-91)	37.0µm

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range

b - Mean length of 35 germ tubes

c - Control growth in SDW

\- No data available, droplets soaked into fibre glass disc

\+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.002)

** - Significantly different from control (p = 0.001)

diffusates. These results indicate that conidia may cause the release of an antifungal principle from redeposited wax and that wax also appears to contain stimulatory compounds.

Further studies on the inhibitory properties of diffusates from wax were carried out using a suspension of wax particles in liquid shake culture. Wax solution or chloroform control (5 ml) was added to 5 ml SDW in a conical glask (50 ml) and the solvent removed by bubbling a stream of OFN through the liquid until the characteristic smell was no longer detectable. Aliquots (5 ml) of a suspension of conidia of B. cinerea were added to flasks to give the spare concentration required. Two replicate flasks were prepared for each of the treatments described in Table 13. The flasks were then placed on an orbital incubator (60 rpm) at 20°C. After incubation for 24h aliquots of the suspension were removed, membrane filtered and bioassayed against conidia of B. cinerea. Results (Table 13) confirmed the proposed release of inhibitory activity from wax by conidia of B. cinerea. Increasing concentrations of conidia causing an increasing inhibitor activity, but conidia and wax alone giving rise to stimulatory diffusates.

(iii) Extraction of diffusate from wax with diethyl ether

Half of a sample of diffusate collected from wax coated slides 24h after inoculation with droplets (10 µl) of B. cinerea conidial suspension was shaken three times with twice its volume of diethyl ether. The water and ether soluble phases were prepared for bioassay as described for leaf diffusates and the inhibitory properties of the two fractions compared with those of total cell free diffusate. The results, given in Table 14, are similar to those obtained with leaf diffusate. Ether extraction removed inhibitory activity from

TABLE 13 The antifungal activity of diffusates obtained from suspensions of wax incubated with water or conidia of B. cinerea

Source of diffusate	Growth of B. cinerea conidia in bioassays		
	% germination[a]		Germ tube length[b]
SDW control	83.0	(78-80)	35.1
Chloroform control	81.8	(77-86)	34.8
10^5 conidia ml^{-1} + chloroform	92.6	(85-95)	47.2***
5×10^5 conidia ml^{-1} + chloroform	96.2	(92-100)+	53.1***
10^6 conidia ml^{-1} + chloroform	100+		55.7***
Water and wax	96.2	(92-100)+	56.5***
10^5 conidia ml^{-1} + wax	71.0	(67-75)+	30.0*
5×10^5 conidia ml^{-1} + wax	63.2	(59-67)+	28.1*
10^6 conidia ml^{-1} + wax	59.8	(56-65)+	27.6**

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.01)

** - Significantly different from control (p = 0.002) *** - Significantly different from control (p = 0.001)

TABLE 14 The antifungal activity of a diffusate obtained from wax incubated with B. cinerea conidia and of its ether and water soluble phases

Source of diffusate	Growth of B. cinerea conidia in bioassays	
	Percentage germination[a]	Germ tube length[b]
Control[c]	87.8 (80-91)	35.3
Ether extracted control[d]	89.0 (83-92)	37.2
Total diffusate	56.8 (50-59)+	23.0*
Water solubles	100+	67.4*
Ether solubles	89.2 (84.91)	37.8

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (μm)

c - Control growth in SDW

d - Growth in SDW following extraction with Et_2O

\+ - Range of replicates outside range of control

* - Significantly different from control ($p = 0.001$)

the water phase, which became stimulatory, however, the redissolved ether soluble phase did not inhibit germination and germ tube growth of B. cinerea conidia. This suggests that the inhibitor(s) released from wax may also be volatile or unstable.

(iv) UV absorption spectrum of diffusate obtained from redeposited wax

The UV absorption spectrum of a cell free diffusate collected 24h after inoculation of wax coated glass slides with conidia of B. cinerea was recorded. Above 200 nm no peaks of UV absorption were detected. The spectrum obtained was similar to that recorded for diffusates from leaves, illustrated in Fig.15.

(v) Detection of antifungal activity on TLC plate bioassay

The detection of an inhibitor(s) in diffusate obtained from a suspension of wax 24h after inoculation with conidia of B. cinerea (5×10^5 conidia/ml) was attempted using a TLC plate bioassay using C. herbarum. Plates were loaded at a rate of 1-5 ml diffusate per cm of origin as described for leaf diffusates, and bioassayed before and after development in the range of solvents also described for TLC plate bioassay of diffusate from leaves. No inhibitory zones were detected, once again indicating the volatile or unstable nature of the inhibitory principle associated with diffusate obtained from extracted leaf wax.

(vi) Analysis of bean wax by thin layer chromatography

Data described so far suggested that a water soluble inhibitor(s) was released from redeposited leaf wax by conidia of B. cinerea. The release of the inhibitor and its properties closely paralleled that of the inhibitor recovered from intact bean leaves. An attempt

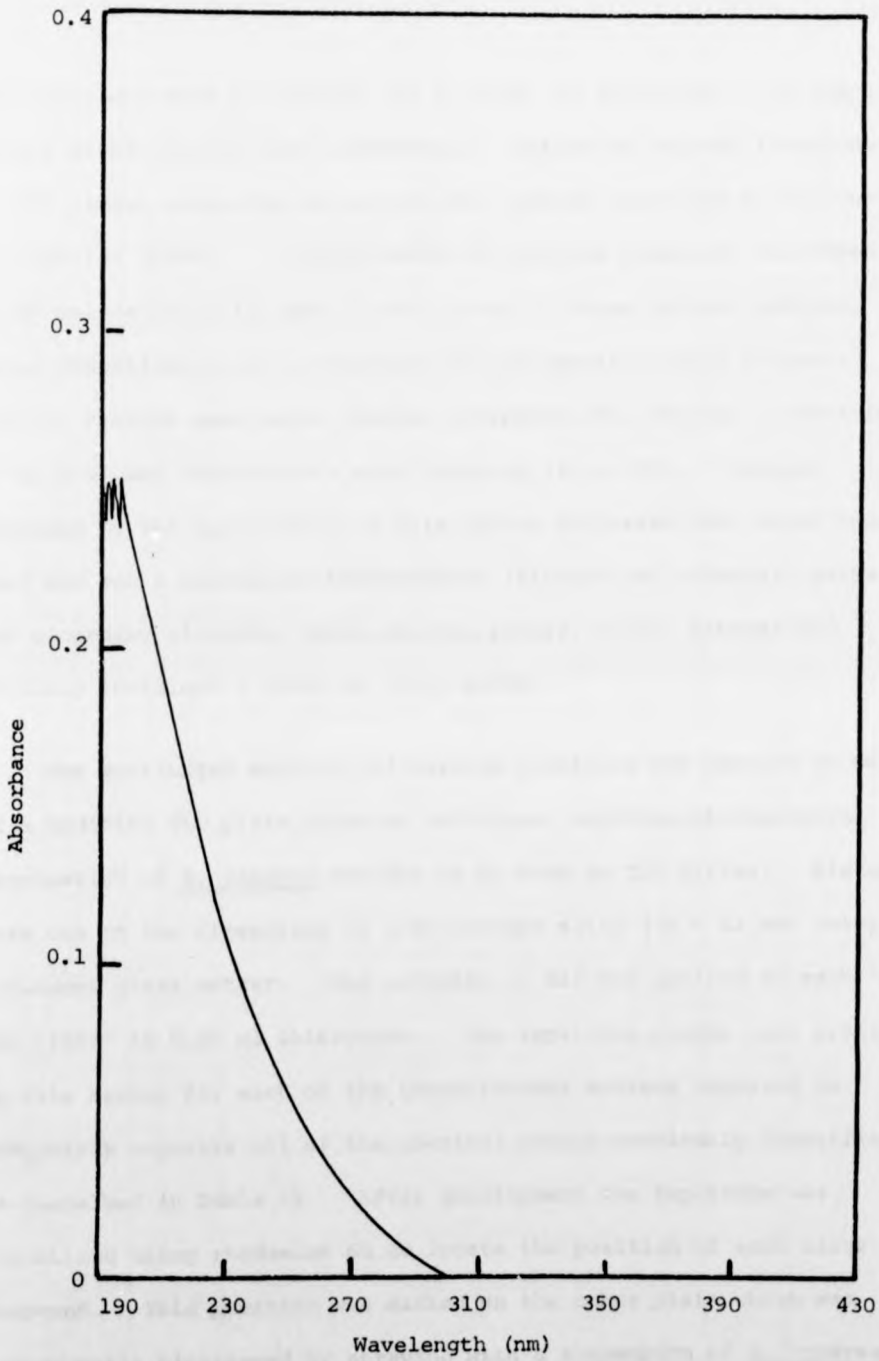


Fig.15 UV absorption spectrum of diffusate collected from lesion free sites on growth room grown bean leaves 24h after inoculation with B. cinerea

was therefore made to analyse wax in order to determine which components of the crude extract were inhibitory. Extracted wax was fractionated on TLC plates using the chromatographic system described by Holloway and Challon (1966). Identification of various fractions was based on Rf values on silica gel coated plates in three solvent systems, after visualization with rhodamine 6G, or specific spray reagents. Solvent systems used were: carbon tetrachloride, benzene : chloroform (7:3, v/v) and chloroform : ethyl acetate (1:1, v/v). Results obtained by the application of this system suggested that broad bean leaf wax was a mixture of hydrocarbons (alkanes and alkenes), primary and secondary alcohols, diols, sterols, esters, alkyl, ketones and possibly contained a trace of fatty acids.

The antifungal activity of various fractions was assayed by means of a modified TLC plate bioassay technique, enabling microscopical examination of B. cinerea conidia to be made on TLC plates. Plates were cut to the dimensions of a microscope slide (76 × 22 mm) using a diamond glass cutter. Wax solution (1 ml) was applied to each 'mini TLC plate' in 0.05 ml chloroform. Two replicate plates were prepared in this manner for each of the three solvent systems required to adequately separate all of the chemical groups previously identified, as described in Table 15. After development one replicate was visualized using rhodamine 6G to locate the position of each class of compound. This position was marked on the other plate which was subsequently bioassayed by spraying with a suspension of B. cinerea conidia (5×10^5 conidia/ml) in SDW. After incubation in humid conditions for 24h development of conidia at the location of each of the groups was assessed by microscopical examination after staining with cotton blue lactophenol. The results obtained (Table 15)

TABLE 15 Germination and germ tube growth of B. cinerea conidia on components of broad bean leaf wax separated by TLC

Class of compound bioassayed	Solvent system[a]	R_F Range	Growth of B. cinerea condia in bioassays	
			Percentage germination[b]	Germ tube length[c]
Control[d]			78.2 (74-95)	33.7
Alkanes/alkenes	I, III	0.86 - 0.91	100+	60.4**
Alkyl ketones	I	0.19 - 0.24	92.6 (87-96)+	37.8
Diols	III	0.16 - 0.20	95.8 (92-100)+	43.7*
Fatty acids	III	0.62 - 0.66	100+	44.0*
Primary alcohols	II	0.17 - 0.23	51.0 (47-53)+	24.2**
Secondary alcohols	II	0.49 - 0.53	93.4 (90-97)+	35.0
Sterol esters	I	0.41 - 0.46	100+	56.3**

a - Solvent systems used; I = carbon tetrachloride, II - Benzene = chloroform (7:3, v/v) and III = chloroform = ethyl acetate (1:1, v/v)

b - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range

c - Mean length of 35 germ tubes (µm)

d - Control growth on TLC plates after development in systems I, II and III without wax application

+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.002) ** - Significantly different from control (p = 0.001)

suggested that the only class of compounds separated from crude bean leaf wax which inhibited germination and germ tube growth of conidia of B. cinerea was the primary alcohols. All other groups located stimulated fungal growth.

(d) Effect of glucose on leaf surface inhibition of B. cinerea

(i) Effect of glucose solutions on the development of lesions on broad bean leaves following inoculution with B. cinerea

The effect of increasing concentrations of glucose, added to inoculum droplets of B. cinerea conidia, on the development of visible symptoms was examined. Half leaflets were inoculated, on the adaxial surface with droplets (10 µl) of a suspension of B. cinerea conidia containing 0.01, 0.1, 1.0 and 10% (w/v). Control leaves were inoculated with conidia in SDW alone. After incubation for 24h lesion development was recorded and the data obtained are given in Table 16. Increasing concentrations of glucose caused an increase in the percentage of sites at which symptoms were recorded. A concentration of 0.1% glucose was required to cause lesion formation at all inoculation sites.

(ii) Effect of glucose on the antifungal activity of diffusates from inoculation sites on broad bean leaves

The effect of glucose on the inhibitory principles associated with inoculum droplets removed from lesion free sites on broad bean leaves was examined. Diffusate was collected from broad bean leaves 24h after inoculation with droplets (10 µl) of a conidial suspension of B. cinerea. Half the diffusate was sterilized by membrane filtration and a bacterial suspension was prepared by washing the membrane filter in the usual way. Aliquots of total and filtered diffusate and of filter washings were then mixed with SDW or glucose

TABLE 16 The effect of glucose on lesion development 24h after the inoculation of broad bean leaves with a suspension of B. cinerea conidia

Infection grade	Percentage of inoculation sites in grades in presence of concentration of glucose[a]				
	0%	0.01%	0.1%	1.0%	10.0%
0	91	20	0	0	0
3.5	4	8	7	0	0
6.5	3	10	10	0	0
9.5	2	31	12	5	0
19		20	30	19	12
38		11	21	28	25
63			15	36	35
87.5			5	8	14
100				4	17

a - Glucose concentrations expressed as percentage (w/v)

solutions in a ratio of 3:1 to give final glucose concentrations of 0, 0.01, 0.1, 1.0 and 10% (w/v). This procedure was adopted to reduce dilution of the diffusates. The diffusates ± glucose were bioassayed against conidia of B. cinerea and the results illustrating the effect of the sugar on antifungal activity are given in Table 17.

The activity of total diffusate obtained from inoculation of leaves with conidia was overcome by glucose, a concentration of 1.0% being required to stimulate germination and germ tube growth of conidia to a level above that of SDW controls. The inhibitory effect of sterilized diffusate was overcome by 0.1% glucose, whereas that of filter washings was still slightly antifungal at this concentration, 1.0% glucose being required to cause stimulation of conidial growth. It is clear from the results obtained that the cause of inhibition of B. cinerea conidia on leaves of the broad bean in situ and the inhibitory activity associated with inoculum droplets can both be overcome by the presence of glucose.

(iii) Effect of glucose on the inhibition associated with redeposited wax

An examination was carried out on the effects of glucose on the inhibition of B. cinerea observed on redeposited wax, and on the inhibitory activity of diffusates obtained from the interaction between wax and conidia (5×10^5 conidia/ml) in liquid culture.

Glass slides coated with a layer of extracted wax were inoculated with droplets (10 µl) of a suspension of conidia of B. cinerea containing 0.01, 0.1, 1.0 and 10% (w/v) glucose. Control slides (coated and uncoated) were inoculated with conidia in SDW alone. After incubation for 24h percentage germination and germ tube growth

TABLE 17 The effect of glucose on the antifungal activity of unfiltered and filtered diffusates and of filter washings, collected 24h after inoculation of broad bean leaves with B. cinerea (Adapted from Rossall, 1974)

Source of diffusate	Growth of B. cinerea (39) conidia in bioassays in the presence of glucose[a]									
	0%		0.01%		0.1%		1.0%		10%	
	%G[b]	GTL[c]	% G	GTL	% G	GTL	% G	GTL	% G	GTL
Conidia on leaf (Total)	18.2 (14-22)	18.1**	27.6 (20-32)	25.9**	47.0 (41-51)	27.1**	100	80.1**	100	>200
Membrane filtered conidia on leaf	21.6 (19-25)+	20.8**	39.4 (34-46)+	46.0*	82.2 (79-87)	64.7**	100	200	100	>200
Membrane filter washings	27.2 (24-31)+	19.4**	47.0 (43-51)+	25.4**	69.2 (66-73)+	28.9*	100	95.4**	100	>200

	% G	GTL
Growth in SDW control	85.0 (80-91)	35.7

a - Glucose concentration expressed as percentage (w/v)

b - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

c - Mean length of 35 germ tubes (μm)

\+ - Range of replicates outside range of control

* - Significantly different from control growth in SDW (p = 0.01)

** - Significantly different from control growth in SDW (p = 0.001)

were recorded and the results obtained are given in Table 18. The inhibitory effect of the wax was overcome by the presence of glucose, 0.1% supporting conidial growth in excess of that of control growth in the absence of wax.

Aliquots of wax diffusate were mixed with glucose solutions or SDW in a ratio of 3:1 (diffusate:glucose) to give final glucose concentrations of 0, 0.01, 0.1, 1.0 and 10% (w/v). This procedure was adopted to reduce dilution of the diffusate. Diffusates ± glucose were bioassayed against conidia of B. cinerea and the results (Table 19) show that the antifungal activity of the diffusate was readily overcome by the sugar, 0.01% glucose being sufficiently concentrated to overcome the inhibitory effect. It is clear from these results that the inhibitory activity associated with extracted broad bean leaf wax is overcome by glucose both on redeposited wax in situ and in diffusates obtained from the wax.

TABLE 18 The effect of glucose on germination and germ tube growth of conidia of B. cinerea inoculated onto redeposited wax extracted from broad bean leaves

Concentration of glucose (µg/ml)	Percentage germination[a]		Germ tube length[b]
Control[c]	84.8	(82-88)	37.9
0	51.2	(44-55)[+]	20.1*
0.01	72.8	(68-79)[+]	25.7
0.1	96.2	(92-100)[+]	89.9*
1.0	100[+]		> 200*
10.0	100[+]		> 200*

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - Control growth on clean glass slides in SDW

\+ - Range of replicates outside range of control

* - Significantly different from control ($p = 0.001$)

TABLE 19 The effect of glucose on the antifungal activity of membrane filtered diffusate obtained by incubating wax suspension with B. cinerea conidia for 24h

Concentration of glucose (µg/ml)	Percentage germination[a]		Germ tube length[b]
Control[c]	88.0	(84-91)	38.7
0	61.2	(55-65)[+]	25.1*
0.01	94.0	(89-96)	42.0
0.1	100[+]		169*
1.0	100[+]		>200*
10.0	100[+]		>200*

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (µm)

c - Control growth in SDW

+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.001)

CHAPTER 4

STUDIES ON THE INHIBITION OF *B. CINEREA* CONIDIA ON LEAVES FROM FIELD GROWN *V. FABA* PLANTS

In order to assess the significance of the inhibition of *Botrytis* on leaf surfaces to the expression of disease resistance in the field, it was considered important to examine infection development in field grown leaves.

(a) Lesion development in field grown leaves following inoculation with *B. cinerea*

Twenty fully expanded bifoliate leaves were detached from young field grown plants with between four and six mature leaves. Leaves were carefully examined and particles of soil on their adaxial surfaces removed by gentle washing with distilled water. After drying where necessary with tissue paper, leaves were inoculated on the adaxial surface with six droplets (10 µl) of *B. cinerea* (39) conidial suspension, and SDW on separate leaflets. Following incubation for 24 and 48h the leaves were examined for lesion development using the system described in Fig.7. No symptoms developed at sites inoculated with water alone. The symptoms produced on the leaflets inoculated with *B. cinerea* are described in Table 20. In contrast to results obtained with growth room grown leaves (Table 1) lesions had developed at nearly all sites 48h after inoculation. Microscopical examination 24h and 48h after inoculation showed that germination of *B. cinerea* was less than 20% at sites without symptoms, but >95% over lesions.

TABLE 20 Lesion formation on the adaxial surface of field grown leaves 24h and 48h after inoculation with B. cinerea (39)

Infection grade	Percentage of inoculation sites in infection grades after:	
	24h	48h
0	40	16
3.5	20	12
6.5	18	9
9.5	13	8
19	12	10
38	7	16
63		19
87.5		10
100		

(b) Time course studies on the accumulation of inhibitory activity in inoculum droplets incubated on field grown leaves

The biological activity of diffusates recovered from field grown leaves was examined. Leaves were detached from plants and prepared as described previously. They were then inoculated with SDW and conidial suspension on separate leaflets and after incubation for 6, 12, 24 and 48h the droplets were collected to form diffusates. Droplets were only taken from symptomless sites. Lesions were first observed 12h after inoculation. Bioassays of total and membrane filtered diffusates and of membrane filter washings were carried out in the usual way.

Results obtained (Table 21) were generally similar to those reported for growth room grown leaves (Table 7). Inhibitory activity was recorded for unfiltered and filtered diffusates, and filter washings inhibited germination. Diffusates obtained from droplets of conidial suspension were more active than water alone. Unfiltered diffusates were, however, less active from field than growth room grown leaves. This reduced activity was associated with less inhibition in filtered diffusates; those collected from field grown leaves after incubation for 48h supported c. 40% germination of conidia in bioassays, whereas comparable growth room diffusates were totally inhibitory (see Table 7). Filter washings were as active from field as from growth room grown leaves.

In conclusion, results suggest that the inhibitory factors detected in diffusates recovered from growth room grown leaves also accumulate in droplets incubated on leaves from the field and that they may cause the inhibition of B. cinerea on the leaf surface. The reduced activity in filtered diffusates from symptomless sites

TABLE 21 The antifungal activity of unfiltered and filtered diffusates and of filter washings collected at different times after inoculation onto broad bean leaves

	Growth of *B. cinerea* conidia in bioassays of diffusates collected at different times after inoculation							
	6h		12h		24h		48h	
	% G	GTL	% G	GTL	% G	GTL	% G	GTL
Conidia on leaf	57.2 (50-63)+	31.1**	49.8 (43-56)+	29.8*	44.0 (37-51)+	25.6***	25.0 (19-28)+	20.1***
Membrane filtered conidia on leaf	81.8 (77-87)	38.0	76.4 (70-82)	36.5	52.8 (48-57)+	30.0*	39.8 (31-46)+	29.8**
Membrane filter washings	61.4 (57-64)+	28.9**	60.2 (56-66)+	27.9**	34.0 (30-39)+	24.8***	29.4 (21-34)+	25.0**
Water on leaf	77.0 (70-82)	36.3	79.2 (72-80)	37.8	55.8 (50-60)+	27.9**	39.8 (36-45)+	21.2***
Membrane filtered water on leaf	83.6 (80-88)	39.8	88.0 (84-93)	39.6	76.2 (70-83)	36.1	75.0 (72-79)*	35.5
Membrane filter washings	70.8 (66-74)+	35.1	75.2 (70-78)+	35.6	39.4 (36-43)+	31.4*	32.0 (26-37)+	24.3***

	% G	GTL
SDW control growth	86.2 (80-91)	38.9

a - Percentage germination, mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range between replicates

b - Mean length of 35 germ tubes (μm)

\+ - Range of replicates outside range of control

* - Significantly different from control (p = 0.01)

** - Significantly different from control (p = 0.002)

*** - Significantly different from control (p = 0.001)

on field grown leaves may have resulted from less release of the water soluble inhibitor or the presence of more nutrients in droplets on field than on growth room grown leaves. In order to distinguish between these possibilities, further studies were carried out on the effect of nutrients on the inhibition of B. cinerea on leaf surfaces.

(c) Studies on the involvement of nutrients in the formation of lesions by B. cinerea

(i) A comparison between lesion development on adaxial and abaxial surfaces of field and growth room grown leaves following inoculation with B. cinerea

Before attempting to analyse the role of nutrients in influencing the inhibition of germination of B. cinerea on the leaf surface of beans, more detailed comparative studies were carried out on the development of lesions following inoculation of the adaxial and abaxial surfaces of leaves from growth room and field grown plants.

Six leaves were prepared for each of the four treatments described and each leaf was inoculated with six droplets (10 µl) of a conidial suspension of B. cinerea. After incubation for 24h the leaves were examined for lesion formation and the results obtained are recorded in Table 22. It is clear from these results that growth room grown leaves were more resistant than those collected from the field and also that fewer symptoms developed on adaxial leaf surfaces.

(ii) The release of carbohydrates and amino acids into droplets incubated on leaves

During earlier experiments considerable variation in symptom development was observed between different leaves. In some cases all inoculation sites on a leaf remained symptomless, whereas on other leaves lesions were formed at most sites. This variation was most pronounced in batches of leaves collected from growth room

TABLE 22 Lesion formation on the abaxial and adaxial surfaces of field and growth room grown leaves 24h after inoculation with B. cinerea (39)

Infection grade	Percentage of inoculation sites in infection grades following inoculation of			
	Growth room grown leaves		Field grown leaves	
	Abaxial surface	Adaxial surface	Abaxial surface	Adaxial surface
0	67	90	3	30
3.5	9	3	5	22
6.5	5	3	5	18
9.5	5	2	16	14
19	4	2	19	8
38	3		31	8
63	5		19	
87.5	2		2	
100				

grown plants. It was decided to take account of this variation when examining the leakage of carbohydrates and amino-acids into inoculum droplets. This was achieved as follows. Each leaf was inoculated on one leaflet with droplets of SDW and on the other with a suspension of B. cinerea conidia. After incubation for 24h leaves were categorized as 'lesion' or 'no lesion' types depending on the number of sites developing symptoms (see Table 23). Water and B. cinerea diffusates were collected separately from 'lesion' and 'no lesion' leaves and assayed for amino acids and carbohydrates. The experiment was carried out using the abaxial and adaxial surfaces of both field and growth room grown leaves and because of the large number of leaves required amino acid and carbohydrate levels were determined in separate experiments. Diffusates obtained were separated into two aliquots for duplicate analyses.

Results obtained are presented in Table 23. They show that greater levels of both carbohydrates and amino acids leaked into droplets of conidial suspension than into water droplets incubated on corresponding leaves. Differences were greatest on 'lesion' type leaves probably because penetrated cells beneath inoculum droplets on these leaves tended to lose solutes more freely. Leakage of nutrients into water droplets demonstrated that higher levels of carbohydrates and amino acids were detected in leaves which had a greater tendency to produce lesions following inoculation with conidial suspension. Field grown leaves supported greater leakage than leaves detached from growth room grown plants and droplets recovered from the abaxial leaf surface contained higher nutrient levels than those from the adaxial surface. Differentiation between 'lesion' and 'no lesion' type leaves also gave large differences

TABLE 23 Levels of carbohydrate and amino acid in diffusates collected from broad bean leaves after incubation for 24h

Source of leaves	Inoculum droplets		Concentration of amino acids and carbohydrates in diffusate			
			Abaxial surface		Adaxial surface	
			Lesion[a]	No lesion[a]	Lesion	No lesion
Growth room grown plants	Conidial Suspension	(C[b]	58 (53-63)	15 (14-16)	44 (40-48)	9 (9-9)
		(AA[b]	0.31 (0.27-0.35)	0.10 (0.09-0.11)	0.20 (0.18-0.22)	0.08 (0.07-0.09)
	Water	(C	49 (47-51)	7 (6-8)	19 (17-21)	3 (3-3)
		(AA	0.08 (0.07-0.09)	0.03 (0.03-0.03)	0.06 (0.06-0.06)	0.02 (0.02-0.02)
Field grown plants	Conidial Suspension	(C	65 (60-70)	23 (20-26)	51 (47-55)	16 (15-17)
		(AA	0.40 (0.36-0.44)	0.22 (0.20-0.24)	0.34 (0.30-0.38)	0.14 (0.12-0.16)
	Water	(C	54 (52-56)	18 (17-19)	27 (24-30)	11 (11-11)
		(AA	0.12 (0.11-0.13)	0.05 (0.05-0.05)	0.08 (0.06-0.10)	0.04 (0.03-0.05)

a - 'Lesion' type leaves defined as: growth room grown, >50% inoculation sites developed symptoms; field grown, >90% developed symptoms. 'No lesion' type defined as: growth room, no inoculation sites developed symptoms; field grown <10% sites developed symptoms.

b - C refers to carbohydrate level expresses as µg glucose/ml; AA refers to amino acid level expresses as µM valine/ml. Figures in parentheses are range of two replicate determinations.

between concentrations of carbohydrates and amino acids detected in water diffusates. With growth room grown leaves a 6-7 times increase in total carbohydrage level was detected in water droplets between 'lesion' and 'no lesion' leaves and amino acids gave an approximately three fold difference. These results suggest that in leaves which have a propensity to develop symptoms following inoculation with B. cinerea conidia high levels of nutrients (carbohydrates and amino acids) probably tend to leak into inoculum droplets.

(iii) The effect of the addition of nutrients to droplets of conidial suspension incubated on growth room grown leaves

It is possible that the reason many sites on field grown leaves developed lesions following inoculation with B. cinerea related to the observed high levels of nutrients which leaked into water droplets following incubation on such leaves. The nutrients could overcome the inhibitory factors within infection droplets. Further studies to test this hypothesis were carried out by inoculating growth room grown leaves with B. cinerea in the presence or absence of solutions of carbohydrates and amino acids at concentrations detected in water droplets removed from leaves 24h after inoculation. Carbohydrate was supplied as glucose and amino acids as glutamine (Blakeman, 1975).

The concentrations of glucose and glutamine selected represented the levels of carbohydrates and amino acids detected in water diffusates from 'lesion' and 'no lesion' type growth room grown leaves following inoculation on the abaxial and adaxial surfaces (Table 23). Suspensions of conidia of B. cinerea were prepared in each of the four nutrient levels being assayed and ten leaves each for the upper and lower surfaces were inoculated with six droplets

(10 µl) of a different suspension on separate half leaflets. Control leaves were inoculated with conidia in SDW alone. After incubation for 24h the percentage of inoculation sites at which lesions had developed was calculated for each treatment and the results are given in Table 24. It can be seen from these results that when levels of glucose and glutamine, equivalent to the levels of carbohydrate and amino acids detected in water droplets collected fron 'lesion' type leaves, were supplied to inoculum droplets of B. cinerea, visible symptoms were produced on growth room grown leaves at most sites by 24h after inoculation. The stimulation was most pronounced on leaves inoculated on the abaxial surface.

In conclusion, the greater ability of B. cinerea to cause lesions on field rather than growth room grown leaves appears to relate to the greater release of nutrients from field grown material. These results also suggest that in the field, the inhibition of conidia on the leaf surface may be of less significance to disease resistance than mechanisms of resistance effective after lesion formation.

TABLE 24 The effect of glucose and glutamine on lesion formation on growth room grown leaves 24h after inoculation with B. cinerea

Nutrient level with inoculum droplet[a]	Percentage of inoculation sites at which lesions developed	
	Abaxial leaf surface	Adaxial leaf surface
Control[b]	15	5
(1) 3 µg/ml glucose) 0.02 µM/ml glutamine)	64	21
(2) 7 µg/ml glucose) 0.03 µM/ml glutamine)	97	37
(3) 19 µg/ml glucose) 0.06 µM/ml glutamine)	100	61
(4) 49 µg/ml glucose) 0.08 µM/ml glutamine)	100	82

a - Nutrient levels (1) - (4) respectively refer to carbohydrate and amino acid concentrations measured in water diffusates from growth room grown leaves: adaxial surface 'no lesion', abaxial surface 'no lesion', adaxial surface 'lesion' and abaxial surface 'lesion'.

b - Lesion development with B. cinerea in SDW alone.

SECTION II

THE INHIBITION OF BOTRYTIS BY PHYTOALEXINS FROM V. FABA

CHAPTER 1

ANTIFUNGAL ACTIVITY OF PHYTOALEXINS

The antifungal activity of phytoalexins from V. faba has previously been determined in a range of culture media. Mansfield (1972) demonstrated that wyerone acid was not antifungal towards B. cinerea in distilled water. Deverall and Rogers (1972) showed that the activity of the phytoalexin was affected by pH and by unknown components of natural media. Pod nutrients, comprising the residual water phase recovered after ether extraction of inoculum droplets collected 24h after the inoculation of pod seed cavities with B. cinerea, have also been used as a medium for bioassays (Deverall, 1967; Mansfield & Deverall, 1974b). However, Hargreaves (1976) concluded that batches of pod nutrients differed in their abilities to support growth of Botrytis and developed a defined medium for bioassays named 'synthetic pod nutrients' (SPN) (Hargreaves, Mansfield & Coxon, 1976).

In all work on the antifungal activity of phytoalexins reported here SPN was used to support fungal growth. The pH of the medium was adjusted to the required level by addition of galacturonate buffer as previously described.

(a) Antifungal activity of phytoalexins against conidia of B. cinerea and B. fabae

In initial experiments antifungal activity of the phytoalexins was assayed against conidia. A range of concentrations of wyerone (5-80 µg/ml), wyerone acid (2.5-40 µg/ml) and wyerone epoxide (2-20 µg/ml) were prepared in SPN buffered to pH 4.0. Solutions of wyerone acid (10-300 µg/ml) were also prepared at pH 5.0. Activity of the phytoalexins was examined against both B. cinerea and B. fabae. Results obtained are illustrated in Figs. 16-19 as graphs of germ tube length against phytoalexin concentration. From these graphs it was possible to estimate ED_{50} and minimum inhibitory dose (MID) concentration of each phytoalexin against both fungi. Values obtained are given in Table 25, from which it can be seen that all the phytoalexins tested were more active against B. cinerea than B. fabae. At pH 4.0 an approximate two-fold difference in ED_{50} existed between the fungi. The inhibitory activity of wyerone acid was greatly reduced at pH 5.0, confirming the findings of Deverall & Rogers (1972). The most active compound tested at pH 4.0 was wyerone epoxide.

As it is unlikely that ungerminated conidia came into contact with high levels of phytoalexins in vivo, the activity of phytoalexins against short term tubes, comparable to the infection hyphae first exposed to the inhibitors within infected tissue, was examined in subsequent experiments.

(b) Antifungal activity of phytoalexins against sporelings of B. cinerea and B. fabae

A range of concentrations of wyerone (2.5-60 µg/ml), wyerone epoxide (1-20 µg/ml), wyerol (20-300 µg/ml) and medicarpin (5-100 µg/ml) were prepared in SPN buffered to pH 4.0 and 5.0. Solutions of wyerone acid were prepared from 2.5-30 µg/ml at pH 4.0 and from 20

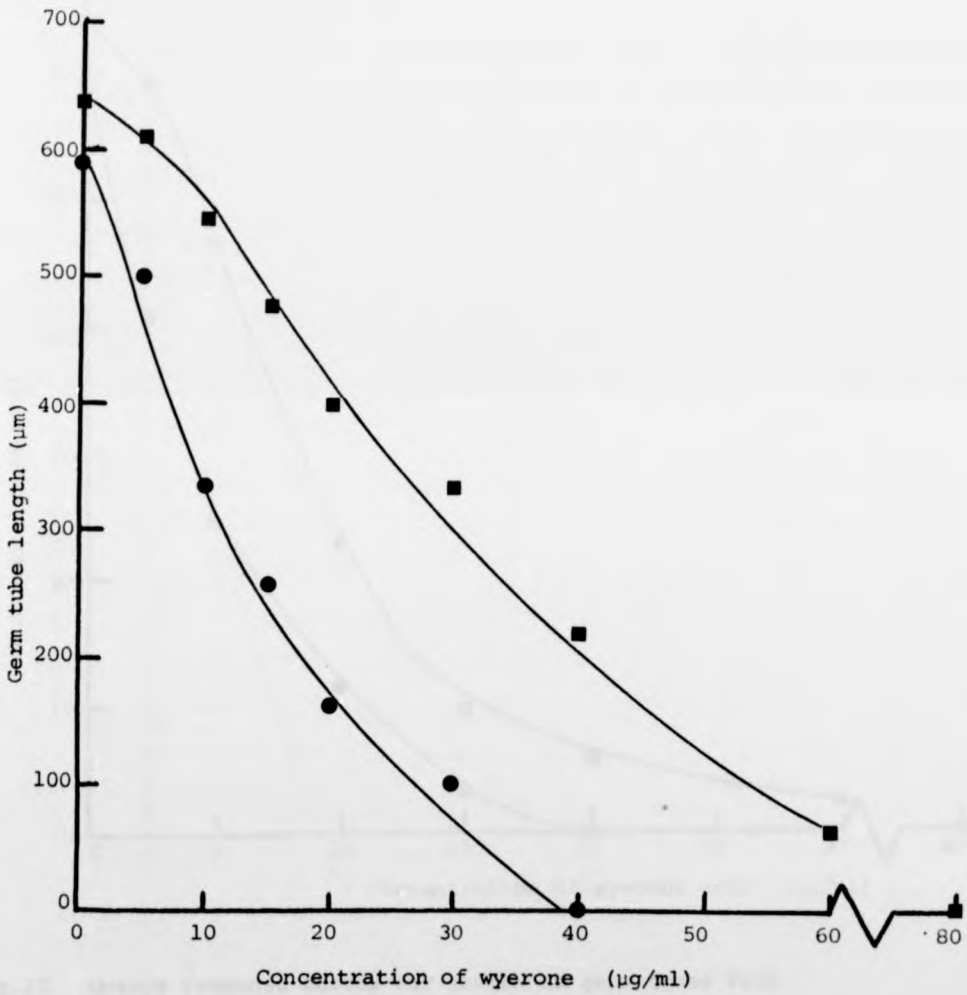


Fig.16 Dosage response curves for growth of germ tubes from conidia of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone at pH 4.0

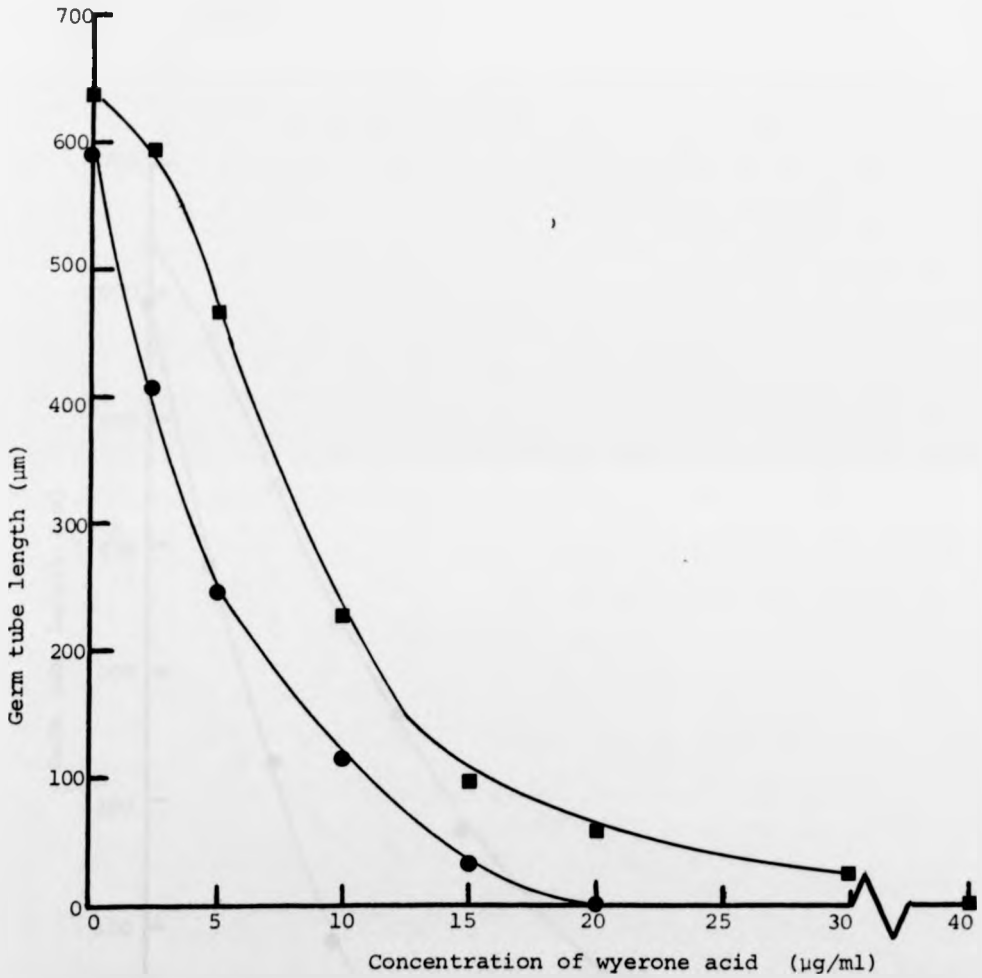


Fig.17 Dosage response curves for growth of germ tubes from conidia of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone acid at pH 4.0

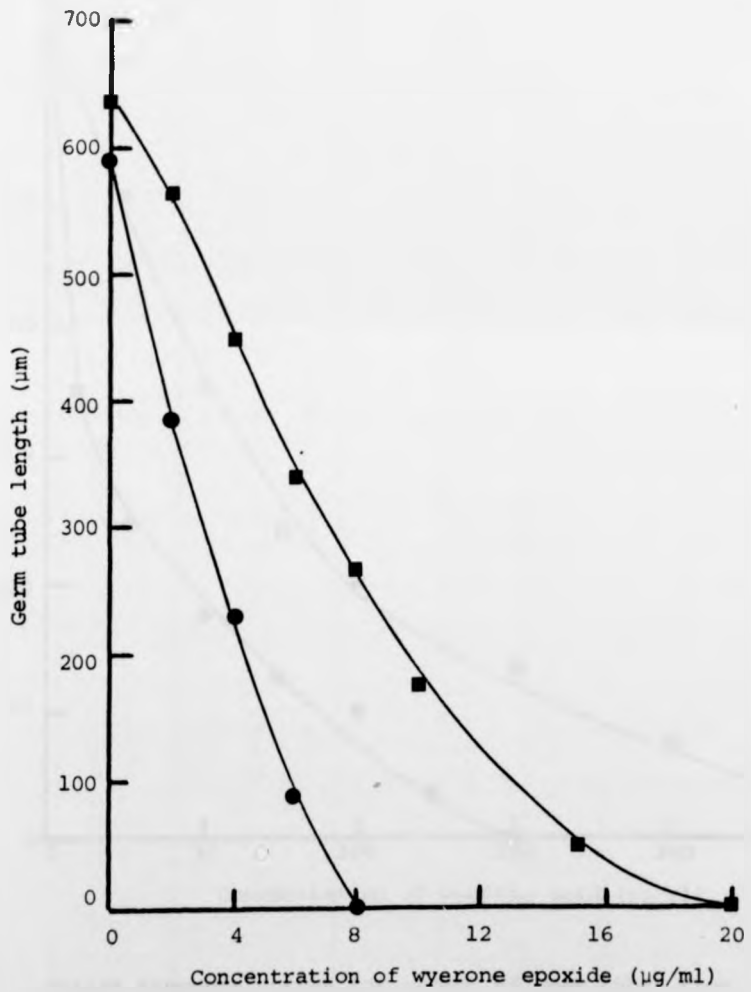


Fig.18 Dosage response curves for growth of germ tubes from conidia of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone epoxide at pH 4.0

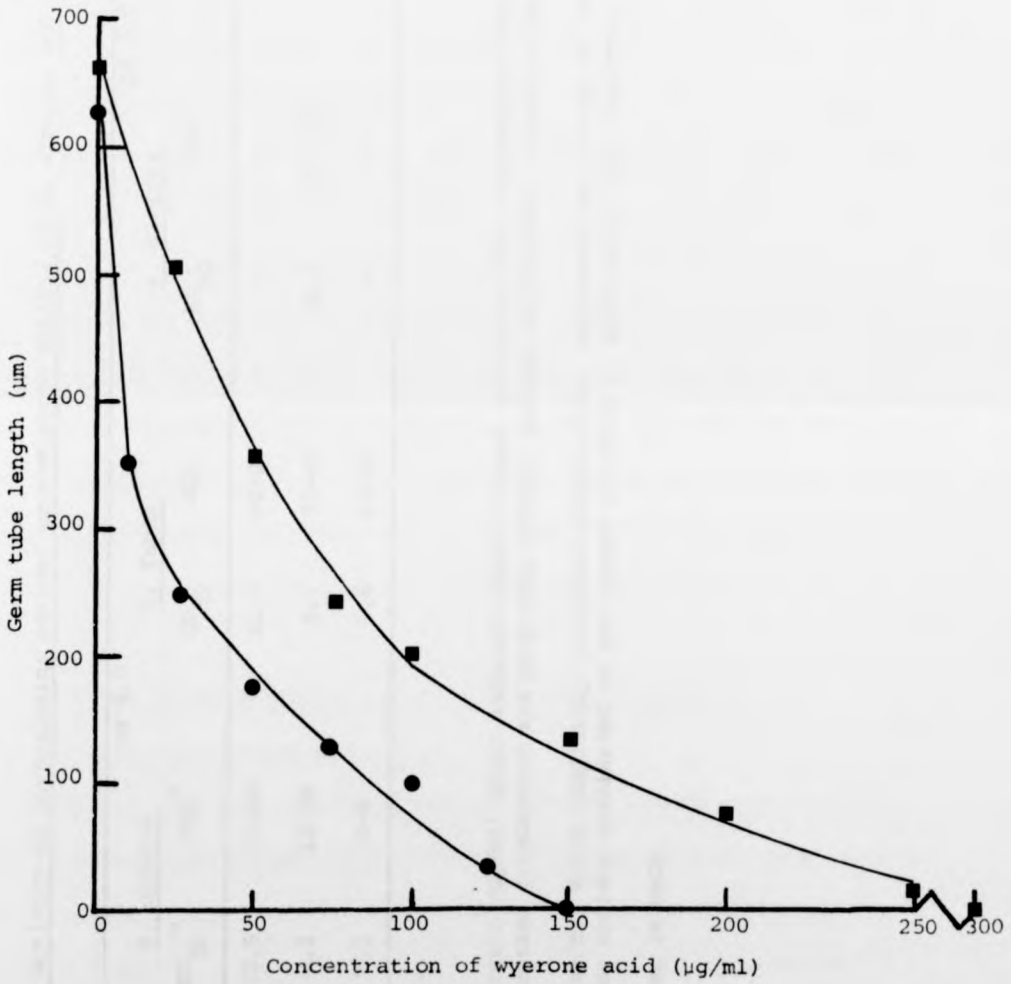


Fig.19 Dosage response curves for growth of germ tubes from conidia of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone acid at pH 5.0

TABLE 25 The antifungal activity of phytoalexins against ungerminated conidia of B. cinerea and B. fabae

Phytoalexin	pH 4.0				pH 5.0			
	B. cinerea		B. fabae		B. cinerea		B. fabae	
	ED_{50}[a]	MID[b]	ED_{50}	MID	ED_{50}	MID	ED_{50}	MID
Wyerone[c]	12.5	30-40	31.0	60-80	-	-	-	-
Wyerone acid	4.2	15-20	8.0	30-40	16.0	125-150	56.0	250-300
Wyerone epoxide[c]	3.1	6-8	6.6	15-20	-	-	-	-

- Not determined

a Concentration of phytoalexin (µg/ml) which reduced overall fungal growth by 50%; determined from graphs of fungal growth (produce of percentage germination and germ tube growth) against phytoalexin concentration.

b Minimum concentration (µg/ml) which completely prevented germination; expressed as a range of concentrations from the highest at which some conidia germinated to the lowest at which no germination was observed.

c Assayed in SPN containing 2% MeOH.

to 300 µg/ml ar pH 5.0. Activity of the phytoalexins was examined against germ tubes (sporelings) of B. cinerea and B. fabae. Results obtained at pH 4.0 are illustrated in Figs. 20-24, and a summary of ED_{50} and MID concentrations, determined at both pH values is given in Table 26.

All the inhibitors assayed were more active against B. cinerea than B. fabae, but differential activity was much less apparent with medicarpin than the wyerone derivatives. Wyerone epoxide was the most active compound tested against sporelings, whereas wyerol possessed comparatively little antifungal activity. The effect of wyerone acid was once again greatly diminished at the higher pH, but pH had little effect on the other inhibitors. The addition of methanol did not significantly alter the activity of the acid. A comparison between Tables 25 and 26 suggests sporelings were more sensitive to wyerone derivatives than ungerminated conidia.

(c) Effect of pH on antifungal activity of wyerone acid

(i) In relation to the pKa of the acid

Preliminary experiments on the antifungal activity of wyerone acid against conidia and sporelings of Botrytis confirmed the findings of Deverall & Rogers (1972) that activity is reduced at high pH. A more detailed study of the effect of pH on activity of wyerone acid towards sporelings was therefore carried out.

The pKa of wyerone acid was determined to be 4.2. By use of the 'Henderson-Hasselbach' equation it was therefore possible to calculate the percentage of the acid which existed in an undissociated state at a given pH (Fig.25).

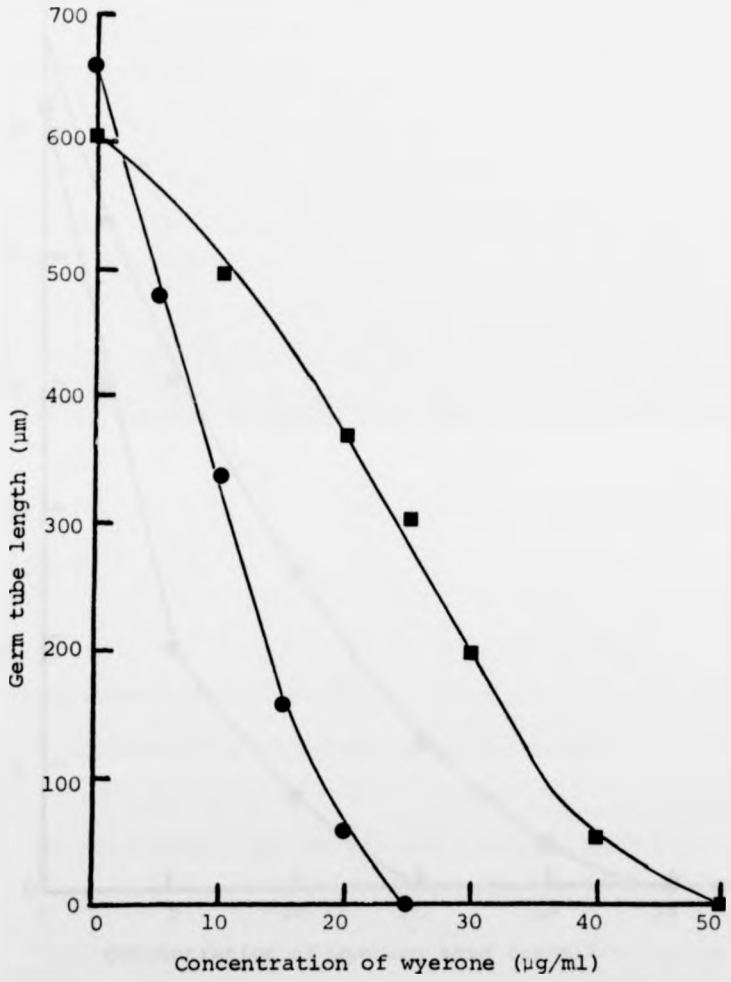


Fig.20 Dosage response curves for the growth of germ tubes from sporelings of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone at pH 4.0

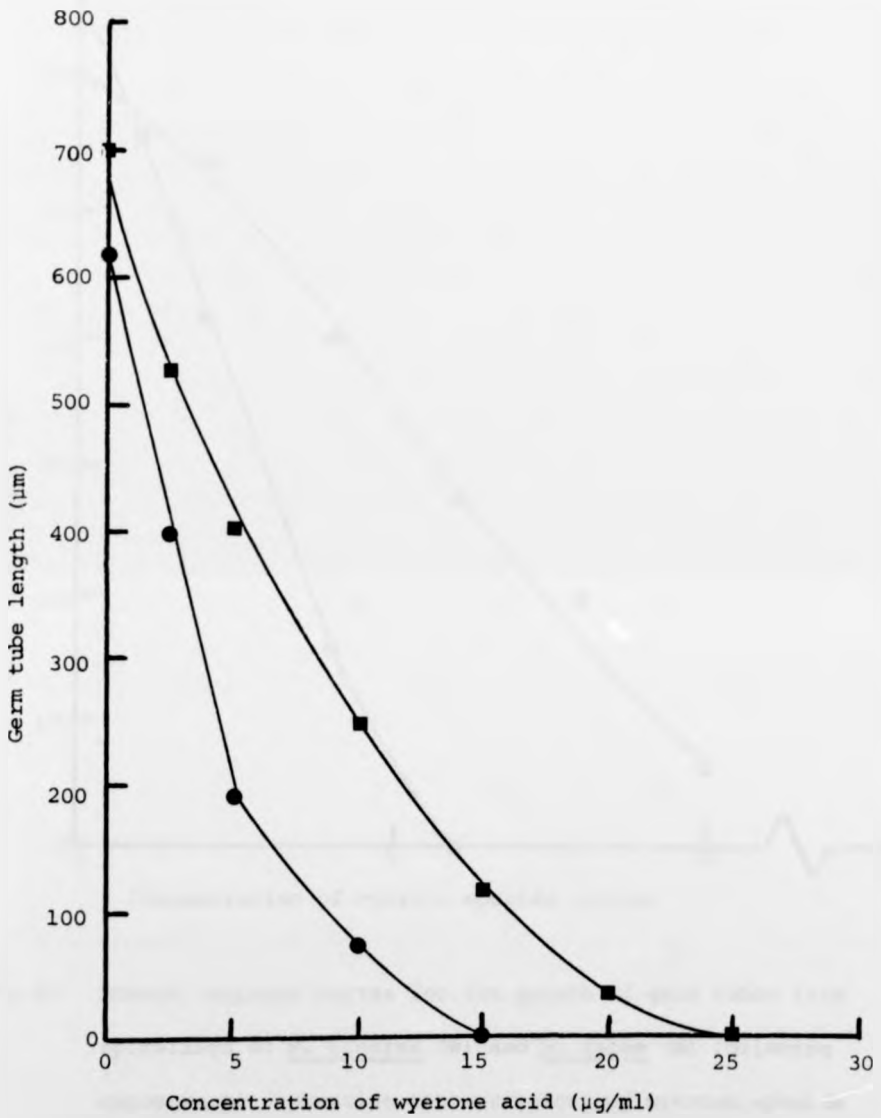


Fig.21 Dosage response curves for the growth of germ tubes from sporelings of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone acid at pH 4.0

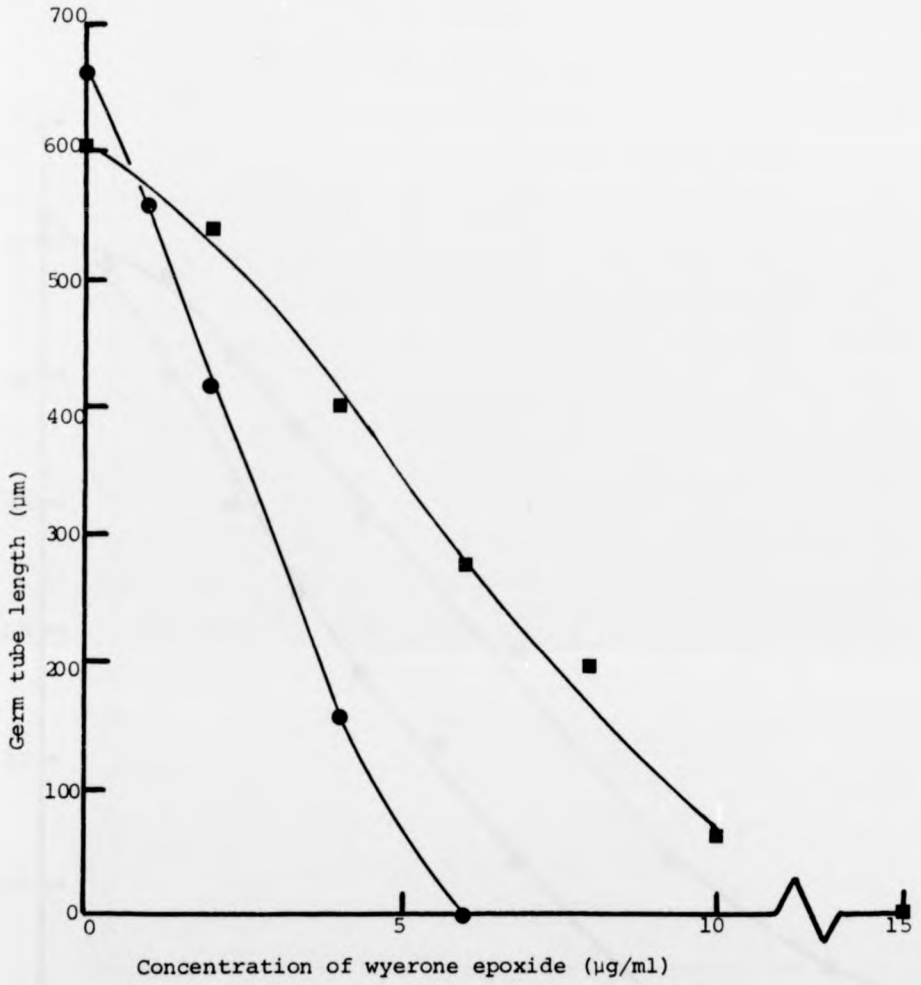


Fig.22 Dosage response curves for the growth of germ tubes from sporelings of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone epoxide at pH 4.0

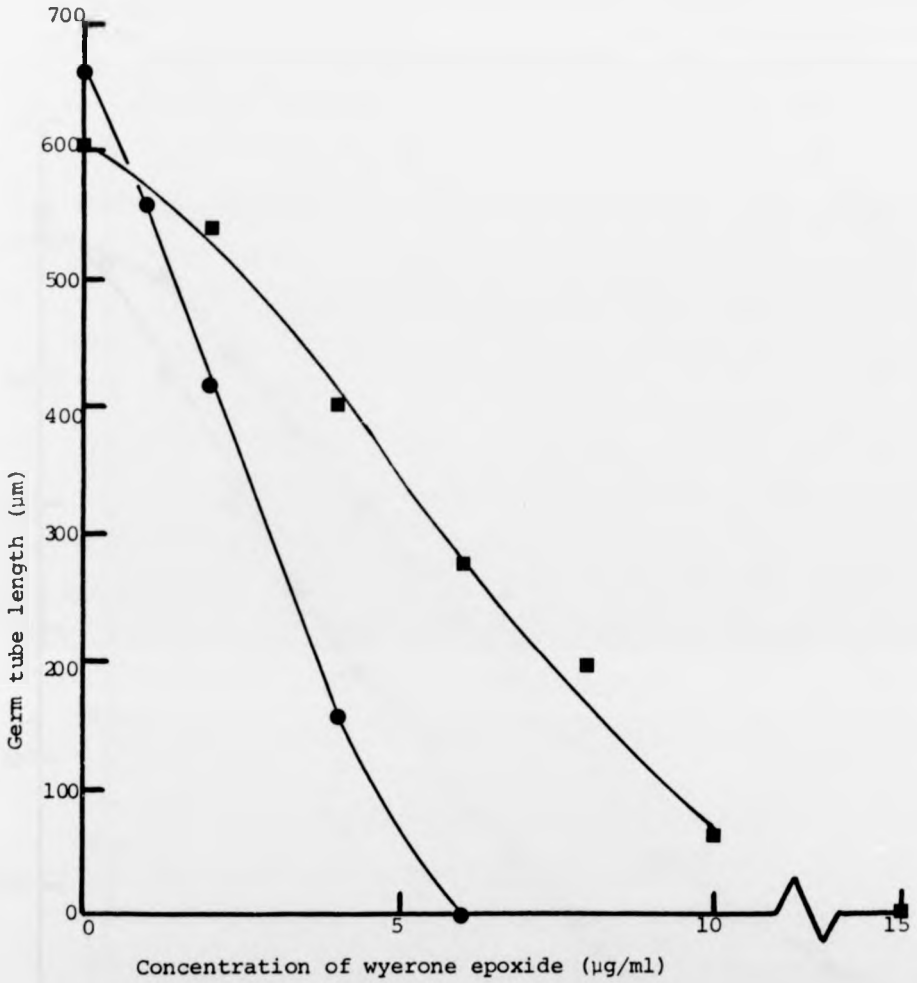


Fig.22 Dosage response curves for the growth of germ tubes from sporelings of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerone epoxide at pH 4.0

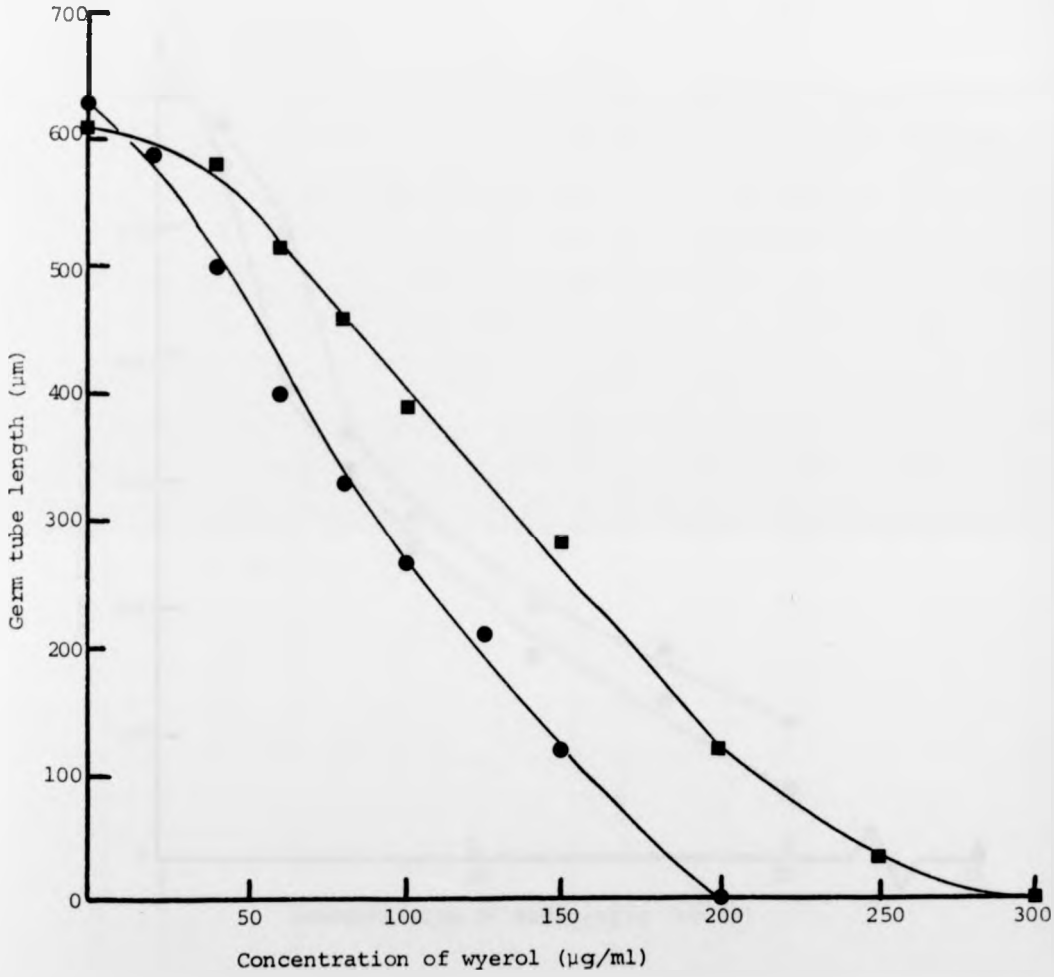


Fig.23 Dosage response curves for the growth of germ tubes from sporelings of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of wyerol at pH 4.0

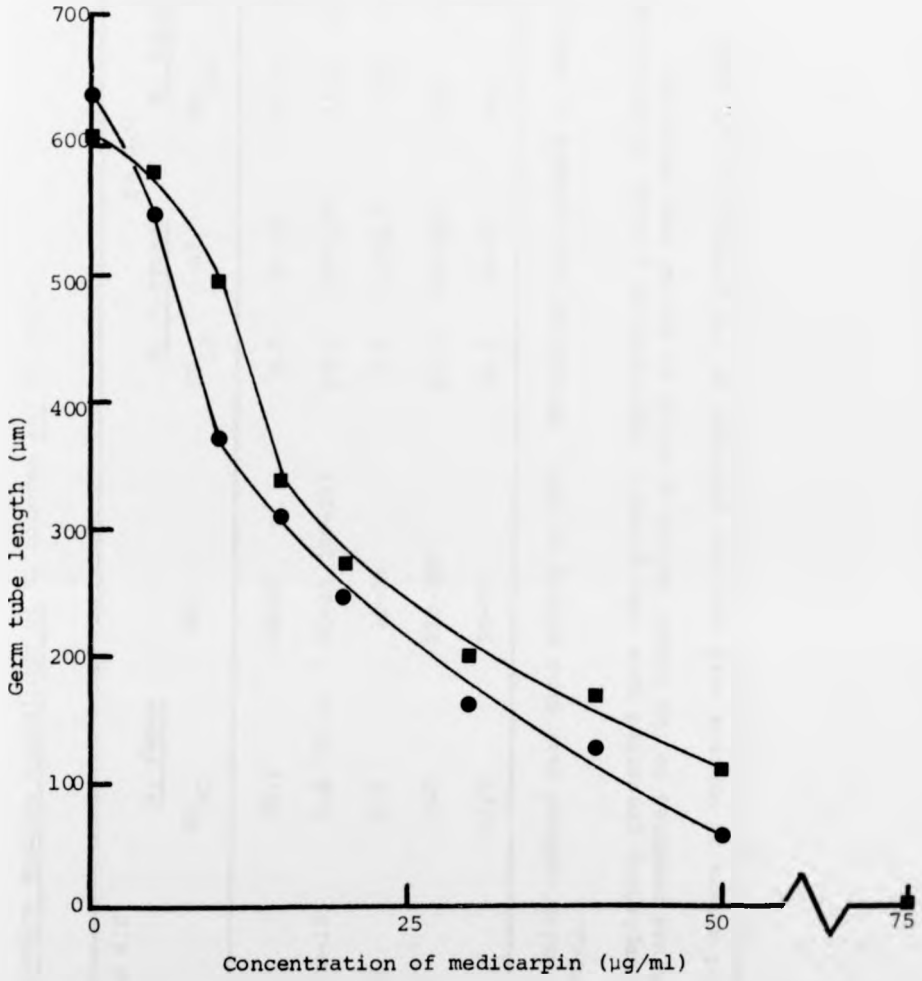


Fig.24 Dosage response curves for the growth of germ tubes from sporelings of B. cinerea (●) and B. fabae (■) following exposure to increasing concentrations of medicarpin at pH 4.0

TABLE 26 Antifungal activity of phytoalexins against sporelings of B. cinerea and B. fabae

Phytoalexins	pH 4.0				pH 5.0			
	B. cinerea		B. fabae		B. cinerea		B. fabae	
	ED_{50}[a]	MID[b]	ED_{50}	MID	ED_{50}	MID	ED_{50}	MID
Wyerone	10.1	20-25	25.0	40-50	9.0	20-25	19.0	40-50
Wyerone acid[c]	3.5 (3-3)	10-15 (10-15)	6.8 (7.0)	20-25 (20-25)	13.0	100-125	42.0	200-250
Wyerone epoxide	2.7	4.0-6.0	5.6	10-15	2.2	4.0-6.0	4.8	10-15
Wyerol	85.0	150-200	140	250-300	83.0	150-200	145	250-300
Medicarpin	14.0	50-75	17.5	50-75	16.2	50-75	23.0	50-75

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

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

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

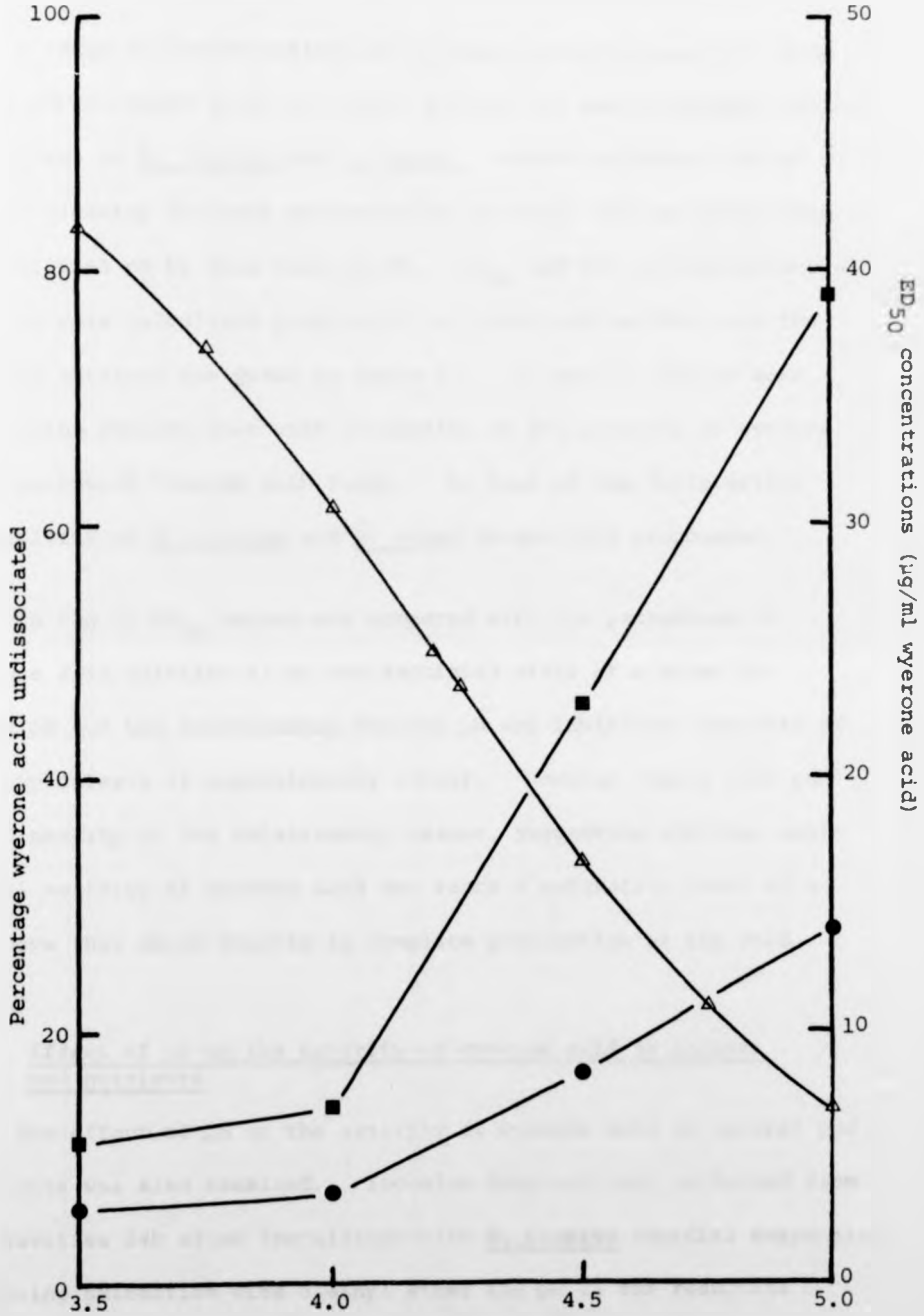


Fig.25 The relationship between pH and ED_{50} concentrations of wyerone acid towards sporelings of B. cinerea (●) and B. fabae (■) and between the percentage of the acid undissociated (Δ)

A range of concentrations of wyerone acid solutions were made up in SPN buffered to pH 3.5, 4.0, 4.5 and 5.0 and bioassayed against sporelings of B. cinerea and B. fabae. After incubation the pH of bulked bioassay droplets was measured and found not to differ from the original pH by more than ±0.05. ED_{50} and MID concentrations at each pH were calculated graphically as described earlier, and the results obtained are given in Table 27. It can be clearly seen from these results that with increasing pH the activity of wyerone acid decreased towards both fungi. At high pH the differential sensitivity of B. cinerea and B. fabae become more pronounced.

In Fig.20 ED_{50} values are compared with the percentage of wyerone acid existing in an undissociated state at a given pH. Above pH 4.0 the relationship between pH and inhibitory activity of the phytoalexin is approximately linear. However, below this pH the linearity of the relationship ceases, suggesting that the anti-fungal activity of wyerone acid may reach a saturation level at a pH above that which results in complete protonation of the acid.

(ii) Effect of pH on the activity of wyerone acid in natural pod nutrients

The effect of pH on the activity of wyerone acid in natural pod nutrients was also examined. Inoculum droplets were collected from pod cavities 24h after inoculation with B. cinerea conidial suspension. Following extraction with diethyl ether the pH of the resultant water phase (pod nutrients) was measured to be 4.2. Aliquots were removed and the pH adjusted to 4.0 and 5.0 by the addition of 0.02M H_3PO_4 or NaOH respectively. A range of concentrations of wyerone acid were prepared in the three solutions and bioassayed against sporelings of B. cinerea and B. fabae as described earlier.

TABLE 27 The effect of pH on the antifungal activity of wyerone acid against sporelings of B. cinerea and B. fabae

Phytoalexins	pH of bioassay droplets							
	3.5		4.0		4.5		5.0	
	ED_{50}[a]	MID[b]	ED_{50}	MID	ED_{50}	MID	ED_{50}	MID
B. cinerea	3.0	5-10	3.6	10-15	8.4	30-40	14.0	100-125
B. fabae	5.6	15-20	7.0	20-25	23.0	75-100	39.0	200-250

a - Concentration of wyerone acid (µg/ml) which reduced germ tube growth by 50%; determined from graphs of germ tube growth against wyerone acid concentration.

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

ED_{50} and MID concentrations were estimated graphically for each pH, and results obtained are given in Table 28. Comparison between these results and those for antifungal activity of wyerone acid in SPN (Tables 26 and 27) shows that the phytoalexin is slightly less active in the synthetic than in the natural medium at pH 4.0 and 5.0.

(d) Antifungal activity of wyerone acid towards a range of species of Botrytis

The activity of wyerone acid was examined against sporelings of a range of Botrytis species. ED_{50} and MID concentrations of the acid were determined graphically from plots of germ tube growth against wyerone acid concentrations at pH 4.0 as previously described and are given in Table 29. Sporelings of approximately 50 µm in length were used as a substrate for bioassays.

The least sensitive of the species tested was the aggressive bean pathogen B. fabae, the weakly pathogenic B. cinerea being the second least sensitive. B. paeoniae was the most readily inhibited species of the non-pathogens tested.

(e) Antifungal activity of wyerone acid towards a range of isolates of B. cinerea and B. fabae

Comparative studies were performed on the sensitivity of four isolates of both B. cinerea and B. fabae to wyerone acid, and their pathogenicities towards leaves of field grown bean plants. Sporelings were used in bioassays, and ED_{50} and MID concentrations of the acid were determined graphically for each of the isolates tested as described previously. Results obtained are given in Table 30. Isolates of B. cinerea and B. fabae differed in their sensitivity to wyerone acid but the most sensitive B. fabae isolate (SS1) was less

TABLE 28 The effect of pH on the antifungal activity of wyerone acid in natural pod nutrients

Fungus tested	pH of bioassay droplets					
	4.0		4.2		5.0	
	ED_{50} [a]	MID	ED_{50}	MID	ED_{50}	MID
B. cinerea	3.1	10-15	4.0	15-20	12.0	100-125
B. fabae	6.3	20-25	8.5	25-30	37.0	200-250

a - Concentration of wyerone acid (µg/ml) which reduced germ tube growth by 50%; determined from graphs of germ tube growth against wyerone acid concentration.

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

TABLE 29 Antifungal activity of wyerone acid at pH 4.0 against sporelings of species of Botrytis

Species tested	Time to produce germ tube of c. 50 µm(h)	ED_{50} [a]	MID [b]
B. allii	12	2.1	5-7.5
B. cinerea	6	3.5	10-15
B. elliptica	6	2.7	7.5-10
B. fabae	6	6.8	20-25
B. narcissicola	8	2.0	5-7.5
B. paeoniae	10	1.2	2.5-5
B. tulipae	7	2.9	10-15

a - Concentration of wyerone acid (µg/ml) which reduced germ tube growth by 50%; determined from graphs of term tube growth against wyerone acid concentration.

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

TABLE 30 Antifungal activity of wyerone acid at pH 4.0 against sporelings of a range of isolates of *B. cinerea* and *B. fabae*

Isolates tested		ED_{50} [a]	MID [b]
B. fabae	(PBI6	10.5	25-30
	(VF2	6.8	20-25
	(Wye	4.2	15-20
	(SS1	3.9	15-20
B. cinerea	(Vn2	3.5	10-15
	(39	3.0	10-15
	(vine	2.8	10-15
	(rose	1.9	5-10

a - Concentration of wyerone acid (µg/ml) which reduced germ tube growth by 50%; determined from graphs of germ tube growth against wyerone acid concentration.

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

inhibited than the most tolerant B. cinerea (Vn 2) (Table 30).

Pathogenicity tests were carried out by inoculating the abaxial surface of leaves detached from field grown plants. Droplets (c. 10 µl) of conidial suspensions were placed on separate half leaflets with a sterile pasteur pipette, six droplets per half leaflet. After incubation for one and three days lesion development caused by isolates of both fungi was recorded using the scheme illustrated in Fig.7. Results obtained are given in Tables 31 and 32 for B. fabae and B. cinerea inoculations respectively. All isolates of B. fabae were more pathogenic than B. cinerea. On the basis of lesion development pathogenicities were ranked as follows:

	Pathogenic						Non-Pathogenic
B. fabae	VF2	>	PBI6	>	Wye	>	SS1
B. cinerea	Vn2	>	39	>	vine	>	rose

With the exception of B. fabae isolates VF2 and PBI6, these rankings closely parallel the comparative sensitivities of different isolates to wyerone acid (Table 30). B. cinerea (Vn2) produced lesions at all inoculation sites, being resistant to leaf surface inhibition.

(f) Effect of wyerone derivatives on rates of growth of B. cinerea and B. fabae

In previous experiments antifungal activity was recorded after exposure to phytoalexins for 18h. A series of experiments was designed to examine the patterns of growth of germ tubes in the presence of wyerone, wyerone acid and wyerone epoxide over a 24h period. Time course studies were carried out on the growth of B. cinerea and B. fabae in the presence of concentrations of each phytoalexin causing 50% reduction in germ tube growth after 18h.

TABLE 31 Lesion development on the abaxial surface of field grown leaves after inoculation with a range of isolates of B. fabae

Infection grade	Percentage of inoculation sites in infection grades							
	BFVF2		BFPBI6		BF Wye		BF SS1	
	1d	3d	1d	3d	1d	3d	1d	3d
0								
3.5							2	
6.5					6		2	
9.5			1		8		4	
19			2		6		10	2
38			7		12		16	2
63	10		10		15		51	2
87.5	57		56		52	5	14	13
100	33		24	16	1	24	1	31
S		4		17		52		32
S1		24		45		19		18
S2		76		22				
S3		2						

TABLE 32 Lesion development on the abaxial surface of field grown leaves after inoculation with a range of isolates of B. cinerea

Infection grade	Percentage of inoculation sites in infection grades							
	Bc Vn 2		Bc 39		Bc vine		Bc rose	
	1d	3d	1d	3d	1d	3d	1d	3d
0			3		6	1	27	6
3.5	1		5		5	1	19	5
6.5	0		5	1	13	1	17	7
9.5	2		16	2	12	2	9	6
19	12	1	19	5	20	7	19	14
38	29	2	31	10	24	9	8	10
63	46	19	19	18	16	22	1	22
87.5	10	67	2	60	4	54		30
100		9		3		3		
S		2		1				

Results obtained are expressed graphically in Figs.26-28.

At the concentrations of inhibitor selected both B. cinerea and B. fabae had similar growth patterns, however, clear differences existed between patterns of growth of Botrytis in the presence of wyerone acid compared to wyerone and wyerone epoxide. With the latter methylated derivatives a lag in growth over the first 6h incubation was observed, whereas with wyerone acid growth of germ tubes appeared to begin from the onset of incubation.

(g) Relationship between metabolism of phytoalexins and growth patterns of germ tubes

Experiments were designed to examine the relationship between the growth of Botrytis germ tubes and metabolism of phytoalexins. Disappearance of phytoalexins was monitored in bathing fluids collected from petri dishes in which fungal growth was subsequently measured. Metabolism of wyerone derivatives is associated with the reduction of the keto group to an alcohol, causing a shift in λ max of the UV spectrum. In SPN solution the shifts are from 360, 366 and 350 nm to c. 315 nm for wyerone, wyerone acid and wyerone epoxide respectively, enabling loss of the phytoalexins to be measured spectrophotmetrically.

Preliminary experiments were carried out to determine the concentration of each phytoalexin allowing germ tube growth of c. 280 µm after 24h. Replicate dishes of sporelings of both B. cinerea and B. fabae were incubated with each of the phytoalexins in SPN solution. At 4h intervals the concentration of remaining phytoalexin and germ tube length were estimated in two replicate dishes. Percentage loss of inhibitor was calculated allowing correction for changes detected in control petri dishes in the absence

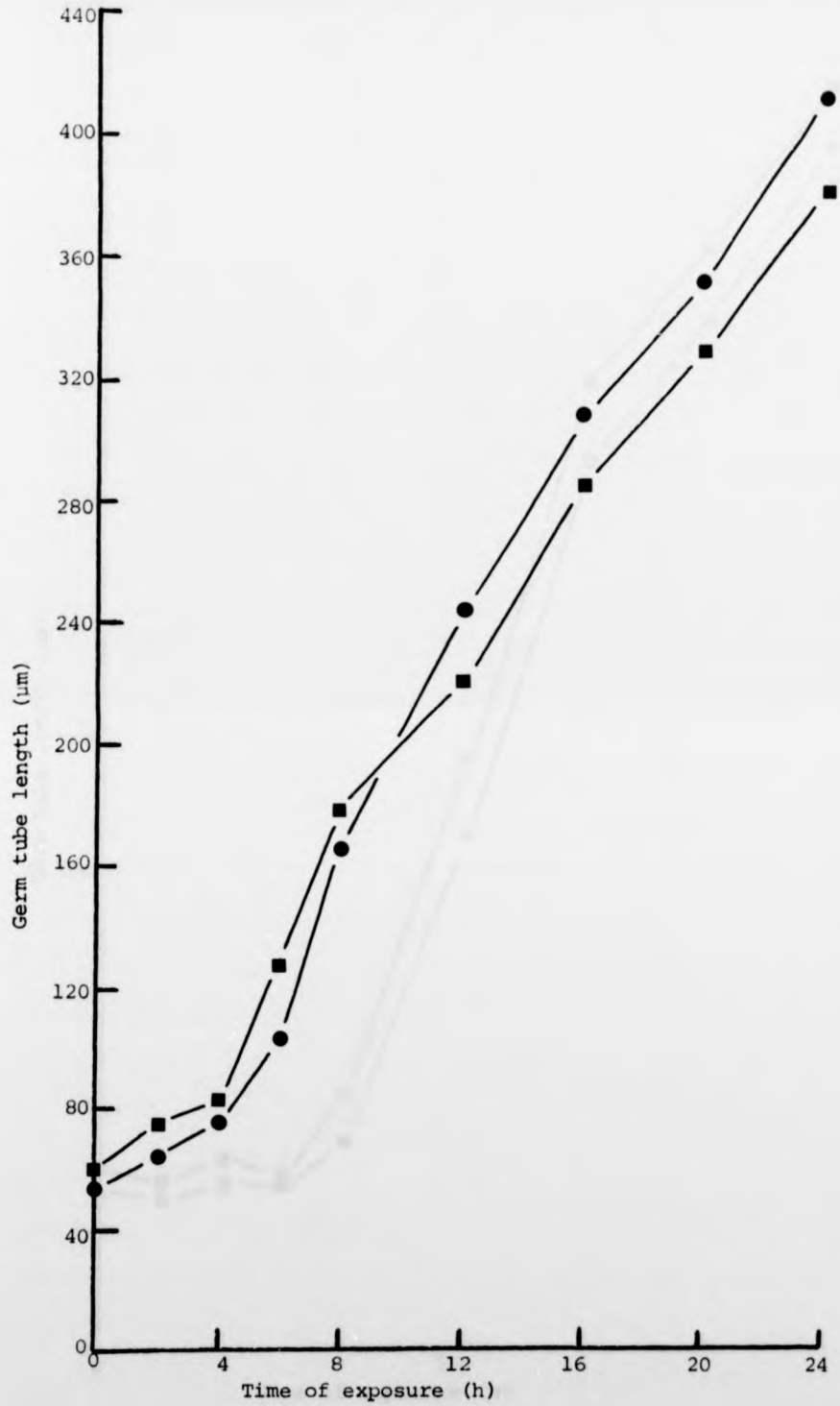


Fig.26 Rates of growth of B. cinerea (●) and B. fabae (■) in the presence of 3.4 and 6.9 µg/ml wyerone acid respectively

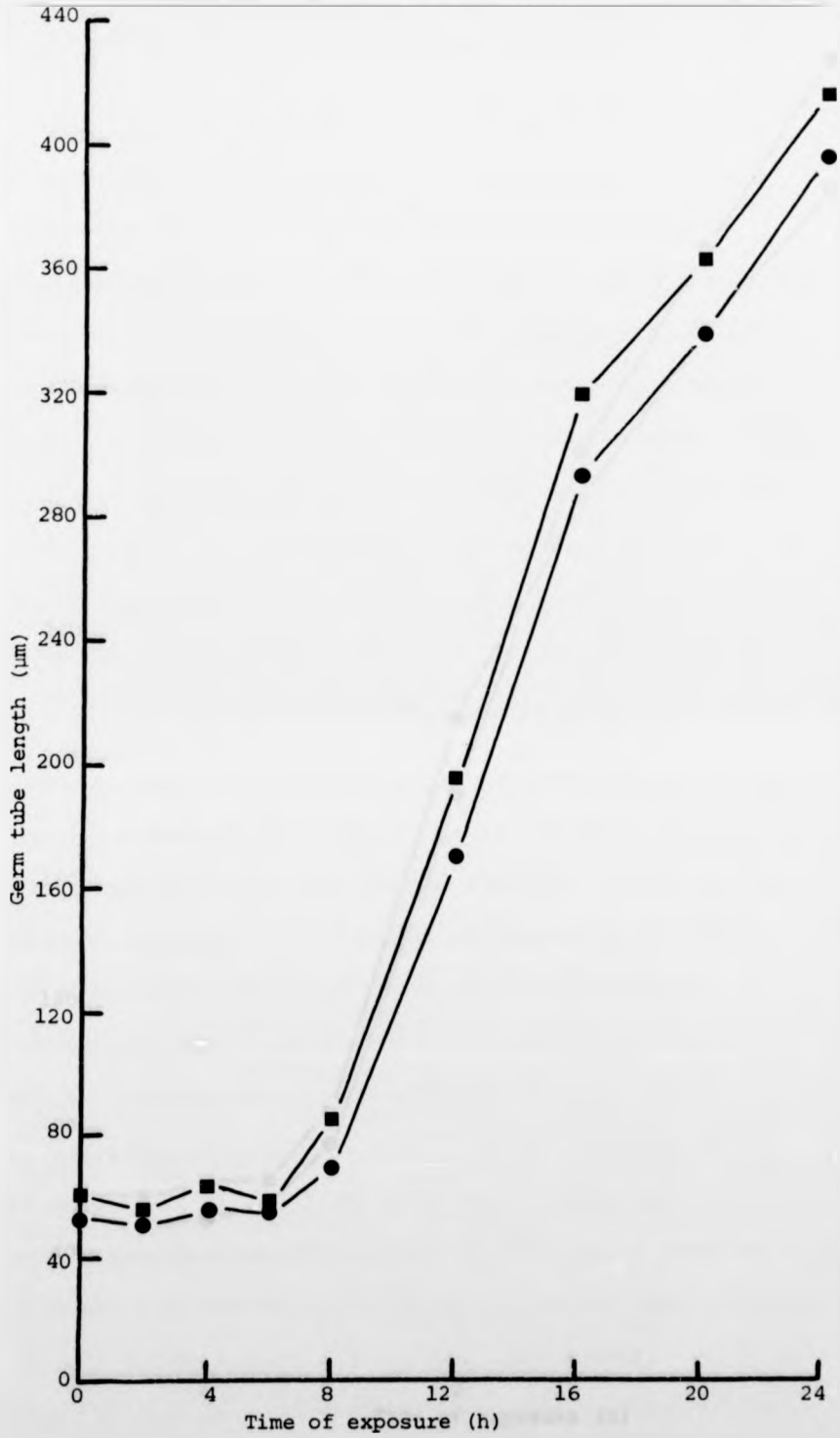


Fig.27 Rates of growth of B. cinerea (●) and B. fabae (■) in the presence of 9.6 and 21.0 µg/ml wyerone respectively

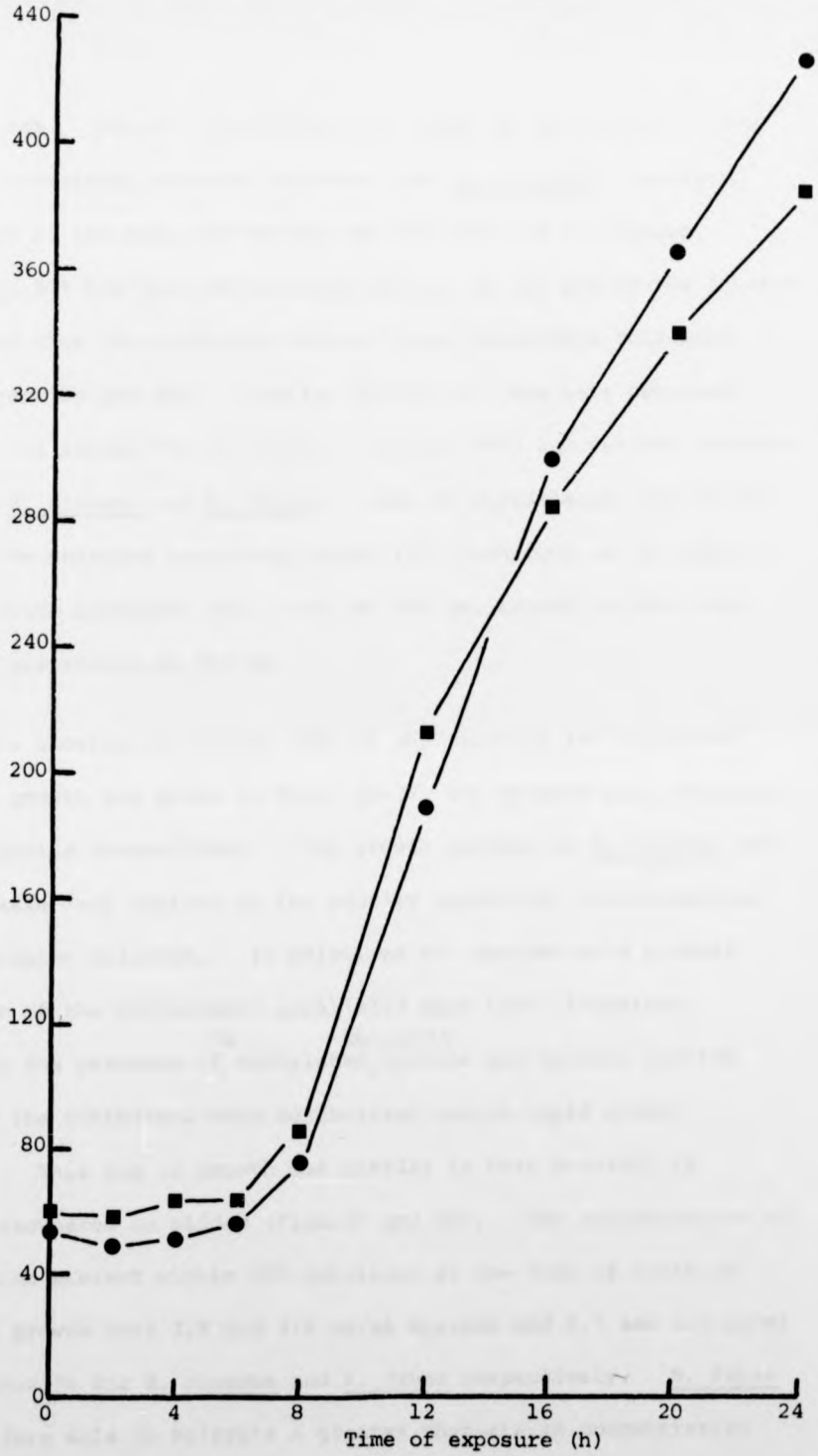


Fig.28 Rates of growth of B. cinerea (●) and B. fabae (■) in the presence of 2.5 and 5.2 µg/ml wyerone epoxide respectively

of sporelings. Fig.29 illustrates the change in absorption of SPN solutions containing wyerone incubated with B. cinerea. Spectrum (a) is that of the pure phytoalexin at the onset of incubation, (b) when c. 50% had been metabolized and (c) at the end of the incubation period when the inhibitor was no longer detectable following ether extraction and TLC. Similar shifts in λ max were obtained following the incubation of wyerone, wyerone acid and wyerone epoxide with both B. cinerea and B. fabae. Loss of phytoalexins beyond 80% could not be measured accurately using this technique as the edge of the metabolite spectrum, with λ max at 315 nm, tended to give high values of absorption at 360 nm.

Graphs showing percentage loss of phytoalexins and concurrent germ tube growth are given in Figs. 30-32, for wyerone acid, wyerone and the epoxide respectively. The growth pattern of B. cinerea and B. fabae were very similar at the equally inhibitory concentrations of phytoalexins selected. In solutions of wyerone acid gradual metabolism of the phytoalexin paralleled germ tube elongation, whereas in the presence of the methylated derivatives, wyerone and wyerone epoxide, c. 60% of the inhibitors were metabolized before rapid growth occurred. This lag in growth was similar to that observed in droplets incubated on slides (Figs.27 and 28). The concentration of phytoalexins present within SPN solutions at the time of onset of germ tube growth were 2.9 and 4.2 µg/ml wyerone and 0.7 and 3.0 µg/ml wyerone epoxide for B. cinerea and B. fabae respectively. B. fabae was therefore able to tolerate a greater phytoalexin concentration at the onset of rapid germ tube elongation. The results suggest that different relationships exist between sensitivity to and ability to metabolize wyerone acid and the methylated phytoalexins, wyerone and wyerone epoxide.

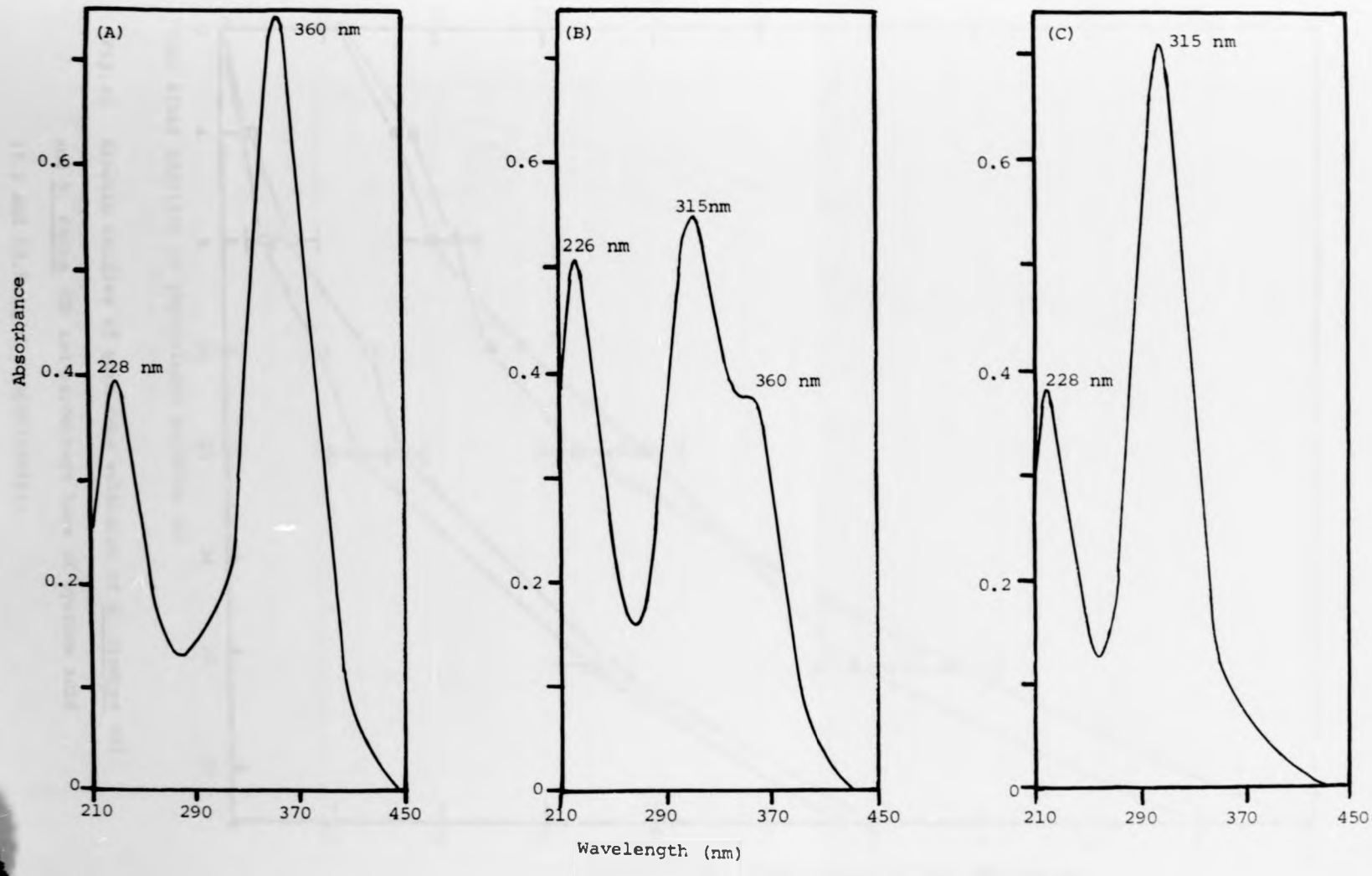
Fig.29

UV absorption spectra of wyerone after exposure to sporelings of B. cinerea:

(A) Oh exposure

(B) 8h exposure

(C) 16h exposure


(A)
360 nm
228 nm
(B)
315nm
226 nm
360 nm
(C)
315 nm
228 nm
Absorbance
0.6
0.4
0.2
0
210
290
370
450
Wavelength (nm)

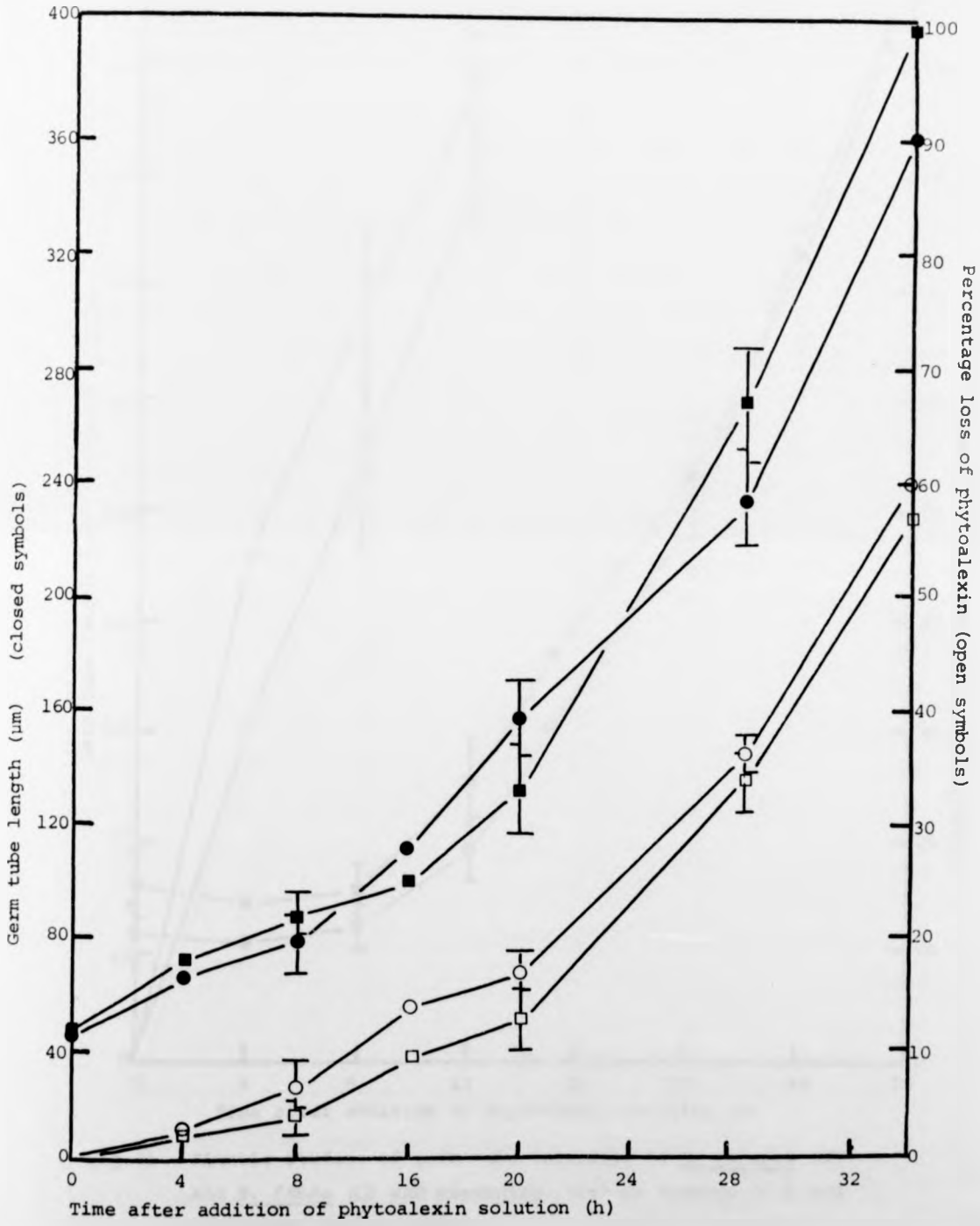


Fig.30 Kinetic studies of germ tube extension of B. cinerea (O) and B. fabae (□) and percentage loss of wyerone acid (3.5 and 13.5 µg/ml respectively).
Bars refer to 95% confidence limits for germ tube lengths and range between replicates for phytoalexin loss.

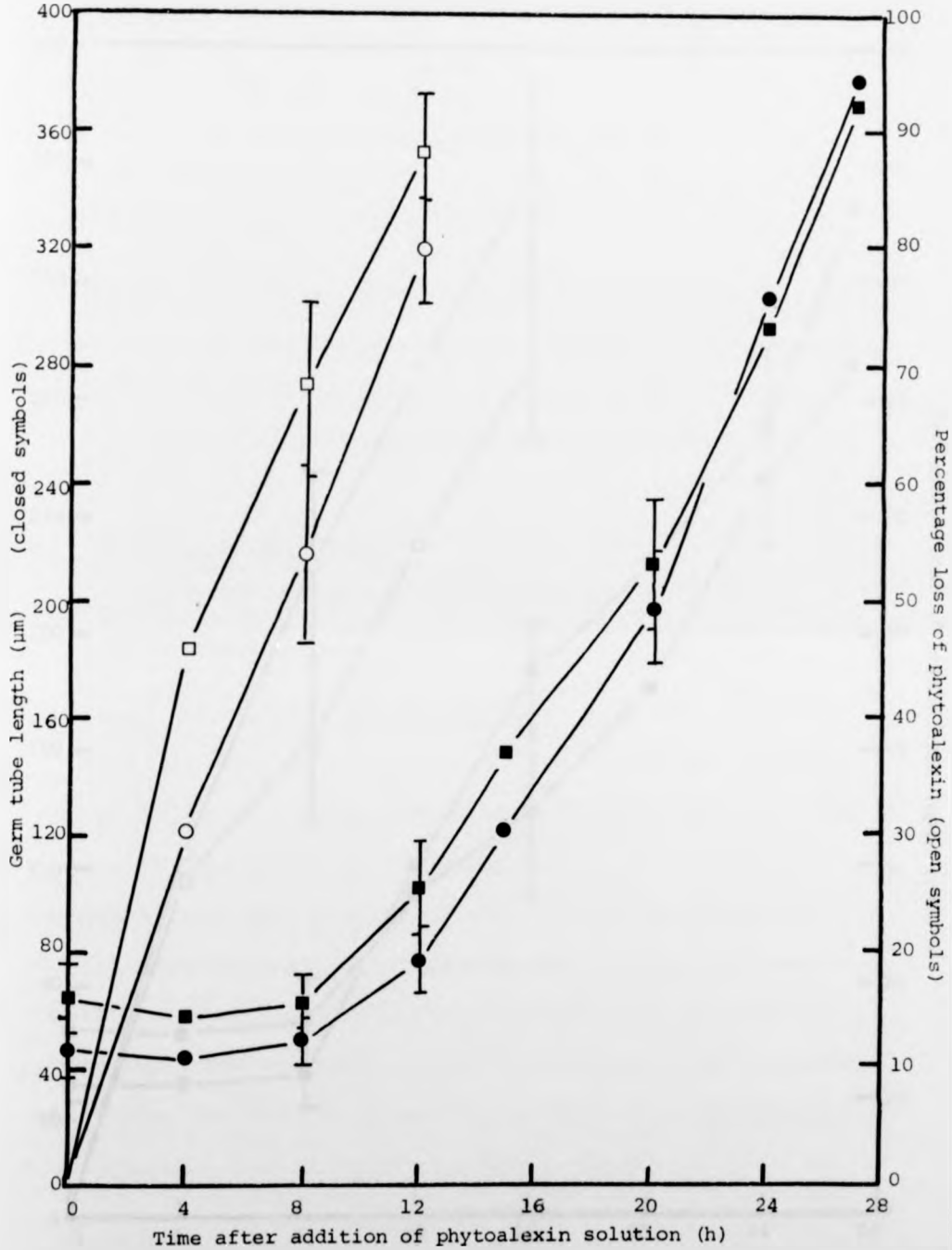


Fig.31 Kinetic studies of germ tube extension of B. cinerea (O) and B. fabae (□) and percentage loss of wyerone (6.5 and 13.0 µg/ml respectively)

Bars refer to 95% confidence limits for germ tube lengths and range between replicates for phytoalexin loss.

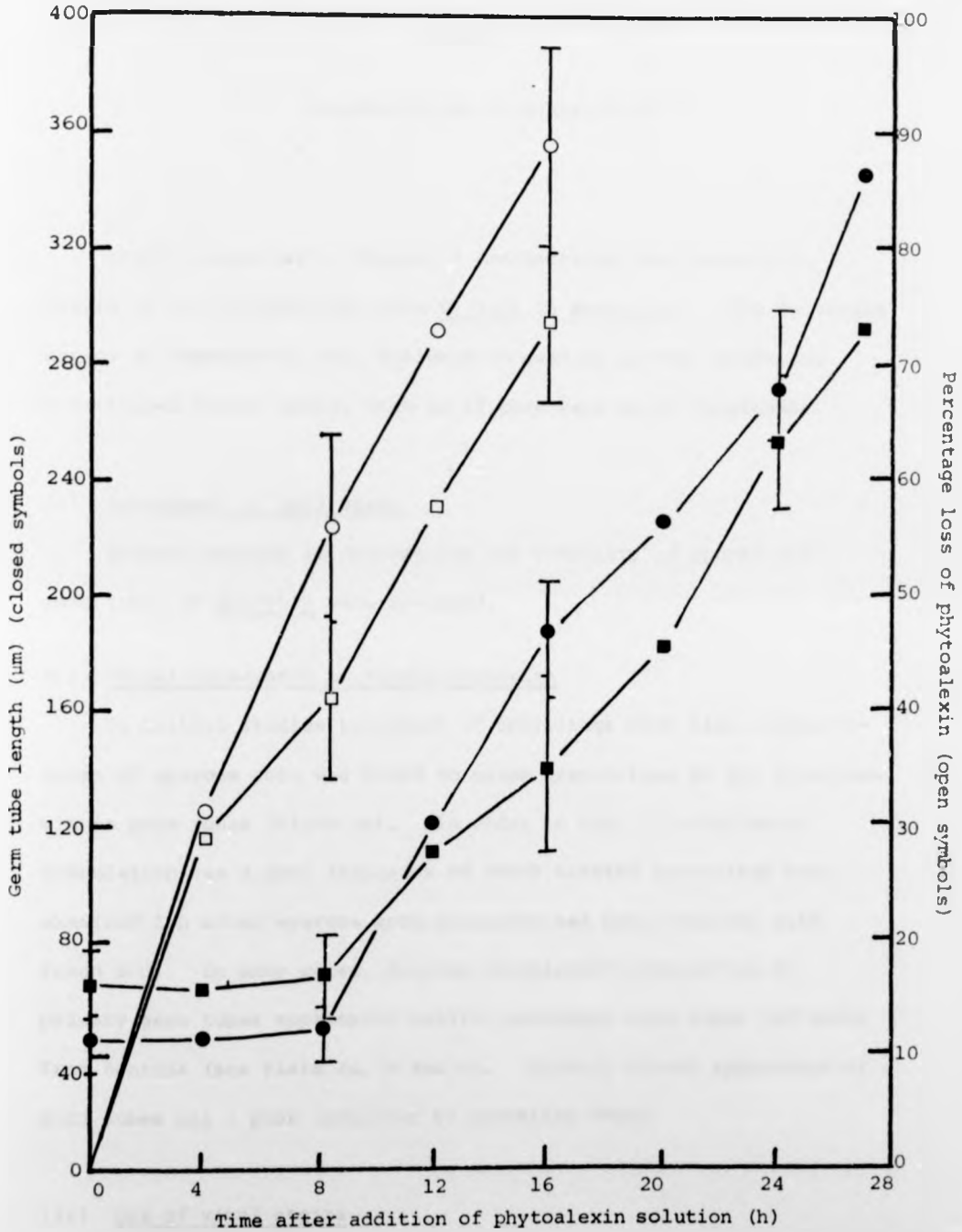


Fig.32 Kinetic studies of germ tube extension of B. cinerea (O) and B. fabae (□) and percentage loss of wyerone epoxide (1.6 and 5.0 µg/ml respectively).

Bars refer to 95% confidence limits for germ tube lengths and range between replicates for phytoalexin loss.

CHAPTER 2

FUNGITOXICITY OF PHYTOALEXINS

Results reported in Chapter 1 demonstrated the fungistatic nature of the phytoalexins from V. faba to Botrytis. The following series of experiments were designed to examine if the inhibitors also killed fungal cells, that is if they were truly fungitoxic.

(a) Assessment of cell death

Several methods of determining the viability of spores and germ tubes of Botrytis were assessed.

(i) Visual appearance of fungal structure

In initial studies treatment of sporelings with high concentrations of wyerone acid was found to cause granulation of the cytoplasm within germ tubes (Plate 2b). In order to test if cytoplasmic granulation was a good indicator of death treated sporelings were examined 10h after wyerone acid solutions had been replaced with fresh SPN. In many cases, despite cytoplasmic granulation of primary germ tubes apparently healthy secondary germ tubes had grown from conidia (see Plate 2a, b and c). Clearly visual appearance of germ tubes was a poor indicator of sporeling death.

(ii) Use of vital stains

An unequivocal method of determining sporeling death was to replace phytoalexin solution with fresh SPN and examine the subsequent continued growth of surviving sporelings. However, this technique was extremely time consuming, and the prolonged

Plate 2

Sporelings of *B. fabae*:

(a) healthy

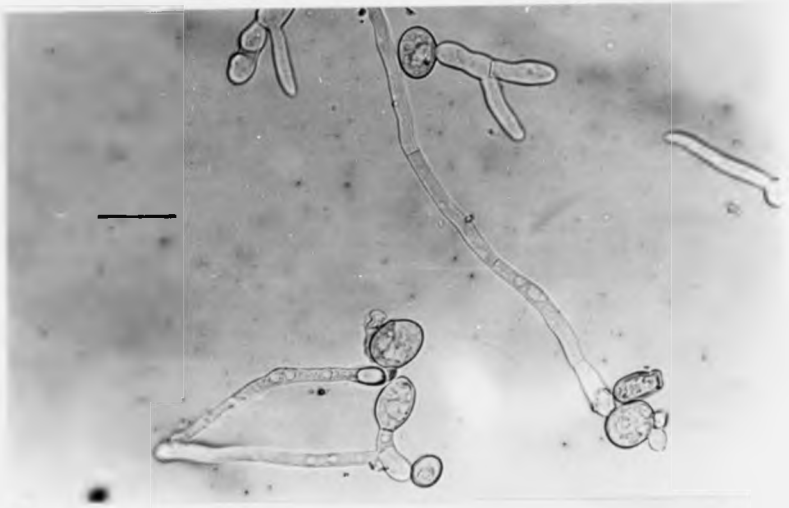
(b) 6h after exposure to 20 µg/ml wyerone acid (note cytoplasmic granulation)

(c) 16h after exposure to 20 µg/ml wyerone acid (note regrowth of apparently healthy germ tube from conidium)

Bar = 25 µm.

wyerone acid

wyerone acid
ealthy germ



a

b

c

Plate 2

incubation period required also increased the likelihood of bacterial contamination of bioassay droplets. Of the range of stains used, Evan's blue, neutral red and phenosafranine were found to stain both living and dead fungal cells and data obtained using fluorescein diacetate were variable. Trypan blue, however, stained only dead tissue and a detailed comparison of viability, assessed using this stain and solution replacement, was therefore carried out.

Sporelings of B. cinerea and B. fabae were grown in droplets of SPN on glass then exposed to 10, 20 and 40 µg/ml wyerone acid and 20, 40 and 60 µg/ml wyerone acid respectively. After incubation for 8, 16 and 24h, the percentage of sporelings alive was assessed for each treatment. Four replicate droplets were tested for each technique. Results obtained, expressed as percentages of sporelings killed, are given in Table 33.

Results suggested that trypan blue staining is an accurate indication of fungal cell viability. SPN replacement tended to give slightly higher figures for percentage kill but this may have been a reflection of the presence of residual phytoalexin around sporelings after washing. At any given concentration B. cinerea was more readily killed than B. fabae and the percentage mortality increased with time of exposure for both fungi. In subsequent experiments trypan blue vital staining was used to assess the fungitoxicity of phytoalexins.

TABLE 33 A comparison between trypan blue vital staining and solution replacement as methods of assessing death of sporelings

Fungus	Time of incubation (h)	Percentage kill at phytoalexin concentrations ± standard error							
		10 µg/ml		20 µg/ml		40 µg/ml		60 µg/ml	
		Stain	Replace	Stain	Replace	Stain	Replace	Stain	Replace
B. cinerea	8	0	0	18.5 ± 1.3	19.8 ± 1.4	42.8 ± 2.2	46.3 ± 1.9	-	-
	16	4.3 ± 0.6	4.8 ± 1.0	49.3 ± 2.3	53.3 ± 5.0	83.8 ± 4.7	88.0 ± 3.3	-	-
	24	8.5 ± 1.0	8.5 ± 1.2	61.3 ± 4.0	64.0 ± 3.6	100	100	-	-
B. fabae	8	-	-	13.0 ± 1.7	14.5 ± 1.6	29.8 ± 3.8	34.0 ± 3.2	60.3 ± 4.4	59.0 ± 2.7
	16	-	-	19.3 ± 3.4	26.0 ± 3.9	57.0 ± 4.5	55.8 ± 3.6	88.5 ± 4.7	90.0 ± 3.0
	24	-	-	23.0 ± 2.6	26.8 ± 3.3	70.5 ± 4.6	74.3 ± 2.3	100	100

- Not determined

(b) Fungitoxicity of wyerone, wyerone acid and wyerone epoxide

A range of concentrations of wyerone, wyerone acid and wyerone epoxide were prepared in SPN buffered to pH 4.0. Sporelings of B. cinerea and B. fabae were treated with the inhibitors and incubated for 24h, four replicate droplets being prepared for each assay. After incubation the mean percentage kill was calculated for each treatment from four replicate counts of 100 sporelings. A dead sporeling was recognized either by its failure to grow in SPN or by deep staining of both the germ tube and conidium with trypan blue.

Results obtained, given in Fig.33, have been plotted as linear regressions, permitting rapid comparison between treatments. In all cases B. fabae was less readily killed than B. cinerea and the minimum lethal dose (MID) concentrations to give 100% kill by 24h are summarized in Table 34. Those for B. fabae are c. 40% higher than those for B. cinerea. MID concentrations were higher than those causing complete inhibition of fungal growth (Table 26). The most toxic inhibitor tested was wyerone epoxide, followed by wyerone acid and wyerone, but the difference in toxicities between wyerone epoxide and acid was less than exhibited for fungistatic activity.

(c) Effect of time of exposure on the toxicity of wyerone acid to Botrytis

The effect of time of exposure on the toxicity of wyerone acid to sporelings of B. cinerea and B. fabae was examined. Sporelings of B. cinerea were exposed to 10, 20 and 40 µg/ml. At 4h intervals the percentage of sporelings dead was assessed in four replicate droplets for each treatment. Mean values calculated are given in Fig.34. For each concentration of wyerone acid tested the percentage of sporelings killed increased with time of incubation, and B. cinerea was killed more rapidly than B. fabae.

Fig.33

Effect of increasing concentrations of phytoalexin on the percentage of sporelings of *B. cinerea* and *B. fabae* killed after incubation for 24h.

(A) wyerone: *B. cinerea* (O) $y = 1.62x - 11.71$

B. fabae (●) $y = 1.05x - 14.86$

(B) wyerone acid: *B. cinerea* (□) $y = 3.54x - 20.57$

B. fabae (■) $y = 2.15x - 21.90$

(C) wyerone epoxide: *B. cinerea* (Δ) $y = 3.20x + 2.58$

B. fabae (▲) $y = 2.23x - 17.50$

Bars refer to range between four replicates

lexin on the

fabae

1.71

4.86

- 20.57

- 21.90

20x + 2.58

23x - 17.50

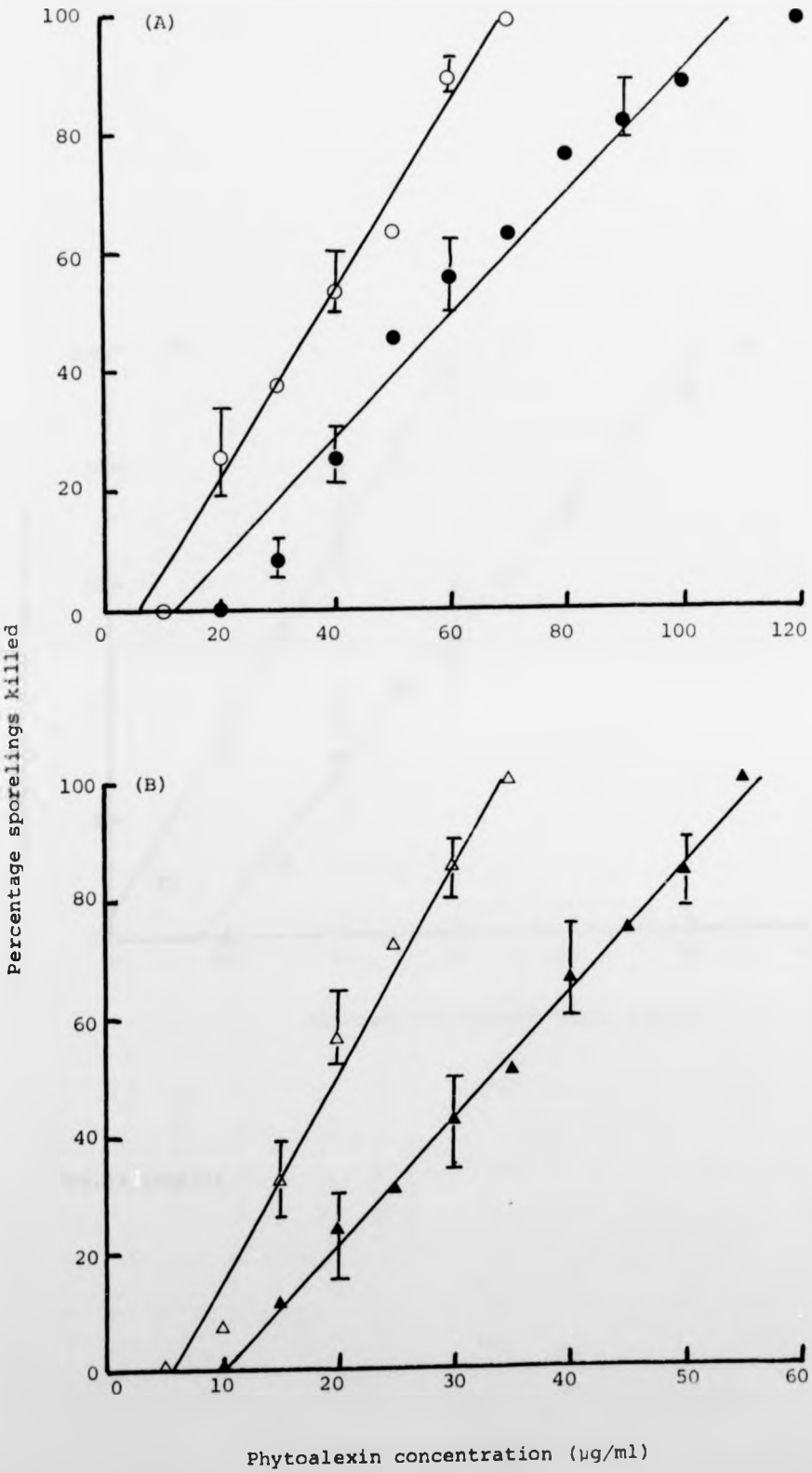


Fig.33

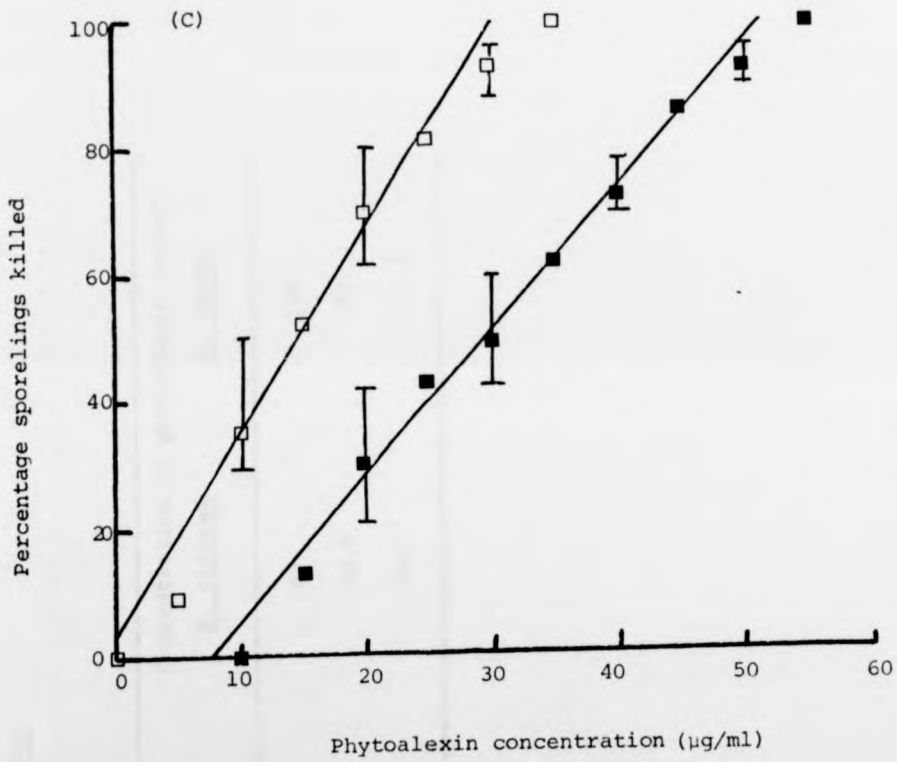

(C)
100
80
60
40
20
0
Percentage sporelings killed
0
10
20
30
40
50
60
Phytoalexin concentration (µg/ml)

Fig.33 (cont.)

TABLE 34 Minimum concentrations of phytoalexins to kill 100% of a population of B. cinerea and B. fabae sporelings after 24h incubation

Phytoalexin	Concentration of phytoalexin (µg/ml)	
	B. cinerea	B. fabae
Wyerone	69	109
Wyerone acid	34.5	57
Wyerone epoxide	30.5	51.5

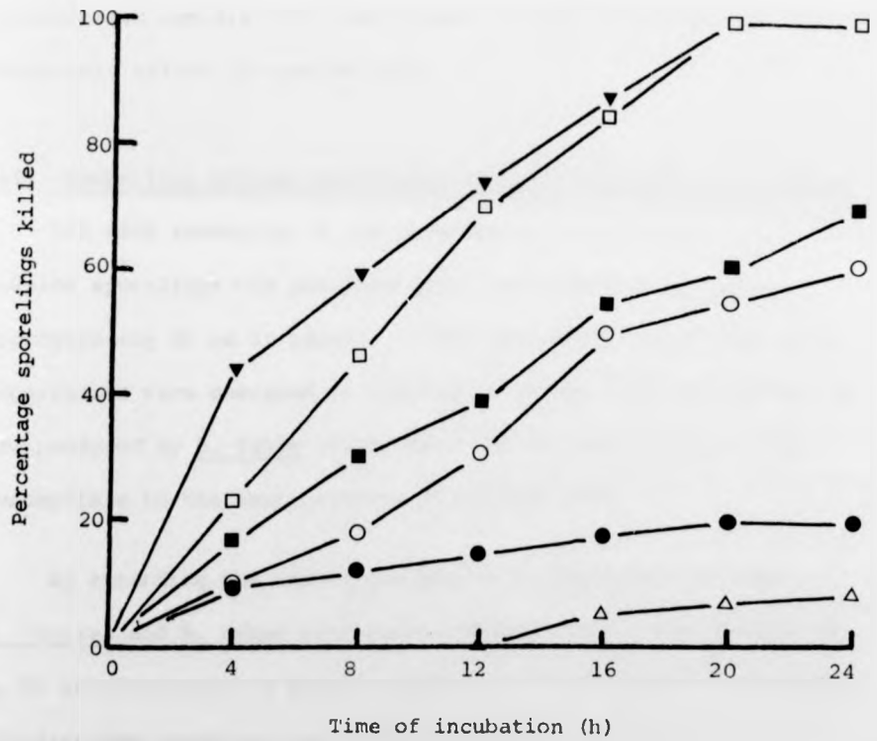


Fig.34 Effect of time of exposure on toxicity of wyerone acid against sporelings of B. cinerea (open symbols) and B. fabae (closed symbols);
Δ = 10 µg/ml, O = 20 µg/ml, □ = 40 µg/ml and
▽ = 60 µg/ml/

(d) Effect of stage of fungal growth on toxicity of wyerone acid to Botrytis

(i) Comparison between sporelings and ungerminated conidia

Sporelings and conidia of both B. cinerea and B. fabae were treated with a range of concentrations of wyerone acid. After incubation for 24h the mean percentage kill was calculated from four replicate counts for each treatment. Results obtained (Fig.35) confirmed the greater tolerance of B. fabae and also indicated that ungerminated conidia were less sensitive than sporelings to the fungitoxic effect of wyerone acid.

(ii) Comparison between sporelings of differing germ tube lengths

All data referring to the fungitoxicity of wyerone derivatives towards sporelings was obtained using germ tubes of Botrytis approximately 50 µm in length. Such germ tubes are single cells. Experiments were designed to examine if larger, septate hyphae, as are produced by B. fabae within bean leaves, were more or less susceptible to the toxic effects of wyerone acid.

By extending the incubation period in SPN from 6-9h both B. cinerea and B. fabae produced sporelings with a mean length of c. 80 µm, mostly with a single septum in the non-branched germ tube, dividing the sporeling into conidium, cell 1 and cell 2. A comparison between the effect of wyerone acid on the death of sporelings with germ tubes of one or two cells was carried out. Sporelings of B. cinerea were exposed to 10, 20 and 40 µg/ml and those of B. fabae to 20, 40 and 60 µg/ml wyerone acid, and the mean percentage killed calculated from four replicated counts after incubation for 8, 16 and 24h. Results obtained are given in Table 35, and once more confirm that B. cinerea is more easily killed than B. fabae

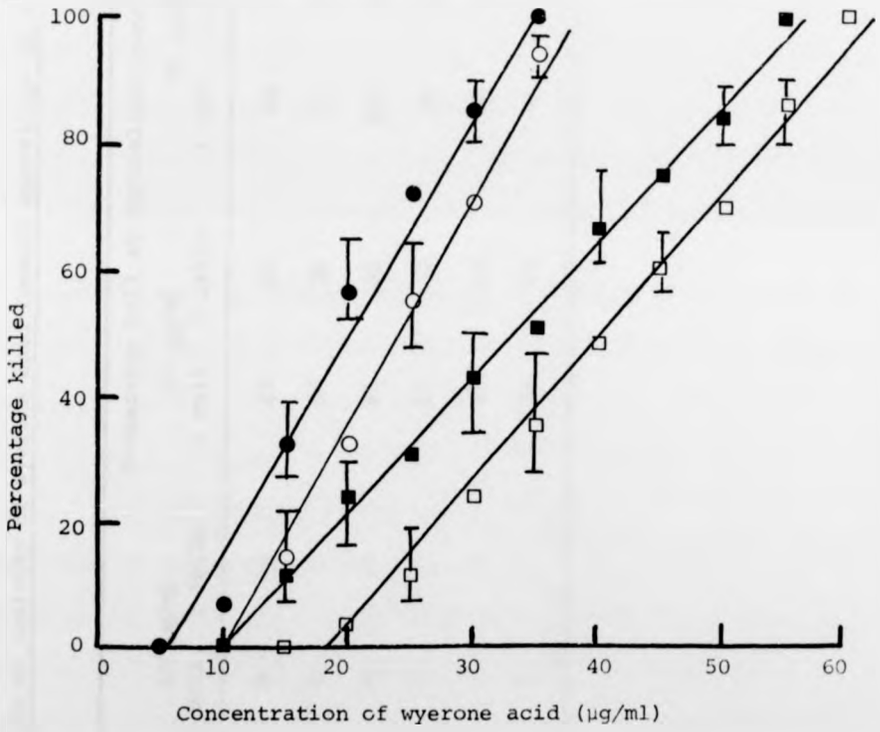


Fig.35 Comparison between the fungitoxicity of wyerone acid against sporelings (closed symbols) and ungerminated conidia (open symbols) of B. cinerea (O) and B. fabae (□).

● y = 3.54x - 20.57 O y = 3.56x = 36.82

■ y = 2.15x - 21.90 □ y = 2.29x - 42.11

Bars refer to range between four replicates.

TABLE 35 Effect of germ tube length on toxicity of wyerone acid towards sporelings of B. cinerea and B. fabae

Fungus	Time of incubation (h)	Percentage kill at phytoalexin concentrations							
		10 µg/ml		20 µg/ml		40 µg/ml		60 µg/ml	
		1 cell	2 cells	1 cell	2 cells	1 cell	2 cells	1 cell	2 cells
B. cinerea	8	0	0	23	11	48	33	-	-
	16	6	0	54	36	81	62	-	-
	24	8	0	66	39	100	92	-	-
B. fabae	8	-	-	15	4	30	26	63	42
	16	-	-	22	14	56	31	82	65
	24	-	-	24	15	71	49	100	94

- Not determined

by wyerone acid. In all cases two celled germ tubes were less readily killed than single celled structures.

(e) Tolerance of 'secondary' germ tubes to wyerone acid

During the development of a system to assess viability of sporelings the observation was made that following exposure to sub-lethal concentrations of wyerone acid new 'secondary' germ tubes could grow from conidia which appeared dead (Chapter 2(a)). Kinetic studies suggested that little metabolism of wyerone acid would be likely until the onset of growth of the germ tube (Fig.30), therefore the possibility that secondary germ tubes may have a greater tolerance of the phytoalexin was examined.

Sporelings of B. cinerea and B. fabae were grown to produce germ tubes of c. 50 µm in length then exposed to 10 and 20 µg/ml wyerone acid respectively. Following incubation for 18h 100 sporelings in four replicate droplets for each treatment were examined microscopically. Secondary germ tubes had been produced by 68% and 57% of sporelings of B. cinerea and B. fabae, having mean lengths of 65 µm and 59 µm respectively. The experimental procedure was repeated but after incubation for 18h the bathing fluids of both fungi were removed, sporelings washed in SPN and fresh wyerone acid solutions added, 5 µg/ml for B. cinerea and 10 µg/ml for B. fabae. Subsequent growth of secondary hyphae was compared to growth of fresh germ tubes c. 65 µm in length (7h sporelings). Results obtained are given in Table 36. Secondary germ tubes of pre-treated sporelings were less sensitive to wyerone acid than those of untreated sporelings of both B. cinerea and B. fabae.

TABLE 36 Effect of wyerone acid on growth of pre-treated secondary germ tubes and untreated germ tubes

	Growth of germ tubes in bioassays (µm)[a]	
	B. cinerea[b]	B. fabae[b]
Pretreated sporelings	258	491
Untreated sporelings	187	263

a Mean growth of 50 germ tubes

b B. cinerea and B. fabae exposed to 5 µg/ml and 10 µg/ml wyerone acid respectively

CHAPTER 3

STUDIES ON THE MODE OF ACTION OF PHYTOALEXINS

(a) Effect of reduced wyerone acid on the antifungal activity of wyerone acid

Within infected tissues *B. fabae* is exposed to the antifungal phytoalexin wyerone acid and also the weakly active metabolites of the inhibitor produced by the fungus. The effect of the metabolite reduced wyerone acid on the activity of wyerone acid against *Botrytis* was therefore examined.

Sporelings (6h) of *B. cinerea* and *B. fabae* were exposed to 0 and 20 µg/ml wyerone acid in SPN containing 0, 20 and 40 µg/ml reduced wyerone acid. Subsequent germ tube growth was measured after incubation for 18h. A minimum of 50 germ tubes were measured from four replicate sites and the growth of germ tubes recorded is given in Table 37. Neither 20 nor 40 µg/ml reduced wyerone acid caused any reduction in germ tube growth recorded after 18h incubation. In the absence of reduced wyerone acid, the phytoalexin completely inhibited *B. cinerea* and greatly limited growth of *B. fabae*. However, in the presence of the metabolite the activity of wyerone acid was reduced. The effect was more pronounced with *B. fabae* where 20 and 40 µg/ml reduced wyerone acid caused an increase in germ tube length of 250 and 400 µm respectively over growth in the absence of the metabolite. With *B. cinerea* the increase in growth was less pronounced. Subsequent experiments on this effect were carried out using *B. fabae*.

TABLE 37 Effect of reduced wyerone acid on the antifungal activity of wyerone acid against sporelings of *B. cinerea* and *B. fabae*

Fungus	Concentration of wyerone acid (µg/ml)	Germ tube growth in SPN containing reduced wyerone acid		
		0 µg/ml	20 µg/ml	40 µg/ml
B. cinerea	0	688	678	680
	20	0[a]	16	28
B. fabae	0	679	687	707
	20	30	280	430

a - No significant increase in germ tube length recorded

(b) Effect of a range of structurally related compounds on the antifungal activity of wyerone, wyerone acid and wyerone epoxide towards B. fabae

The previous experiment had shown that the metabolite reduced wyerone acid decreased the activity of its corresponding phytoalexin, wyerons acid. In the following experiments the effects of various phytoalexin metabolites and synthetic structurally related compounds on the antifungal activity of wyerone, wyerone acid and wyerone epoxide was examined. I am indebted to R.O. Cain, Chemistry Department, Stirling University for the synthesis of a range of furan derivatives.

Structures of the metabolites and synthetic furans examined are given in Table 38. The synthetic derivatives (compounds 5-10) ran as single spots visualized under UV radiation (254 nm) and with rhodamine 6G on TLC plates developed on $CHCl_3$, hexane : acetone (2:1, v/v) and Et_2O : MeOH (8:1, v/v). Their structures were confirmed by mass spectrometry. Concentrations of solutions of the synthetic derivatives were adjusted using the molecular extinction coefficients determined by Cain (1977).

The antifungal activities of compounds 1-10 (Table 38) were compared with that of wyerone, wyerone acid and wyerone epoxide. Solutions of all compounds were made up to a concentration of 2×10^{-4}M (c. 50 µg/ml) in SPN buffered to pH. 4.0. B. fabae sporelings were exposed to the compounds using standard slide bioassay techniques. After 18h incubation the growth of the germ tubes was compared with growth in SPN alone. At the concentration selected the three phytoalexins were completely inhibitory to germ tube growth. Wyerol caused a 9.3% reduction in germ tube growth but growth in solutions of other compounds lacking the keto-acetylenic moiety was

TABLE 38 Structures of wyerone derivatives studied

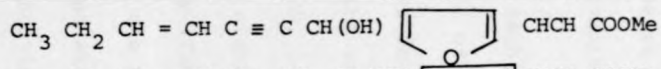
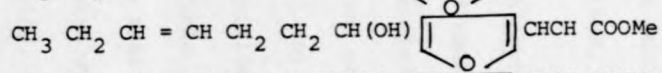
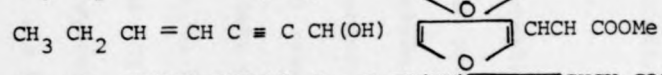
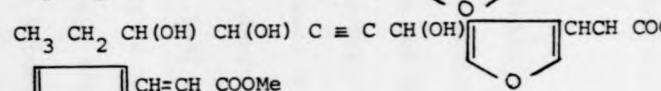
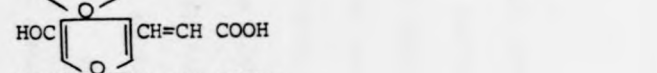
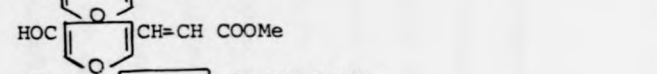
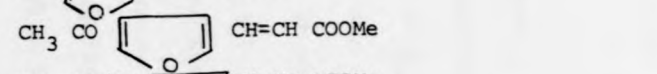
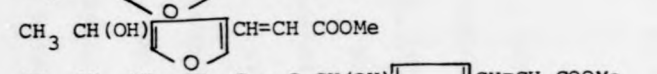
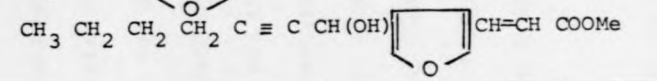
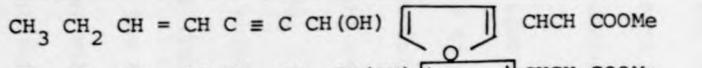
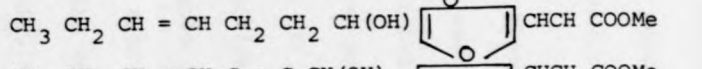
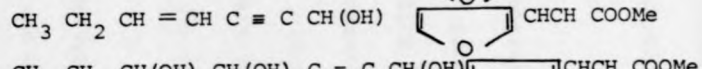
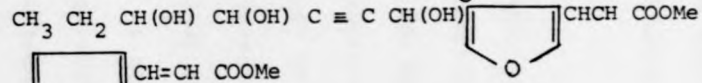
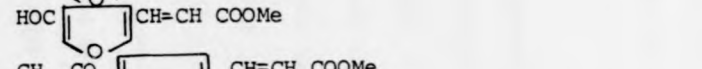
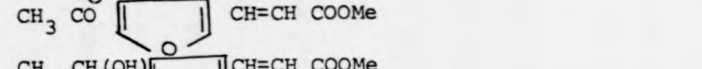
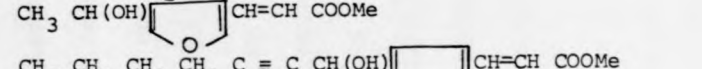
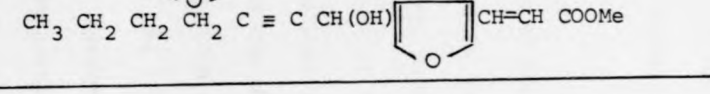
Assigned Number	Common name	Structure
1	Wyerol	
2	Reduced wyerone acid	
3	Wyerol epoxide	
4	Dihydrodihydroxy-wyerol	
5		
6		
7		
8		
9		
10	Dihydrowyerol	

TABLE 38 Structures of wyerone derivatives studied

Assigned Number	Common name	Structure
1	Wyerol	
2	Reduced wyerone acid	
3	Wyerol epoxide	
4	Dihydrodihydroxy-wyerol	
5		
6		
7		
8		
9		
10	Dihydrowyerol	

not significantly different from that in controls. The effect of compounds 1-10 on the antifungal activity of wyerone, wyerone acid and wyerone epoxide at concentrations of 30, 15 and 10 µg/ml respectively was examined.

Solutions of each phytoalexin were prepared in SPN in the presence or absence of each potential phytoalexin inhibitor at 2×10^{-4}M. After incubation for 18h growth of B. fabae germ tubes from sporelings (6h) was measured in the usual way. None of the compounds (Table 38, 1-10) caused an increase in phytoalexin activity, but some caused a significant decrease (Table 39). Of the phytoalexin metabolites (Table 38, 1-4) only reduced wyerone acid was found to decrease the activity of the phytoalexins and the effect was most pronounced with wyerone acid. The synthetic derivatives varied in their activity, the ketone, compound 8, being particularly active against each phytoalexin. The presence of ketone, acetylenic and acidic groups appeared to influence the inhibition of antifungal activity and in general wyerone acid was more affected than the other phytoalexins.

(c) Studies on the mechanism of inhibition of the activity of acid by reduced wyerone acid

Experiments were designed to test the hypothesis that the metabolite may have been competing with the phytoalexin for a specific binding site within the fungus. Studies on enzyme kinetics have shown that inhibition of activity can be caused by the addition of a compound with a similar structure to the substrate. Inhibition is a result of the compound affecting the formation of the unstable enzyme-substrate complex, and can be either non-competitive with the enzyme or competitive, in which the inhibitors form an alternative

TABLE 39 Effect of wyerone derivatives on the antifungal activity of phytoalexins against germ tubes of B. fabae

Compound Number	Percentage increase in germ tube length when compounds added to solutions of Wyerone[a]	Wyerone acid	Wyerone epoxide
1	0[b]	0	0
2	28.4	81.1	19.6
3	0	0	0
4	0	0	0
5	29.6	20.0	22.8
6	0	63.5	0
7	0	0	0
8	50.2	83.3	56.0
9	0	19.5	0
10	0	0	0

a - Activity of wyerone, wyerone acid and wyerone epoxide (30, 15 and 10 µg/ml) respectively were assayed in presence or absence of compounds 1-10 (Table 38) at 2×10^{-4}M. Reduction in antifungal activity expressed as percentage increase in growth compated to controls in absence of compounds 1-10; Wyerone 56.1 - 247.5 µm, wyerone acid 51.9-194.0 µm and wyerone epoxide 52.5-155.2 µm.

b - Germ tube lengths not significantly different ($p = 0.05$) from those in solutions containing phytoalexins alone.

unstable complex, thus competing with the original substrate for sites on the enzyme. If inhibition is of a competitive nature, by increasing the concentration of substrate at a constant enzyme and inhibitor concentration the inhibitory effect is overcome. In the case of non-competitive inhibition, however, inhibitory activity would occur at high substrate concentrations. Analogous studies were carried out using B. fabae, sporelings, wyerone acid and reduced wyerone acid. The effect of increasing concentrations of wyerone acid (substrate) on the inhibition of growth of germ tubes of B. fabae (enzyme) in the presence of constant levels of reduced wyerone acid (inhibitors) was examined.

B. fabae sporelings were exposed to a range of wyerone acid concentrations (1-10 µg/ml) in SPN in the presence or absence of 20 and 40 µg/ml reduced wyerone acid. Incubation time for bioassays was reduced from 18h to 3h to permit a more accurate measurement of the short germ tubes thus produced, and to minimise the possibility of phytoalexin metabolism. In preliminary tests no metabolism of the phytoalexin was detected in solutions of 1 and 2 µg/ml wyerone acid incubated with B. fabae sporelings under these conditions. Growth of germ tubes was recorded with a calibrated eye piece graticule. A minimum of 50 germ tubes were measured from the four replicate droplets of each treatment.

The antifungal activity of increasing concentration of wyerone acid is expressed in Fig.36 as fractional inhibition of germ tube growth in solutions lacking the phytoalexin but containing reduced wyerone acid when applicable. Values of fractional inhibitor of 1.0 and 0, therefore, indicate complete and no inhibition due to wyerone acid respectively.

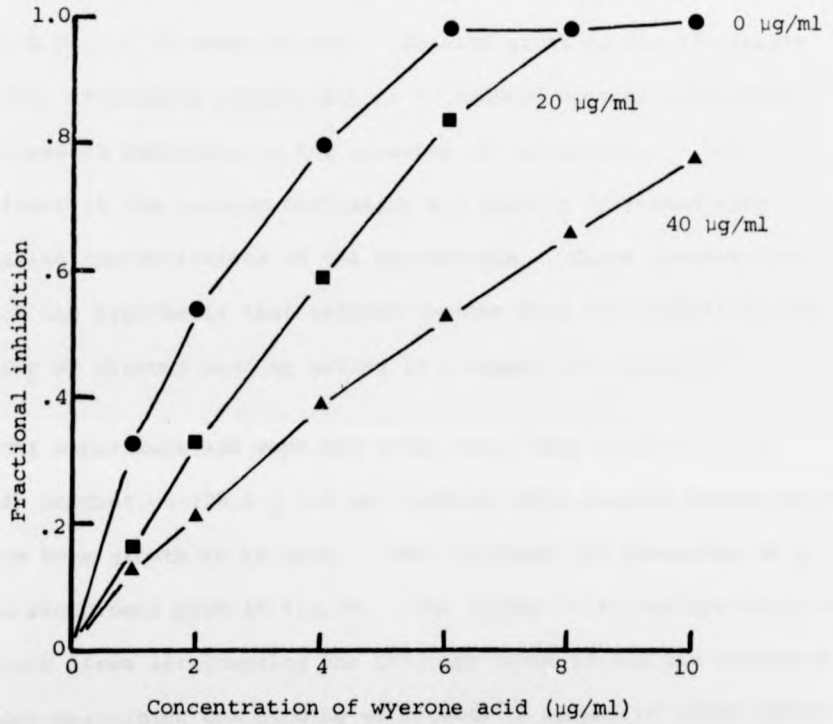


Fig.36 Fractional inhibition of B. fabae germ tube growth by wyerone acid alone (●) or in the presence of reduced wyerone acid 20 (■) and 40 (▲) µg/ml.

Under the bioassay conditions of short incubation period employed, reduced wyerone acid itself was slightly antifungal. Germ tube lengths after incubation in SPN alone or with 20 and 40 µg/ml reduced wyerone acid were 136.4 ± S.E. 8.9, 98.6 ± S.E. 6.5 and 88.0 ± S.E. 5.1 µm respectively. Results given in Fig.29 clearly show that increasing concentrations of reduced wyerone acid caused an increasing reduction in the activity of phytoalexin. However, the effect of the reduced derivative was greatly decreased with increasing concentrations of the phytoalexin. These observations support the hypothesis that reduced wyerone acid was inhibiting the activity of wyerone acid by acting in a competitive manner.

The experiment was repeated using germ tubes produced after only 4h incubation (25.6 ± 2.1 µm) enabling more precise measurements of germ tube growth to be made. Data obtained are presented as a double reciprocal plot in Fig.37. The linear relationships obtained with both lines intercepting the ordinate close to 1.0 are analogous to those describing the binding of ligands to enzymes or other macromolecules (Price & Dwek, 1974). Linearity can be most simply explained by postulating an equilibrium between binding sites in B. fabae, wyerone acid and a binding site/wyerone acid complex, formation of the complex resulting in inhibition of growth of B. fabae. Fractional inhibition of growth would therefore represent fractional saturation of binding sites by the phytoalexin. Furthermore, these studies strongly suggest that reduced wyerone acid competitively inhibits the binding of wyerone acid to a receptor site in B. fabae, probably by the formation of a less harmful binding site/reduced wyerone acid complex from a similar equilibrium. If the metabolite was reducing the activity of the phytoalexin by other

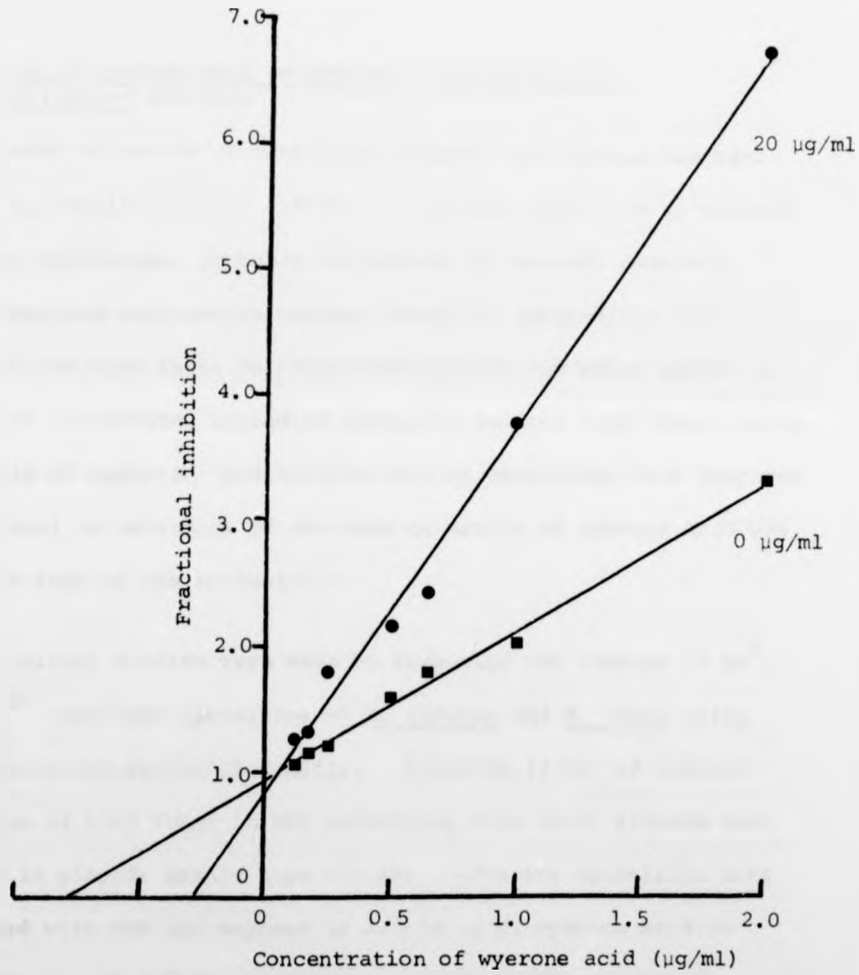


Fig.37 Double reciprocal plot of fractional inhibition of B. fabae germ tube growth by wyerone acid alone (■) (y = 1.195x + 0.927) or in the presence of reduced wyerone acid 20 µg/ml (●) (y = 2.943x + 0.805).

means, the effect would not be overcome at high phytoalexin concentrations (Figs. 36 and 37) and the plot in Fig.37 obtained in the presence of the reduced derivative would not intercept the ordinate at the same point as in its absence.

(d) Effect of wyerone acid on leakage of cations from sporelings of Botrytis

The mode of action of antifungal polyene antibiotics has been reviewed by Hamilton Miller (1973). Organisms sensitive to polyenes bind these substances, probably to sterols in the cell membrane, causing membrane malfunction whereby essential metabolites leak out. "Nystatin" has been shown to cause membrane lesions which result in the loss of metabolites including inorganic cations from fungal cells. The effects of nystatin and wyerone acid on sporelings were compared in an attempt to determine if the mode of action of wyerone acid was similar to that of the antiobiotic.

Preliminary studies were made by measuring the leakage of Na^+, K^+ and Mg^{2+} ions from sporelings of B. cinerea and B. fabae using atomic absorption spectrophotometry. Aliquots (2 ml) of conidial suspensions of both fungi in SDW containing 0.5% (w/v) glucose were incubated in plastic bottle tops for 6h. Adhering sporelings were then washed with SDW and exposed to 2.5-50 µg/ml wyerone acid in SDW adjusted to pH 4.0 with 0.5M HCl. Control dishes were arranged, containing sporelings in acidified water alone and phytoalexin solutions in the absence of sporelings. Under these conditions the antifungal activity of wyerone acid was c. 30% greater than under standard bioassay conditions. The experiment was replicated three times and after 4h incubation bathing fluids were decanted off and the concentrations of K^+, Na^+ and Mg^{2+} determined for each replicate solution.

No detectable leakage of Na^+ was recorded from sporelings of either B. cinerea or B. fabae following incubation with wyerone acid and concentrations of Mg^{2+} in diffusates were too low to measure accurately. However, 20 and 40 µg/ml wyerone acid induced K^+ leakage from sporelings of both fungi. A more detailed analysis of K^+ leakage was therefore carried out, comparing the effects of 20 and 40 µg/ml wyerone acid and 10 µg/ml nystatin on sporelings of B. cinerea and B. fabae.

Nine replicate dishes of sporelings were exposed to SDW, 20 and 40 µg/ml wyerone acid and 10 µg/ml nystatin as described above. Controls were prepared in the absence of fungi. After incubation for 1, 2 and 4h the concentration of K^+ was recorded in three replicates for each treatment. Results given in Table 40 show the leakage of K^+ induced in excess of that recorded in SDW controls and inhibitor controls. By 1h after solution replacement nystatin had caused leakage of K^+ from both B. cinerea and B. fabae but wyerone acid had caused no leakage. After 2h the phytoalexin began to cause loss of K^+ from sporelings, 20 µg/ml being less effective than 40 µg/ml wyerone acid. The concentration of K^+ in bathing fluids continued to rise after 4h following treatment with nystatin, whereas wyerone acid treatment resulted in little change from the levels recorded after 2h.

These results suggest that induced leakage of K^+ ions from sporelings treated with wyerone acid is probably not a result of the same change induced by the polyene antibiotic nystatin. The mode of action of the phytoalexin is therefore unlikely to be one of direct membrane disruption, ionic leakage being a secondary response from dead or damaged fungal cells.

TABLE 40 Leakage of potassium into incubation fluids of Botrytis induced by wyerone acid and the polyene antibiotic 'nystatin'

	Time of incubation (h)	Induced leakage of potassium (K^+) into incubation fluids (ppm)		
		Wyerone acid		Nystatin
		20µg/ml	40 µg/ml	10 µg/ml
B. cinerea	1	0[a]	0	0.25
	2	0	.17	0.30
	4	0.07	.16	0.40
B. fabae	1	0	0	.99
	2	.14	.23	1.15
	4	.15	.23	1.46

a - No increase from control detectable

CHAPTER 4

FUNGAL DEVELOPMENT AND PHYTOALEXIN ACCUMULATION IN BEAN LEAVES

In earlier work on the role of phytoalexins in resistance of V. faba to Botrytis no detailed studies were made on the timing of cessation of fungal growth within epidermal tissues. A series of experiments were designed to clarify this point.

(a) The growth and death of infection hyphae of B. cinerea and B. fabae within epidermis of bean leaves

Previous experiments clearly demonstrated that the furano-acetylenic phytoalexins from V. fabae are toxic towards conidia and sporelings of B. cinerea and B. fabae. It was therefore of interest to investigate if infection hyphae died within limited lesions. In the experiments reported here, the growth and death of infection hyphae was recorded from 1-3d after inoculation of leaves with droplets containing a range of spore concentrations.

For each inoculation concentration six leaves were used, each leaf being inoculated with 24 droplets of conidial suspension. At daily intervals a minimum of three epidermal strips from different leaves were examined microscopically. At all inoculum concentrations tested <5% of conidia failed to produce infection hyphae, and hyphal length was estimated by measuring 20 hyphae with a calibrated eyepiece graticule. Percentage viability of infection hyphae was recorded after examination of a total of 80 hyphae in four replicate counts. Dead hyphae were recognised by their intense staining with trypan blue (see Plate 4a and b). Results from repeated experiments

carried out in mid June and late August are given separately in Tables 41 and 42 respectively.

In general infection hyphae were shorter and more were killed in leaves collected early in the season. These effects were particularly marked with B. cinerea inoculations at 5×10^5 conidia/ml. The effect of spore concentration on length of infection hyphae was most pronounced with B. fabae, shorter hyphae being produced at low inoculum concentrations, but even with only ten spores per droplet (5×10^2/ml) the percentage of B. fabae infection hyphae killed was still less than 50% by 3d after inoculation. With the exception of leaves inoculated with 5×10^5 conidia/ml, particularly late in the season, all B. cinerea infection hyphae were dead by 3d after inoculation. Little hyphal growth was observed between 1 and 3d. Results suggest that at spore concentrations of 10^5 conidia/ml or less infection hyphae of B. cinerea were restricted by 1d and died by 3d after inoculation. At concentrations of 10^4 conidia/ml or above, B. fabae infection hyphae grew rapidly, causing spreading lesions to develop.

Typical infection hyphae formed by B. cinerea and B. fabae are shown in Plate 3 (a and b) respectively. The avirulent species had produced a short infection hyphae compared to that formed by B. fabae. Plate 4 (a and b) shows similar inoculation sites following staining with trypan blue.

Plate 3

Infection hyphae within epidermal cells of *V. faba* 2d after inoculation:

(a) *B. cinerea*, note short infection hyphae (arrowed)

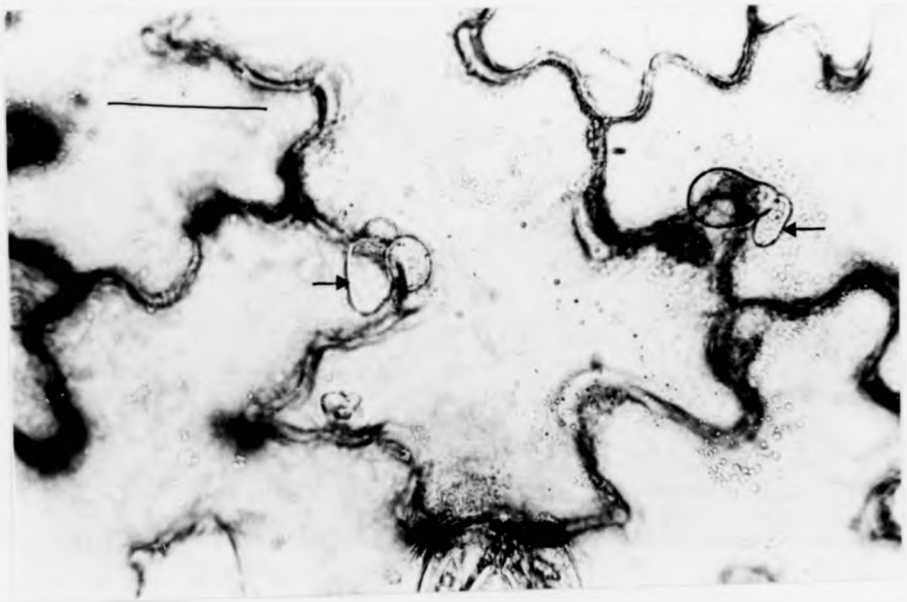
(b) *B. fabae*, note ramifying growth of intracellular hyphae, and disruption of host cell walls

Bar = 25 µm.

of V. faba 2d after

on hyphae (arrowed)

h of intracellular
cell walls



a

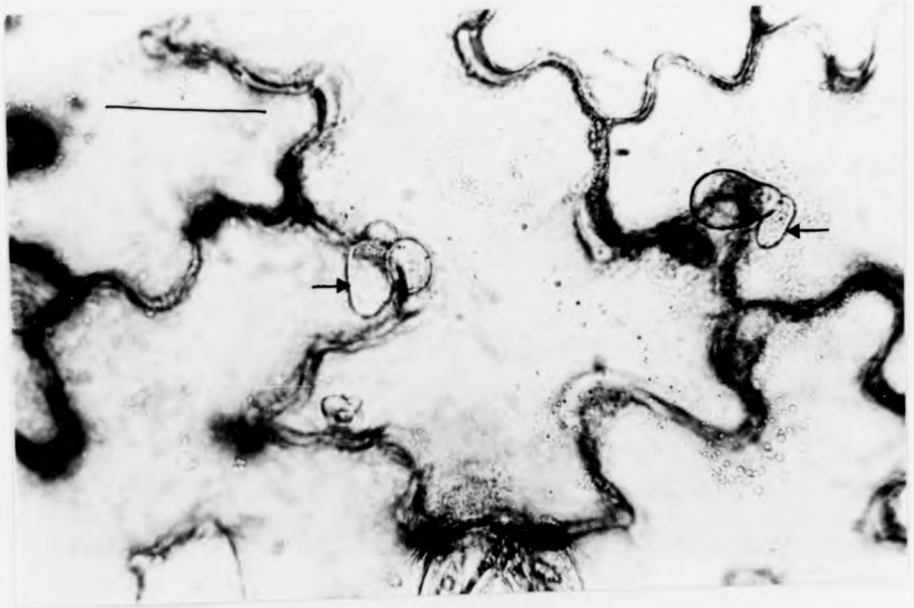
b

Plate 3

f V. faba 2d after

hyphae (arrowed)

of intracellular
cell walls



a

b

Plate 3

Plate 4

Botrytis infection hyphae stained with trypan blue within bean epidermis.

(a) *B. cinerea*; note deep stained hyphae (arrowed) in contrast with unstained one.

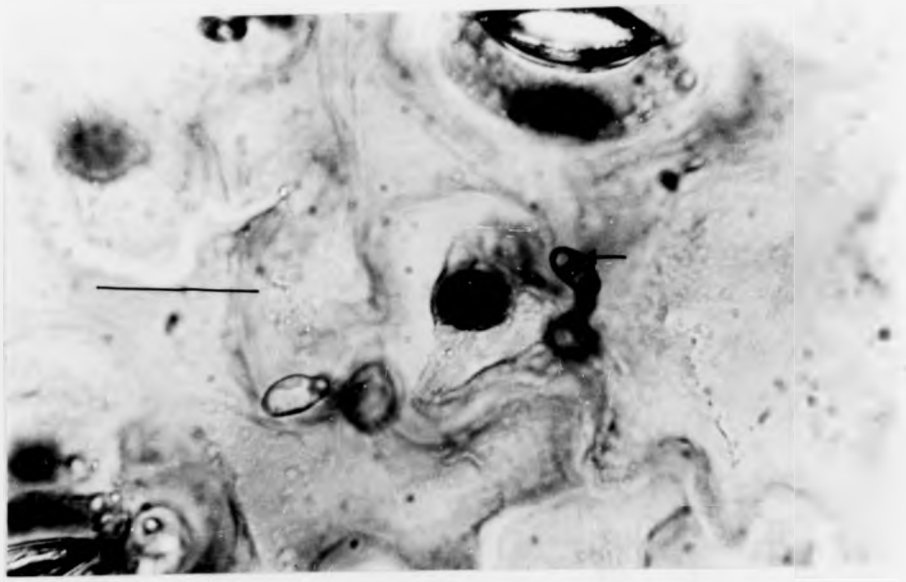
(b) *B. fabae*; note unstained ramifying hyphae (arrowed) in contrast to deeply stained host wall and cytoplasm.

Bar = 25 µm

ypan blue within

hyphae (arrowed)

fying hyphae
stained host



a

b

Plate 4

TABLE 41 Growth and death of infection hyphae of B. cinerea and B. fabae in epidermis of bean leaves[a]

Species	Concentration of conidia (/ml)	Time after inoculation (d) 1 % killed[b]	1 length[c]	2 % killed	2 length	3 % killed	3 length
B. cinerea	5×10^5	25	16	80	21	>95	33
	10^5	27	15	89	21	100	19
	10^4	47	9	95	14	100	13
	10^3	55	11	100	8	100	14
B. fabae	10^5[d]	-	-	-	-	-	-
	10^4[d]	-	-	-	-	-	-
	10^3	10	53	22	80	32	>150
	10^2	14	40	45	63	42	120

a - Leaves harvested in mid-June, 1977

b - Percentage of infection hyphae dead assessed with trypan blue vital stain

c - Mean length of 20 infection hyphae (µm)

d - <5% dead infection hyphae by 3d, mean length by 1d > 100 µm

TABLE 42 Growth and death of infection hyphae of B. cinerea and B. fabae in epidermis of bean leaves

Species	Concentration of conidia (/ml)	Time after inoculation (d) 1 % killed[b]	1 length[c]	2 % killed	2 length	3 % killed	3 length
B. cinerea	5×10^5	11	21	20	40	23	103
	10^5	21	16	67	28	795	35
	10^4	50	16	78	22	100	19
	10^3	47	15	90	21	100	22
B. fabae	10^5	<5	>200	>5	mycelium	<5	mycelium
	10^4	<5	92	<5	>150	<5	>200
	10^3	9	61	12	121	11	>150

a - Leaves harvested in late August, 1977

b - Percentage of infection hyphae dead assessed with trypan blue vital stain.

c - Mean length of 20 infection hyphae (µm).

(b) Comparative studies on the growth of infection hyphae of B. cinerea and B. fabae and the accumulation of phytoalexins within epidermal tissues and inoculum droplets

In further studies on the development of infection hyphae in relation to phytoalexin accumulation an inoculum concentration of 10^5 conidia/ml was selected, as this was shown to cause a maximum response in underlying epidermal cells and gave clear differentiation between B. cinerea and B. fabae. Previous results suggested that infection hyphae of B. cinerea ceased growing by 1d after inoculation. The following experiments were designed to examine the growth of infection hyphae of B. cinerea and B. fabae within the first hours after inoculation in relation to the onset of phytoalexin accumulation within epidermal tissue.

(i) Growth of infection hyphae in the epidermis in relation to phytoalexin accumulation

The growth of infection hyphae and accumulation phytoalexins in epidermal tissue of field grown leaves was examined during the first 14h after incubation. Bifoliate leaves, detached from field grown plants in late June 1977, were inoculated on the abaxial epidermis with inoculum droplets (20 µl) containing conidia of B. cinerea (c. 2000 conidia/droplet). Leaves were incubated in sandwich boxes in the usual way. Four leaves were randomly selected for microscopical examination. Examination of epidermal strips prepared from inoculation sites was made at 2h intervals. Each time at least three separate sites were stripped from each leaf and the lengths of at least 12 infection hyphae were measured from each with a calibrated eyepiece graticule. Data collected for each leaf are given separately in Fig.38. Although some variation existed between leaves, growth of infection hyphae showed a similar pattern in each leaf. A rapid increase in hyphae length was observed between 4 and 10h after inoculation but thereafter a marked reduction

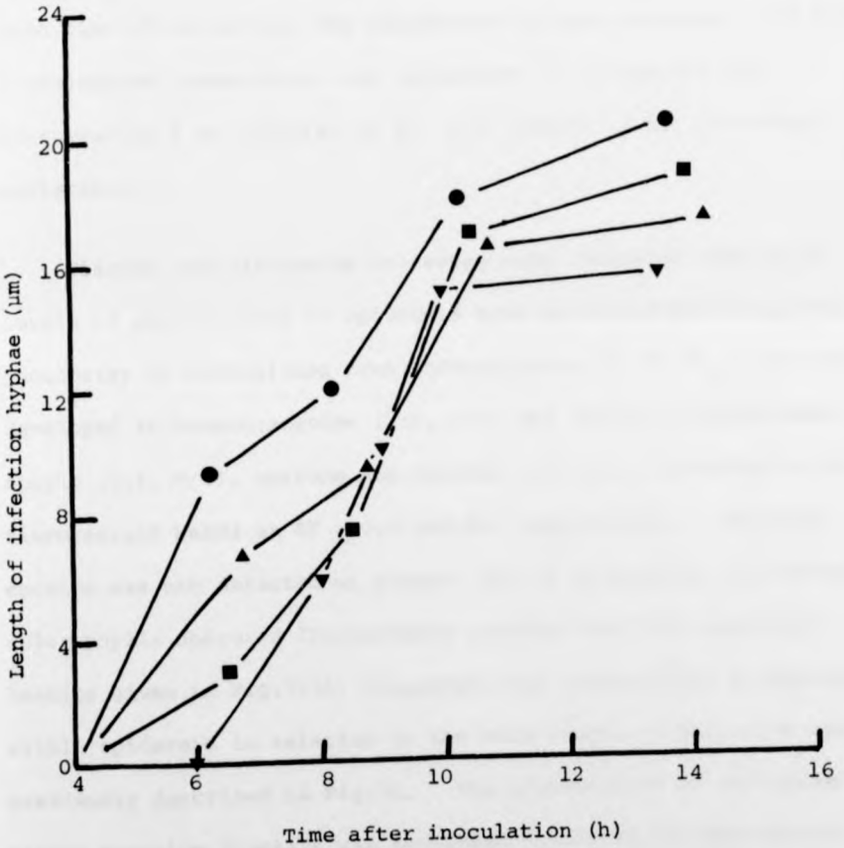


Fig.38 Rates of growth of B. cinerea infection hyphae with bean leaf epidermis.

●, ■, ▲, ▼ refer to individual leaves.

in growth rate was apparent.

At the start of each two hourly microscopical examination, inoculum droplets and epidermal strips were collected from about 200 sites on six replicate leaves, giving c. 2ml diffusate and c. 0.1g tissue. Collection of epidermal strips took 1-1½h and a mean time of collection was calculated on each occasion. To enable microscopical examination and collection to be carried out concurrently I am indebted to Dr. J.W. Mansfield for providing assistance.

Tissues and diffusates collected were extracted with Et_2O. Levels of phytoalexins in epidermis were estimated by UV spectrophotometry of bands eluted from chromatograms of the Et_2O extracts developed in hexane:acetone (2:1, v/v) and chloroform:petroleum spirit (2:1, v/v), wyerone and wyerone acid were recognised as blue flurorescent bands at RF c.0.6 and 0.1 respectively. Wyerone epoxide was not detected on plates, but it is possible contaminating chlorophylls obscured fluorescence arising from this compound. Results given in Fig.39(A) illustrate the accumulation of phytoalexins within epidermis in relation to the mean length of infection hyphae previously described in Fig.38. The accumulation of phytoalexins within inoculum droplets was monitored following the development of chromatograms of diffusate extracts in Et_2O:MeOh (8:1, v/v). Wyerone acid (RF c. 0.3) was the only phytoalexin detected, and reached concentrations of 0.2, 0.3 and 0.6 µg/ml after incubation for 8, 10 and 13h respectively.

The experiment was repeated using leaves inoculated with B. fabae and comparative data for mean length of infection hyphae

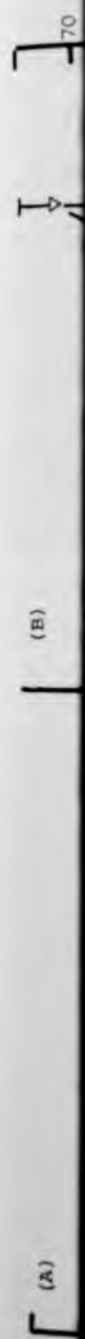


Fig. 39

Growth of infection hyphae of B. cinerea (A) and B. fabae (B) in epidermis of bean leaves, in relation to accumulation of wyerone acid (●) and wyerone (■).

Bars refer to 95% confidence limits.

(A) and

in relation

erone (■).

Fig.39

and accumulation of phytoalexins within epidermis are given in Fig.39(B). Epidermal tissue beneath B. fabae inoculation sites was considerably more disrupted than comparable tissue beneath B. cinerea sites. Removal of strips of pure epidermis for extraction was therefore more difficult and there was far greater contamination of epidermal tissue with underlying mesophyll.

Results obtained suggest that the cessation of growth of B. cinerea infection hyphae within epidermis is associated with the accumulation of phytoalexins, especially wyerone acid, which had reached levels of over 40 µg/g FW by 14h after inoculation. Growth of B. fabae infection hyphae was much more rapid and no check in growth rate was observed. Lower levels of phytoalexins accumulated in B. fabae infected tissues and it is possible that the inhibitors that were detected were present in contaminating mesophyll cells.

The accumulation of phytoalexin in B. fabae inoculum droplets was similar to that described for B. cinerea. Wyerone acid was again the only inhibitor detected. However, its level of accumulation was lower than that observed for B. cinerea. It was not detected until 10½h after inoculation (0.3 µg/ml). At this time, examination of chromatograms did not disclose the presence of any known metabolites of wyerone acid.

(ii) Accumulation of phytoalexins and phytoalexin metabolites in epidermal tissue and inoculum droplets of bean leaves inoculated with B. cinerea and B. fabae

Earlier experiments suggested a good correlation between cessation in growth of B. cinerea infection hyphae and the accumulation of phytoalexins within infected tissue. Inhibited infection hyphae died within 3d of inoculation. The accumulation of phytoalexins

in inoculum droplets and underlying epidermis from 1-3d has therefore been examined to determine whether the inhibitors could be responsible for death of infection hyphae *in vivo*.

Bifoliate field grown leaves collected in mid June and late August 1977, were inoculated on the abaxial surface with ten droplets (*c.* 20 µl) of conidial suspension of *B. cinerea* and *B. fabae* on separate leaflets. At intervals of 1, 2 and 3d inoculum droplets were removed from *c.* 200 infection sites of both fungi. The underlying epidermis at each site was carefully peeled off and collected to give *c.* 0.1g tissue for each treatment. Diffusates and tissues were separately extracted with Et_2O and extracts subjected to TLC for analysis of wyerone derivatives.

Chromatograms of diffusate extracts were developed in Et_2O: MeOH (8:1, v/v). Wyerone acid (R_F *c.* 0.3) was the only phytoalexin to accumulate to measurable concentrations in inoculum droplets, although wyerone epoxide R_F *c.* 0.9 was present in trace amounts in some diffusates. In *B. fabae* diffusates wyerone acid metabolites were also detected, reduced wyerone acid at R_F *c.* 0.4 and wyerone acid metabolite 1 (tentatively identified as wyerol acid) at R_F *c.* 0.7. The chromatographic procedures used to separate components of tissue extracts are summarized in Fig.40. After elution of isolated bands, the concentration of eluted phytoalexins and metabolites were determined by UV spectrophotemetry in the usual way. The accumulation of the phytoalexins and fungal metabolites of wyerone acid in leaves harvested in mid June and late August 1977, are given in Tables 43 and 44 respectively.

The most abundant phytoalexin in epidermal tissue was wyerone acid, which reached a maximum concentration in excess of 500 µg/g

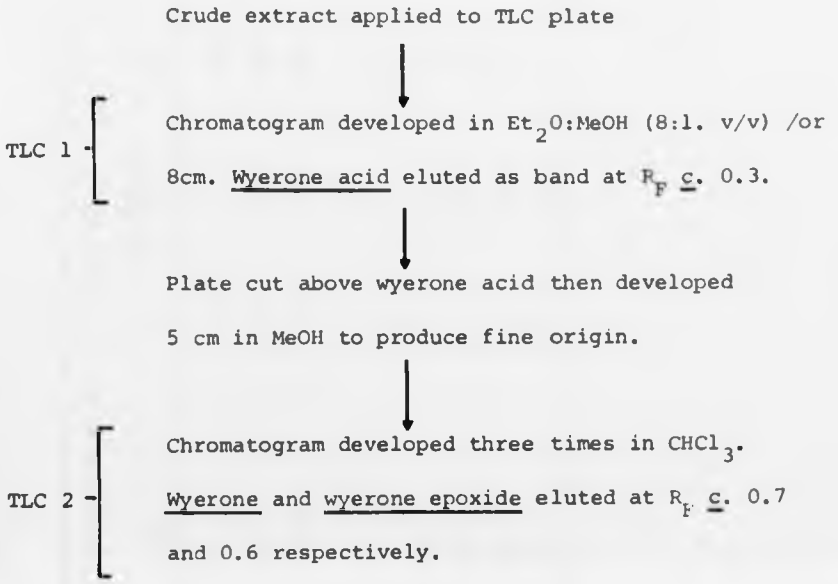


Fig.40 Summary of chromatographic procedure to separate wyerone, wyerone acid and wyerone epoxide from epidermal tissue extracts.

Metabolites of wyerone derivatives could not be separated from contaminating chlorophylls using this system.

TABLE 43 Phytoalexin and phytoalexin metabolite accumulation in bean leaves[a] inoculated with Botrytis

Source of extract	Phytoalexin or metabolite	Yield of phytoalexin (µg/g FW or µg/ml)					
		B. cinerea			B. fabae		
		1d	2d	3d	1d	2d	3d
Epidermal strips	(Wyerone	10.0	42.5	57.4	7.9	4.4	ND[b]
	(Wyerone acid	163	384	515	49.3	25.7	Tr[c]
	(Wyerone epoxide	5.5	88	12.5	4.7	ND	ND
Inoculum droplets	(Wyerone	ND	ND	ND	ND	ND	ND
	(Wyerone acid	7.6	14.2	39.0	2.0	1.7	2.1
	(Wyerone epoxide	Tr	Tr	Tr	ND	ND	ND
	(Wyerone acid metabolite 1[d]	ND	ND	ND	0.8	10.3	26.7
	(Reduced wyerone acid	ND	ND	ND	1.0	10.0	9.9

a - Leaves harvested in mid June, 1977

b - Not detectable on TLC plate

c - Trace, insufficient to measure spectrophotometrically

d - Tentatively identified as wyerol acid (Hargreaves, 1976)

TABLE 44 Phytoalexin and phytoalexin metabolite accumulation in bean leaves[a] inoculated with Botrytis

Source of extract	Phytoalexin or metabolite	Yield of phytoalexin (µg/g FW or µg/ml)					
		B. cinerea			B. fabae		
		1d	2d	3d	1d	2d	3d
Epidermal strips	(Wyerone	4.4	24.1	29.3	4.1	ND[b]	ND
	(Wyerone acid	102	157	223	30.0	Tr[c]	ND
	(Wyerone epoxide	Tr	4.8	6.1	ND	ND	ND
Inoculum droplets	(Wyerone	ND	ND	ND	ND	ND	ND
	(Wyerone acid	4.8	8.1	14.7	4.1	ND	ND
	(Wyerone epoxide	ND	ND	ND	ND	ND	ND
	(Wyerone acid metabolite 1[d]	ND	ND	ND	5.6	14.1	27.1
	(Reduced wyerone acid	ND	ND	ND	4.1	11.3	16.7

a - Leaves harvested in late August, 1977

b - Not detectable on TLC plates

c - Trace, insufficient to measure spectrophotometrically

d - Tentatively identified as wyerol acid (Hargreaves, 1976)

FW 3d after inoculation with B. cinerea. Much lower levels of wyerone and wyerone epoxide were recorded.

Accumulation of furano-acetylenic compounds in leaves detached from plants early and late in the season showed similar trends, except that in general lower levels of inhibitors were present in older leaves.

Phytoalexin concentrations 1d after inoculation were lower in leaves inoculated with B. fabae than with B. cinerea. With B. cinerea concentrations of phytoalexins, particularly wyerone acid, continued to rise from 1-3d whereas with B. fabae the levels diminished. Loss of wyerone acid from such sites was associated with accumulation of the known metabolites of wyerone acid within inoculum droplets. Yields of the metabolites increased with time of incubation, suggesting that wyerone acid was constantly being produced and metabolized from 1-3d in lesions caused by B. fabae.

(iii) The antifungal activity of inoculum droplets removed from leaves

Earlier results (Tables 43 and 44) illustrate the accumulation of phytoalexins, primarily wyerone acid, in inoculum droplets recovered from above lesions in leaves from field grown plants. The antifungal activity of bulked inoculum droplets from B. cinerea and B. fabae inoculations against sporelings of B. cinerea was therefore examined. Bifoliate leaves were inoculated on the abaxial epidermis with droplets (c. 20 µl) of B. cinerea and B. fabae conidial suspensions (10^5 conidia/ml) on separate leaflets. At 1, 2 and 3d after inoculation droplets from ten leaves were collected for each treatment. Aliquots were sterilized by membrane filtration and the pH of diffusates determined. Membrane filtration was shown

to have no effect on the concentration of wyerone acid in test solutions. Diffusates were bioassayed against sporelings of B. cinerea. Results obtained are given in Table 45.

The results clearly show that bacteria removed from diffusates by membrane filtration contributed to inhibitory properties of all diffusates. After filtration B. fabae diffusates collected 1-3d after inoculation become highly stimulatory. The stimulatory effect of unfiltered diffusates diminished with time of incubation, probably due to an increase in bacterial numbers within inoculum droplets rich in nutrients released from infected cells.

B. cinerea diffusates collected 1d after inoculation were more antifungal before sterilization but by 2d sterile diffusates also became totally inhibitory. Increasing activity correlated with the increase in wyerone acid concentrations recorded in Tables 43 and 44, suggesting the phytoalexin may inhibit growth of hyphae on the leaf surface.

The pH of inoculum droplets did not fall as low as that described for diffusate from pods (Table 28). The antifungal activity of wyerone acid within inoculum droplets on leaves would therefore probably not be as great as in droplets in pod seed cavities.

(c) Estimation of pH within infected plant cells

Earlier results showed that wyerone acid was the most abundant phytoalexin to accumulate in epidermal tissue and inoculum droplets following the inoculation of leaves with B. cinerea. The activity of this inhibitor has been shown to be dependent on the pH of the solution in which it is dissolved. The pH within infected cells would therefore have a large bearing on the involvement of wyerone

TABLE 45 Antifungal activity of diffusates from leaves[a] inoculated on the abaxial surface before and after membrane filtration

	Time after inoculation (d)	pH of diffusate[b]	Growth of germ tubes in bioassays (µm)	
			Not filtered	Filtered
B. cinerea	1	4.80	25	52
	2	4.80	NFG[c]	NFG
	3	4.85	NFG	NFG
B. fabae	1	4.55	604	>1000
	2	4.9	420	>1000
	3	-[d]	162	>1000

Control growth in SDW 82 µm

a - Leaves harvested late July, 1977

b - pH of conidial suspensions at onset of incubation; 7.1, B. cinerea and 7.3 B. fabae

c - No significant increase in germ tube growth

d - Insufficient diffusate to record pH

acid in the restriction and death of infection hyphae. Attempts were therefore made to estimate the pH at which infection hyphae were exposed to phytoalexins with infected tissues.

Further studies were made on the pH of inoculum droplets collected from pod seed cavities and leaves. The pH of individual drops collected from pods was recorded with a semi-micro electrode 1-3d after inoculation of endocarp with conidial suspensions of B. cinerea and B. fabae (10^5 conidia/ml). The mean pH for each treatment was calculated and results obtained are given in Table 46. The pH decreased with time of incubation, becoming less than 4.0 by three days after inoculation with either fungus.

The pH of bulked droplets collected from leaves was determined following inoculation with conidial suspensions of B. cinerea and B. fabae (5×10^5 conidia/ml). The higher spore concentration was used in order to maximise host response. By 1d the pH values of B. cinerea and B. fabae droplets were 4.35 and 4.25 respectively.

The indicator methyl red was also used in attempts to determine the pH at microsites within infected tissue. Epidermal strips were stained with methyl red 12h after inoculation with B. cinerea or B. fabae. Cytoplasm in live cells in close proximity to dead cells containing infection hyphae stained red, indicating the pH was ≤ 4.5. The pH of penetrated cells could not be determined as they had assumed a brown colouration. Healthy cells distant from penetration sites stained yellow, suggesting the cytoplasmic pH was ≥ 6.0.

These results strongly suggest that the pH at infection sites falls below that of healthy tissue. It seems probable that the pH at which infection hyphae are exposed to wyerone acid within leaf tissue is ≤ 4.5.

TABLE 46 *The pH of droplets of* B. cinerea *and* B. fabae *conidial suspension following incubation in pod seed cavities*

Time after inoculation (d)	Mean pH of droplets following inoculation with: *B. cinerea*[a]	*B. fabae*
1	4.70 ± 0.08 [S.E]	4.45 ± 0.10
2	4.20 ± 0.08	4.05 ± 0.08
3	3.90 ± 0.06	3.70 ± 0.06

a - pH of conidial suspensions at onset on incubation: *B. cinerea* 6.8, *B. fabae* 7.0

DISCUSSION

I INHIBITION OF *BOTRYTIS* ON THE LEAF SURFACE OF *V. FABA*

(a) Germination of Botrytis conidia on the leaf surface in relation to symptom development

Conidia of *B. cinerea* (isolates 39 and SR) suspended in SDW rarely caused lesion formation following inoculation of leaves detached from growth room grown plants even after incubation for 48h. This finding was similar to that of Mansfield & Deverall (1974a), but fewer lesions were produced in this study. It is not clear whether this reflects greater resistance of plants grown under growth room as opposed to greenhouse conditions or the lower pathogenicities of isolates of *B. cinerea* used in this research. *B. allii* and *B. elliptica* also failed to produce symptoms at all but a few sites of inoculation on broad bean leaves, whereas the virulent *B. fabae* (isolate PBI6) had produced lesions at all sites within 24h after inoculation.

Microscopical observation confirmed the suggestions of Rossall (1974) and Mansfield & Deverall (1974a) that the failure of conidia of *B. cinerea* to produce a lesion was associated with poor germination and germ tube growth compared to that observed at lesion sites. Conidial development in SDW on glass slides indicated that conidia possessed sufficient endogenous nutrient reserves to germinate well and therefore growth on leaves was probably actively inhibited. Growth of *B. allii* and *B. elliptica* conidia was also greatly reduced at lesion free inoculation sites on leaves.

Purkayastha and Deverall (1965a) suggested that *B. fabae* conidia may have an advantage over *B. cinerea* in infection droplets by

virtue of possessing a greater inherent growth rate resulting from a larger volume of nutrient reserves. Garrett (1970) discussing the findings of Brown (1922) and Last (1960) postulated that the nutrient reserves of B. fabae conidia endowed them with a greater inoculum potential than a similar inoculum of B. cinerea. However, the inhibition of germination of conidia of B. elliptica, which have a similar volume to those of B. fabae, suggests that leaf surface inhibition may not be influenced solely by levels of endogenous nutrients within conidia.

Comparative studies using broad bean, French bean and tomato indicated that the failure of inocula to produce lesions by 24 and 48h after inoculation was always associated with inhibition of germination of conidia of B. allii, B. cinerea (39 and SR), B. elliptica, and B. fabae (PBI6 and SSI). With the exception of B. fabae inoculations, inhibition was most pronounced on broad bean leaves.

(b) The accumulation of inhibitory activity in inoculum droplets collected from lesion free sites

Preliminary attempts to explain the inhibition of germination of conidia on the leaf surface were based on the detection of antifungal activity in droplets removed from leaves. Previous workers have studied the inhibition of B. cinerea on leaves of V. faba. The first report on the biological activity of inoculum droplets was made by Brown (1922), who observed that water droplets incubated on broad bean leaves sometimes became inhibitory to B. cinerea in vitro. Purkayastha & Deverall (1965b) examined the inhibition of B. cinerea on broad bean leaf surfaces. In their research they detected more inhibitor in infection droplets than in water droplets incubated on the leaf surface, and concluded that a phytoalexin was involved in the

inhibitory process. However, they assumed that the same process of inhibition was in operation at non-lesion sites as at sites where lesions developed. Mansfield (1972) and Mansfield & Deverall (1974b) subsequently showed that although a phytoalexin, wyerone acid, was involved in restricting the growth of B. cinerea through broad bean leaves after lesion formation, wyerone acid played no part in the inhibition of conidial development on the leaf surface at inoculation sites where no symptoms were produced. The work reported in this thesis has confirmed that wyerone acid, and other phytoalexins subsequently identified in infected broad bean tissue (Hargreaves, 1976; Hargreaves, Mansfield & Rossall, 1977), were absent from infection droplets removed from sites at which no lesions developed.

Bailey (1969) has shown that the ability of leaf discs of pea (Pisum sativum) to produce the phytoalexin pisatin declined during senescence in vitro. He also suggested that hyphal growth on young leaves may be restricted by phytoalexins produced in response to fungal metabolites diffusing from germinating spores (Bailey, 1971). However, Mansfield, Dix and Perkins (1975) suggested that fungal penetration into the epidermis was an essential prerequisite for the induction of pisatin accumulation in intact pea leaves. It therefore seems unlikely that phytoalexins are generally involved in the inhibition of germination of conidia on leaf surfaces.

Factors other than phytoalexins have been found to cause the inhibition of conidial development on the leaf surface. Epiphytic bacteria have been shown to be strongly involved in the inhibition of B. cinerea conidia. Droplets collected from leaves of broad bean, French bean and tomato were found to be inhibitory, and antifungal activity was always reduced following the removal of bacteria by

membrane filtration. The possibility that this reduction of activity by filtration was caused by an antifungal principle(s) being bound to the membrane was discounted. Bacteria trapped on the filter were shown to be inhibitory to B. cinerea in vitro when resuspended in SDW. Inhibitory activity detected in water droplets by Brown (1922) and in inoculum droplets removed from lesion free sites (Purkayastha & Deverall, 1965b) was probably therefore due at least in part to the presence of bacteria within the droplets.

Results obtained suggested that bacteria did not produce the water soluble inhibitor detected in inoculum droplets and that no such inhibitor arose from possible interactions between bacteria and other components within the inoculum droplet. It seems probable that inhibition resulted from nutrient competition between bacteria and conidia as glucose overcame the effect in vitro and on the leaf surface in situ.

Blakeman & Fraser (1971) and Blakeman (1972) suggested a similar mechanism for the inhibition of B. cinerea on leaves of chrysanthemum and beetroot respectively, and this conclusion closely parallels the hypothesis put forward by Lingappa and Lockwood (1964) to explain soil fungistasis.

A continuously growing population of bacteria on the surface of leaves need not be postulated to explain the inhibition of fungal spores. The initial inoculum of bacteria could come from bacterial cells already on the leaf and from sedimentation of bacteria from the atmosphere into infection droplets. The presence of nutrients exuded into the surface from within the leaf is likely to cause multiplication of bacteria. The presence of B. cinerea conidia has

also been shown to promote bacterial growth in infection droplets as a result of the passage of amino acids from the conidia into the droplets (Fraser, 1971).

The work of Sztejnberg and Blakeman (1973a) provided further evidence to support the concept that B. cinerea conidia may be inhibited by nutrient competition between bacteria and conidia. They demonstrated that conidia leached of their endogenous nutrients exhibited poor germination and germ tube growth, and by incident-light fluorescence microscopy showed that bacteria isolated from beetroot leaves tended to aggregate around conidia. No lysis on damage to conidia was observed.

Brodie & Blakeman (1975 & 1976) suggested that antagonistic isolates of bacteria from beetroot leaves adversely affected germination of B. cinerea conidia by utilizing mutrients derived from conidia and exogenous sources. The capacity of bacteria to deplete levels of amino acids in amino acid/glucose solutions was thought to be the main mechanism whereby they inhibited conidia. Further research on the interaction between bacteria associated with broad bean leaves and B. cinerea conidia using techniques similar to those described by Sztejnberg & Blakeman (1973a) and Brodie & Blakeman (1975 & 1976), would possibly provide more evidence that nutrient competition is the main mechanism by which bacteria inhibit conidial development in this system.

The precise role of bacteria in inhibiting B. cinerea on the surface of the leaf in situ is unclear. Attempts to ascertain their role on beetroot leaves have been made by endeavouring to remove them from the biological system described for the inoculum droplet.

Sztejnberg & Blakeman (1973b) irradiated leaves with high energy short wave UV light to sterilize them prior to inoculation with B. cinerea. However, rapid recolonization of leaves by bacteria protected from direct exposure to radiation overcame the desired effect. Blakeman & Sztejnberg (1974) incorporated anti-bacterial antibiotics within B. cinerea inoculum droplets to try to suppress bacterial multiplication. Data obtained was, however, difficult to interpret as levels of antibiotic (streptomycin) which resulted in satisfactory germination of B. cinerea conidia not only reduced bacterial numbers but also caused an increased leakage of amino acids and carbohydrates. Improved fungal performance may, therefore, have been induced by the presence of excess nutrients. Further evidence to resolve this problem may be obtained from the inoculation of a sterile plant, although growth of a plant in aseptic conditions may affect things other than the bacterial population of its leaf surface.

It is of importance to consider the limitations of the work on the inhibition by bacteria reported in this thesis. As experiments were carried out using detached leaves, it is possible that the accumulation of photosynthetic assimilates in the leaf increased the availability of nutrients for leakage into the infection droplet; and hence promoted bacterial growth and subsequent inhibition of conidial development. Inoculation of intact plants would have been a closer approach to the natural system, although practical problems involved would have been difficult to overcome. The possibility that bacteria may produce an unstable or a highly volatile inhibitor, such as ethylene, cannot be completely overlooked.

Clark & Lorbeer (1976) suggested that bacteria on leaves of onions may be involved in the differential pathogenicities of

B. cinerea and B. squamosa. Subsequently, however, Clark & Lorbeer (1977) concluded that leaf surface bacteria alone probably do not prevent lesion formation by B. cinerea on onion leaves, Yoder & Whalen (1975) reached a similar conclusion for the infection of cabbage by B. cinerea. In the broad bean leaf system described here a water soluble inhibitor remaining after removal of bacteria from infection droplets was also probably involved in leaf surface inhibition.

After membrane filtration, diffusates recovered from broad bean leaves were more antifungal than those from leaves of French bean and tomato. The residual inhibitor within diffusates from broad bean leaves appeared to originate mainly from the interaction between the leaf and conidia. The properties of antifungal diffusates from symptomless leaf inoculation sites were identical to properties of diffusates obtained from the inoculation of redeposited extracted leaf wax, and as inhibitor was also observed in situ following inhibition of wax layers it would appear likely that the water soluble inhibitor detected in diffusates arose from epicuticular wax.

The involvement of cuticular waxes in chemically inhibiting the development of fungal pathogens has been suggested for several other host-parasite systems. An ether soluble acidic fraction of wax from apple leaves was shown to inhibit apple powdery mildew (Podosphaera leuchotricha) and Botrytis fabae (Martin, Batt & Burchill, 1957). This observation was made by treating apple and broad bean leaves with the extracted fraction of apple leaf wax prior to inoculation, and noting the subsequent reduction in germination of powdery mildew conidia and suppression of disease symptoms caused by B. fabae, respectively. Blakemann & Sztejnberg (1973) demonstrated that

reformed waxes extracted from beetroot leaves were inhibitory to conidia of B. cinerea in vitro.

Results reported in this thesis not only demonstrate the inhibitory activity of reformed waxes but also show that a water soluble inhibitor was released from wax into inoculum droplets. As the presence of conidia was necessary to cause release of inhibitory activity from wax it is possible that the released compound(s) may result from a partial degradation of wax. The induced antifungal activity in inoculum droplets cannot be considered a phytoalexin response if the inhibitory principle arises from epicuticular wax as phytoalexins are normally considered to arise from metabolic changes within plant cells.

Although leaves were dipped into chloroform for only one second to extract wax, the possibility that antifungal compounds from underlying plant cells may still have passed into solution cannot be totally disregarded. Blakeman & Atkinson (1975) suggested that a germination inhibitor associated with wax from chrysanthemum leaves may merely be co-extracted with the wax, being of cellular origin. However, chrysanthemum wax was extracted by dipping leaves into chloroform for 5s, thus increasing the likelihood of co-extraction.

Phenolic compounds have been implicated in the inhibition of fungi on the surface of leaves. Dix (1974) demonstrated the presence of antifungal compounds in the leaves of Norway maple (Acer platanoides L.) and tentatively linked the poor colonization by Cladosporium spp. with the presence of trihydroxy benzoic acid (gallic acid) on the surface of leaves. Recently Irvine, Dix & Warren (1978) have suggested that seasonal changes in levels of inhibitors including

gallic acid, which passed into solution from leaves of Norway maple, correlated well with changes in growth of phylloplane fungi. However, it is unlikely that phenolic compounds were responsible for the inhibitory activity associated with broad bean leaves and extracted wax as these could readily have been detected by UV spectroscopy and TLC bioassays.

Although more water soluble inhibitor was detected in droplets containing conidia, than in water incubated on the leaf surface, the release of the inhibitor in the absence of conidia cannot be ignored. It is possible that more than one inhibitory compound was produced; one may be freely available to pass into solution on the leaf surface and the other only pass into solution in the presence of conidia. Results suggested that the inhibitor(s) arising from intact leaves and extracted waxes was volatile or unstable.

The only component of wax separated by TLC shown to be inhibitory to conidia of B. cinerea was the primary alcohol fraction. Fukuda & Brannan (1971) demonstrated the production of the enzyme, alcohol oxidase, by B. cinerea, which oxidised primary alcohols to their corresponding aldehydes and liberated hydrogen peroxide. Hydrogen peroxide would not partition into Et_2O or $CHCl_3$ and removal of residual solvent from the aqueous phase by bubbling OFN through it would be likely to cause its decomposition. At a sufficiently high concentration hydrogen peroxide would be antifungal, as it is widely used as a disinfectant. It would be of interest to examine the presence of hydrogen peroxide in diffusates from leaves and redeposited wax using O-dianisidine dihydrochloride reagent, as desrcibed by Janssen & Ruelius (1968).

(c) The effect of nutrients on leaf surface inhibition

Although the inhibition of germination and germ tube growth on the leaf surface prevented lesion formation at most inoculation sites, symptoms did develop at some sites on growth room grown leaves. As glucose readily overcame the inhibition of conidial development this may reflect the presence of nutrients randomly distributed on the leaf surface before inoculation, either as constituents of the cuticle, or dried exudates from epidermal cells (Martin & Juniper, 1970). A second possibility is that nutrients accumulate after inoculation by exosmosis from the epidermis into the infection droplet, and that the rate of accumulation varies at different sites. The variation may be due to differences in cuticular construction and/or physiological conditions of subtending cells. Brown (1922) studied the significance of exosmosis into droplets on the infection of plants by B. cinerea. Droplets of water incubated on petals become stimulatory to B. cinerea conidia to different degrees depending upon sites. Random variation in exosmosis from healthy tissue is therefore not unknown. Brown (1922) also found that a rapid increase in conductivity in drops containing conidia coincided with the penetration of the epidermis. Therefore the stimulation of fungal growth observed at lesion sites may follow the successful penetration of an epidermal cell, thus releasing nutrients to overcome the fungistatic condition of the conidia. Further investigations are needed before any distinctions can be drawn between these alternative proposals to explain the production of lesions at some inoculation sites.

When inoculated onto field grown leaves in SDW, B. cinerea conidia caused the formation of visible symptoms at more inoculation sites than on corresponding leaves detached from growth room grown

plants. Inhibition of germination and germ tube growth of conidia at lesion free sites was once again associated with the accumulation of antifungal activity within inoculum droplets, although the activity of cell-free diffusates was always less than those from growth room leaves. Reduced inhibitory activity could have resulted from either a lesser production of the antifungal principle(s) or a larger release of nutrients into inoculum droplets from the leaf. It was not possible to examine the former alternative as no method of characterizing and estimating the levels of the water soluble inhibitor was discovered. Increased leakage of nutrients onto the surface of leaves as a result of weathering and microbial attack (such as may be experienced under field conditions) causing a deterioration in the properties of the cuticular surface may occur (Last & Deighten, 1965; Ruinen, 1966). Results obtained suggested that a strong relationship existed between the propensity of a leaf to develop lesions following inoculation with B. cinerea, and its tendency to leach carbohydrates and amino acids into droplets incubated on the leaf surface. When levels of glucose and glutamine, as detected in aqueous diffusates from susceptible leaves, were incorporated with droplets of B. cinerea inoculated onto the surface of growth room grown leaves, lesions developed at most sites.

It seems likely that because of the greater availability of nutrients on field grown leaves, the inhibition of conidia on the leaf surface may be of less importance in the field than order growth room or greenhouse conditions. These experiments also showed that the levels of glucose required to overcome the inhibitory effect were much lower in the presence of glutamine, thus supporting the conclusions of Blakeman & Brodie (1977) that limitation of amino acids rather than carbohydrates is likely to inhibit development of B. cinerea conidia.

(d) Conclusions on the inhibition of Botrytis on leaves of V. faba

It has been found that conidial development of avirulent Botrytis spp. is inhibited at inoculation sites where lesions failed to develop. Inhibition is probably the joint result of two processes. Leaf surface bacteria are inhibitory, and the process of inhibition by bacteria appeared to be one of nutrient competition between bacteria and conidia. A water soluble inhibitor (not a known phytoalexin) which probably arose from cuticular wax was also shown to be involved in the process of inhibition of germination. Results obtained indicated that although some inhibitor passed in solution in water droplets incubated on the leaf surface, more inhibitory activity was detected in droplets of conidial suspension on the leaf. A mechanism whereby conidia could induce the production of an unstable inhibitor from a wax component was suggested.

Both components in the inhibitory system could be overcome by glucose, or more especially by a mixture of glucose and glutamine, which promoted conidial development and subsequently allowed leaf infection. Results showed that B. fabae conidia were also inhibited in vitro by diffusates from leaves. It is probable that the reason this species was not inhibited in situ related to its ability to penetrate rapidly into epidermal cells and thereby release nutrients into the inoculum droplet overcoming any inhibitory factors which may have begun to accumulate.

II ROLE OF PHYTOALEXINS IN THE RESISTANCE OF *V. FABA* TO *BOTRYTIS*

(a) Antifungal activity of phytoalexins from V. faba

(i) Bioassay procedures

The antifungal activity of the five major phytoalexins recognised in tissues of *V. faba* following inoculation with *B. cinerea* and *B. fabae* has been examined (Hargreaves, 1976; Hargreaves *et al*, 1977). *In vitro* determinations of antifungal activity were designed with caution, as the composition of test media can influence the activity of specific compounds (Deverall & Rogers, 1972; Van Etten, 1973). Bioassays using radial growth of mycelium on agar have been used extensively in earlier work (for example Bailey, 1974; Mansfield & Deverall, 1974b; Smith, Van Etten & Bateman, 1975 and Smith, 1976). In such experiments growth of the fungus on plates was compared in the presence or absence of specific phytoalexin concentrations. Bailey, Skipp & Carter (1976), examining the activity of phaseollin against mycelial growth of *Colletotrichum lindemuthianum*, concluded that, as growth rates were non-linear at different phytoalexin concentrations, such assays based on one measurement only may be misleading for this and possibly other phytoalexins.

Use of agar media bioassays was also criticized by Skipp & Bailey (1976) who found that results obtained using this technique were influenced by the composition of agar media, mass, age and race of inocula, the incubation period and the solvents used to solubize the inhibitor. Duczek & Higgins (1976) suggested that discrepancies between the antifungal activity of the phytoalexins medicarpin and maackiain against *Stemphylium botryosum* and *Colletotrichum lindemuthianum* measured using germ tube and mycelial growth bioassays

was probably a reflection of the ability of the fungi to detoxify the inhibitors during the long incubation period required for the latter bioassay. Various types of bioassay used to measure fungitoxicity of isoflavanoid phytoalexins were compared by Skipp & Bailey (1977), who concluded that the most reliable indications of a phytoalexin's fundamental activity were obtained using liquid media bioassays, particularly against sporelings. With the exception of preliminary studies against conidia, this type of bioassay was adopted in the research reported here. The pH of the liquid media was buffered to the value required.

(ii) Relative activity of phytoalexins

Results obtained suggested that all 'wyerone' type phytoalexins tested were more inhibitory against the weakly pathogenic B. cinerea than against the aggressive pathogen B. fabae. Medicarpin (a pterocarpanoid phytoalexin), however, exhibited comparatively little differential activity. Although the accumulation of medicarpin (Harvgreaves et al, 1977) may be of phylogenetic interest (Ingham & Harborne, 1976), the time course of its accumulation and low levels reached during resistant reactions suggest that compared to wyerone derivatives this phytoalexin is not of great significance in the inhibition of growth of invading hyphae.

At pH 4.0, wyerone epoxide was slightly more antifungal than wyerone acid. Both were more active than wyerone and wyerol was much less active than the other phytoalexins tested, presumably due to the reduction of the keto group to an alcohol. Since wyerol was only present at low levels in infected tissue (Hargreaves, 1976), it is likely that it does not play a significant role in the disease resistance of the broad bean.

All the "wyerone type" phytoalexins (except wyerol) possess the keto-acetylene moiety, which is probably responsible for the antifungal activity of these compounds (Fawcett, Spencer & Wain, 1969; Mansfield & Widdowson, 1973). Assuming that the site of action of the phytoalexins is within the fungal cytoplasm, it would be expected that the rate of phytoalexin uptake is an important factor determining antifungal activity. Because both wyerone and wyerone epoxide are relatively lipoid soluble, due to the presence of the methyl ester group, they should move through the fungal cell membrane without hindrance, and it would be expected that wyerone would be as active as wyerone epoxide and wyerone acid. However, as this is not the case, factors other than phytoalexin uptake must be involved. Differences in the activities of the compounds may reflect differing mode of action or rates of metabolism by the fungi or the presence of specific binding sites for each compound. The presence of certain moieties within any structure may facilitate binding to a receptor or enhance the inhibitory properties of a bound molecule.

Structural requirements of antifungal isoflavanoids have been examined by Van Etten (1976) and Van Etten & Pueppke (1976). They were not able to draw firm conclusions pertaining to structural/activity relationships. A common three-dimensional shape was not essential for antifungal activity and it was suggested that compounds with differing structures may have different sites and different modes of action.

(iii) Effect of pH of antifungal activity of phytoalexins

The activity of wyerone acid against conidia and sporelings was influenced by the pH of the medium, but pH had little effect on the other phytoalexins tested. Earlier work (Deverall & Rogers, 1972) showed that a similar effect was observed when the activity of wyerone acid was assayed against conidia only. Brown & Swinborne (1971) also demonstrated that pH influenced the activity of benzoic acid, an antifungal compound extracted from apple fruits.

The action of low pH on the activity of wyerone acid is probably through suppression of dissociation of the acid. The undissociated molecule may be readily taken up by the fungi as discussed for fungicides by Bryde (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 a 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 cells, perhaps by either absorption onto the cell wall or repulsion by groups of a similar charge. It must be stressed that it is not known whether wyerone acid must pass into the cytoplasm to produce growth inhibition 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. It has also been suggested that phaseollin acts either on the plasma membrane or affects some process required for membrane function (Van Etten & Bateman, 1971).

As an alternative explanation for the effect of pH on wyerone acid activity, Deverall & Rogers (1972) suggested that pH may influence fungal membranes directly and facilitate the inhibitory activity of wyerone acid. Below pH 4.0 the increase in activity associated with a drop in pH appears to diminish, suggesting that the phytoalexin may achieve maximum activity at a pH above that at which it is fully dissociated. A precise knowledge of the mode of action of wyerone acid and the location of possible receptor sites within the cytoplasm or on cell membranes is necessary before a conclusive explanation of the effect of pH on wyerone acid activity can be reached.

(iv) Mode of action of phytoalexins

Attempts to determine if wyerone acid acted directly on fungal membranes were made by examining the induced leakage of cations from sporelings of B. cinerea and B. fabae. Although K^+ leakage was detected at fungistatic concentrations, the rate of loss of ions was slower than that obtained using the polyene antibiotic "nystatin", which is known to affect fungal membranes directly. It is therefore unlikely that wyerone acid acts in a similar manner to the polyene antibiotic. Confirmation that wyerone acid acts at a different site from nystatin could be obtained by incorporating sterols, which act at an alternative binding substrate for polyenes, into the bioassay medium. However, the possibility that wyerone acid does act directly on fungal membranes cannot be completely discounted.

The possible existence of a specific binding site or sites for phytoalexins within germ tubes was examined by assessing the effect of non-antifungal compounds with a similar structure to phytoalexins, on the antifungal activity of specific concentrations of the inhibitors.

The effect of reduced wyerone acid on the activity of wyerone acid towards sporelings of B. cinerea and B. fabae suggested that the protective influence of the reduced metabolite was more pronounced with B. fabae. However, this may merely be a reflection of the initial greater antifungal activity of the concentration of wyerone acid towards B. cinerea.

The relationships between the structure of potential phytoalexin inhibitors and their effects on wyerone, wyerone acid and epoxide are not clear. Reduced wyerone acid was the only active metabolite but a synthetic keto-furan (compound 8) was a more effective phytoalexin inhibitor. The presence of ketone, acetylenic and acid groups appeared to influence the inhibitor of antifungal activity and in general wyerone acid was more affected than other phytoalexins. However, all three phytoalexins were affected to varying degrees by some compounds, so the possibility of a common site of action cannot be overlooked. Further research is necessary to elucidate the structures of the most potent phytoalexin inhibitors and their relative effects on different phytoalexins.

Analysis of the relationship between wyerone acid concentration and fractional inhibiton of B. fabae germ tube growth by the phytoalexin in the presence or absence of reduced wyerone acid was carried out (Rossall, Mansfield & Price, 1977).

Results are most easily explained by postulating the existence of a binding site for wyerone acid on or in germ tubes, the formation of binding site, wyerone acid complex resulting in inhibition of germ tube growth. Reduced wyerone acid was thought to act by competing for such sites. The binding site may be the site of action of

wyerone acid or may be a site involved in the uptake of the compounds (wyerone acid in particular) into fungal germ tubes. However, in view of the lipophilic nature of the undissociated molecules it would seem probably that they enter cells passively, and competition for some receptor site within hyphae is therefore more likely. The greater sensitivity of species of Botrytis other than B. fabae to wyerone acid may therefore relate to the presence of more binding sites in the sensitive fungi. The presence of wyerone acid receptor sites in Botrytis requires confirmation by direct binding studies using radioactively labelled wyerone acid.

The possibility that reduced wyerone acid, and other phytoalexin metabolites, may interfere with the action of phytoalexins within infected tissue merits further investigations.

(v) Differential sensitivity of fungi to wyerone acid

The tolerance of B. cinerea and B. fabae to phytoalexins of V. faba in vitro correlated with their pathogenicity towards broad bean, sporelings of B. fabae being less sensitive to each phytoalexin than those of B. cinerea. The effect of wyerone acid, the predominant phytoalexin produced by bean leaves, on a range of Botrytis spp. non-pathogenic to bean was examined. No species other than B. fabae was more tolerant than the weakly pathogenic B. cinerea, including B. elliptica which has a large conidial volume, similar to that of B. fabae. It was also interesting to note that the germ tubes which grew fastest in SPN were not necessarily the least sensitive to the phytoalexin.

Van Etten (1973) also examined the antifungal activity of the phytoalexins, pisatin and phaseollin, towards a range of fungal species.

Apart from occasional discrepancies, he found pea pathogens were more tolerant of pisatin than non pathogens and pathogens of French bean were more tolerant of phaseollin than non-pathogens of bean.

However, Smith et al (1975) suggested that no correlation existed between fungal sensitivity to kievitone and pathogenicity towards French bean, and Bailey, Burden, Mynett & Brown (1977) demonstrated that Septoria nodorum, on non-pathogen of French bean, could metabolize the phytoalexin phaseollin to less antifungal derivatives.

The tolerance of different isolates of B. cinerea and B. fabae to wyerone acid was compared to their pathogenicities towards leaves of V. faba. In general, fungi most tolerant of the phytoalexin were most pathogenic towards bean leaves. However, it is not possible to conclude a causal link between pathogenicity and insensitivity to wyerone acid as other determinants of pathogenicity, such as the production of phytotoxic metabolites may be involved.

(vi) Relationships between growth of fungal germ tubes and metabolism of phytoalexins

The involvement of phytoalexin metabolism in the differential sensitivity of fungi towards wyerone derivatives was examined. Metabolic pathways involved in detoxification are summarized in Fig.4. Preliminary experiments on growth rates of germ tubes of B. cinerea and B. fabae in the presence of wyerone, wyerone acid and wyerone epoxide at approximately ED_{50} concentrations for each indicated that a lag in growth occurred with methyl esterified phytoalexins, but not with wyerone acid.

As suggested by Van Etten & Pueppke (1976) the kinetics of this growth in relation to disappearance of the inhibitor was investigated.

Experiments using wyerone acid suggested that germ tube growth and phytoalexin metabolism were closely parallelled. Apparent differences in the ability of the two fungi to metabolize wyerone acid (Deverall & Vessey, 1969; Mansfield & Widdowson, 1973) therefore probably reflected the difference in the differential sensitivity of both fungi to the phytoalexin. However, results obtained using wyerone and wyerone epoxide indicated that metabolism was a necessary precursor of germ tube growth of B. cinerea and B. fabae. It was therefore considered possible that for these two inhibitors differential rates of metabolism could explain their differing sensitivities. However, the onset of rapid growth by B. fabae always occurred when wyerone and the epoxide were present at levels about twice those allowing rapid growth of B. cinerea. It would seem, therefore, that the sensitivity of B. cinerea and B. fabae to each of the wyerone derivatives is not directly related to their abilities to metabolize the phytoalexins. It may be a reflection of the presence of fewer phytoalexin receptor sites in the virulent pathogen.

(vii) Fungitoxicity of phytoalexins

The fungitoxic properties of "wyerone" phytoalexins as distinct from their fungistatic effects were examined. A phytoalexin was considered fungitoxic if it killed fungal structures rather than merely inhibiting their growth. Some authors, however, do not differentiate between the two effects (e.g. Smith, 1978).

Wyerone, wyerone acid and the epoxide were all shown to be fungitoxic to sporelings of B. cinerea and B. fabae at concentrations slightly greater than those determined to give a complete fungistatic effect. These findings contrasted with those of Skipp & Bailey

(1977) who suggested that medicarpin and kievitone were fungicidal to one day old sporelings at doses which completely inhibited growth. Pisatin, however, was rarely shown to kill fungal cells.

Although wyerone epoxide had been shown to be slightly more inhibitory to germ tube growth than wyerone acid, their fungitoxicities were very similar. Both were more toxic than wyerone. The similarities in the relationships of the fungicidal effects of the three phytoalexins to their inhibitory properties towards both B. cinerea and B. fabae suggests the site of action for toxicity is likely to be the same as that for inhibiton of growth.

Death of sporelings was not instantaneous even at concentrations far greater than those required to completely inhibit growth. Within a population of sporelings some died more quickly than others, suggesting that individuals differed in sensitivity to the inhibitors.

Secondary germ tubes were occasionally formed from surviving conidia following the death of the primary germ tube after exposure to wyerone acid. This observation was similar to that made by Skipp & Bailey (1976) who observed the growth of unaffected hyphae of Colletotrichum lindemuthianum from interstitial cells of ld old germ tubes even after exposure to high concentrations of phaseollin. Higgins (1978) also observed the resumption of growth of Stemphylium botryosum germ tubes after exposure to maackiain, as a branch just behind the apex.

As the concentration of wyerone acid in the medium would not be likely to decrease before the onset of growth, it was likely that the secondary germ tubes produced in this way would be less sensitive to the phytoalexin. Experimental evidence confirmed this hypothesis.

The increased tolerance of secondary hyphae could be an expression of their greater ability to metabolize the phytoalexin or a reduced sensitivity to the inhibitors. A reduction in sensitivity could possibly be caused by the presence of fewer wyerone acid binding sites.

To attempt to elucidate the mechanism of survival of the secondary hyphae described, kinetic studies on the growth of hyphae and phytoalexin metabolism, as described for primary germ tubes, would provide valuable information pertaining to their relative ability to detoxify wyerone acid.

(viii) Effect of fungal growth stage on tolerance of phytoalexins

Results suggested that conidia were less sensitive to both the fungistatic and fungitoxic effects of wyerone acid. This may be a reflection of changes in wall and membrane permeability, propensity to metabolize the phytoalexins, or a change in binding sites, rendering the sporelings more vulnerable to phytoalexin attack.

Older sporelings, with septate germ tubes were also less easily killed by wyerone acid than young ones. The increased tolerance may be due to possible changes described above, or it is also possible that with longer germ tubes the ratio of binding sites to molecules of wyerone acid available reduces sufficiently to diminish the toxicity of the phytoalexins. There was no indication that terminal and subterminal cells differed in their sensitivity to wyerone acid, although spores were usually the last to die. This effect may once again reflect a difference in permeability and/or binding of the phytoalexin.

(b) Phytoalexin accumulation and activity in vivo

In order to be strongly implicated as specific mediators of resistance, phytoalexins must satisfy several fundamental requirements, and Daly (1972) emphasized the need for caution in asserting a role in disease resistance for each antimicrobial compound extracted from an infected plant. He emphasized that phytoalexins must be shown to accumulate within infected tissue to a concentration sufficiently high to cause the cessation in growth of the invading fungus, and be located at the same site as infection hyphae. Inhibitory levels must also be achieved by the time fungal growth is observed to stop.

(i) Localization and timing of phytoalexin accumulation and cessation of fungal growth

Phytoalexins must be localized around the site of inhibited infection hyphae to be considered as a causal agent of inhibition. Following inoculation, B. cinerea was restricted to the production of short infection hyphae within leaf epidermis, whereas B. fabae ramified through leaf tissue.

An an inoculum concentration of 2000 conidia/droplet (10^5 conidia/ml) virtually every epidermal cell was affected, therefore levels of phytoalexins determined in epidermal strips probably accurately reflected the levels to which infection hyphae were exposed in situ. The accumulation of phytoalexins in V. faba was associated with tissue necrosis and cellular browning, but the relationship between necrobiosis and phytoalexin accumulation is far from clear. Microspectrofluorimetric studies have shown that wyerone acid accumulates in live cells adjacent to dead cells in bean leaves infected with B. cinerea (Mansfield, Hargreaves & Boyle, 1974). The other phytoalexins may also accumulate primarily in living cells. Inhibitors may diffuse

out of live cells and/or may be released following their death and the associated disruptions of plasmalemma and tonoplast.

Examination of inoculated epidermis with a fluorescence microscope revealed the presence of many fluorescent cells around sites of attempted penetration. Fluorescence in necrotic cells could have been obscured by UV absorbing substances. As phytoalexins (primarily wyerone acid) also progressively accumulated in inoculum droplets overlying limited lesions caused by B. cinerea, when further growth of infection hyphae was halted, it seems safe to assume the inhibitors were not compartmentalized within cells. Direct fungal contact with phytoalexins in situ is therefore very probable.

Hargreaves & Bailey (1978) have also suggested that phytoalexins in hypocotyls of Phaseolus vulgaris are produced by live cells in close contact with dead cells, and are then taken up and accumulated in the dead cells. Yoshikawa, Yameuchi & Masuago (1978) also concluded that glyceollin is localized around infection hyphae of Phytophthora megasperma var. sojae during the resistant response of soy bean hypocotyls.

The role of wyerone in disease resistance may be more important than is indicated by its comparatively low antifungal activity. Hargreaves et al (1976) suggest wyerone may be a precursor for the more active wyerone acid, and that its activity may be enhanced by binding to cell walls adjacent to invading hyphae. Wyerone deposited on cellulose fibrils effectively inhibited germ tube growth of B. cinerea (Hargreaves, et al, 1976).

Observations on the growth and viability of infection hyphae within broad bean epidermis suggested that B. cinerea ceased growing

within the first day after inoculation and progressively died within the following two days. B. fabae, however, continued to grow throughout the incubation period and only a few infection hyphae died (except at the lowest spore concentration tested).

Detailed studies of the rates of growth of infection hyphae within the first few hours after inoculation suggested the involvement of phytoalexin accumulation (particularly wyerone acid) as a determinant in the cessation of growth of B. cinerea. With B. fabae, much lower levels of phytoalexin accumulation corresponded to rapid growth of hyphae. B. cinerea infection hyphae grew little or not at all from 10h to 3d after inoculation. During this time, however, the concentration of phytoalexins within the tissue continued to rise, probably causing the observed death of the hyphae.

Contradictory findings were, however, made by Jones Unwin & Ward (1975), who suggested that although capsidiol accumulation within papper fruit was sufficiently high to explain the observed cessation in growth of Phytophthora infestans, it was fungistatic only and other unknown factors were responsible for fungal death in plant cells.

Studies examining ultrastructural changes of sporelings exposed to phytoalexins in vitro and changes occurring within inhibited infection hyphae may provide further evidence that phytoalexins were responsible for inhibition observed in vivo. However, as discussed earlier, within a population of sporelings, not all respond at the same rate, therefore it would be difficult to know precisely what effect one was examining at the ultrastructural level.

Differences observed in fungal growth and phytoalexin accumulation in replicate experiments at different times of the growth season may

reflect changes in the physical growth conditions of plants affecting the phytoalexin response, as described by Cruickshank, Veeraraghavan, & Perrin (1974) for medicarpin production by white clover. Bailey (1969) also concluded that pisatin production in pea declined with age.

(ii) Activity of wyerone acid in vivo

Accumulation of phytoalexins within epidermal tissue confirmed that the predominant inhibitor was wyerone acid. In vitro bioassays strongly suggested that the antifungal activity of this phytoalexin was dependent on pH, therefore the intracellular pH of infected tissue must be considered to attempt to confirm the role of wyerone acid accumulation in the observed inhibition of infection hyphae.

It proved difficult to obtain a precise measurement of intracellular pH, but results obtained suggest it was ≤ 4.5. At this pH it is almost certain that the inhibiton and death of B. cinerea infection hyphae can be explained on the basis of phytoalexin accumulation. More accurate measurement of pH could be attempted using a micro-electrode, however, Anderson & Higinbotham (1975) suggested that use of such electrodes can easily lead to erroneous results.

Further evidence to support the conclusion that the pH within infected epidermal cells is sufficiently low to enable accumulated wyerone acid to inhibit B. cinerea may be obtained by considering the results of test on the antifungal activity of diffusates. By 2d after inoculation, bulked droplets from B. cinerea inoculation sites were totally inhibitory to germ tubes after sterilization, although the concentration of wyerone acid within inoculum droplets was probably much less than that within epidermal cells.

(ii) Differentiation between pathogenicities of B. cinerea and B. fabae

Although phytoalexin accumulation can explain the inhibition of fungal hyphae during the development of limited lesion, the mechanisms underlying the pathogenicity of B. fabae are less well defined. The differential abilities of B. cinerea and B. fabae to colonize leaf and pod tissues appear to relate primarily to the interaction between these species and wyerone acid accumulation. Although metabolism of phytoalexins by host tissues may occur, the net accumulation of inhibitors within infected tissue is probably controlled primarily by a balance between phytoalexin production by the plant and degradation by the fungus. It has been suggested that wyerone acid is metabolized more rapidly by B. fabae than by B. cinerea (Deverall, 1967; Mansfield & Widdowson, 1973) and that this may tip the balance in favour of the pathogen. However, apparent differences in the rates of wyerone acid metabolism previously reported probably merely reflect the differential sensitivities of the fungi to the phytoalexin. B. fabae may be predisposed to metabolize wyerone acid because of its comparative tolerance of the inhibitor.

The accumulation of wyerone acid metabolites in diffusates from leaves from 1-3d after inoculation with B. fabae corresponded with the disappearance of the phytoalexin. However, it was not possible to estimate the levels of metabolites in tissue with the chromatographic system used so any indication of phytoalexin turnover cannot be made. The development of a more sophisticated analytical system, possibly using high pressure liquid chromatography (HPLC) may enable such measurements to be made. Within the first 12h after inoculation 'wyerone' phytoalexins accumulated more slowly in B. fabae inoculation sites than in B. cinerea lesions. At this stage no known metabolites

were detected in inoculum droplets. The ability of the pathogen to suppress phytoalexin accumulation is therefore worth consideration. As phytoalexin production is probably associated with live cells, B. fabae may induce less phytoalexin production than B. cinerea simply by killing more host cells before accumulation of the inhibitors can take place. Balusubramani, Deverall & Murphy (1971) suggested that B. cinerea probably produced less polygalacturonase than B. fabae in vivo, and would therefore be less likely to kill epidermal cells. Further support for the hypothesis can be drawn from the results of Purkayastha (1968) who demonstrated that culture filtrates produced by B. fabae were more phytotoxic than those produced by B. cinerea. Preliminary investigations (Mansfield, personal communication) have confirmed that B. fabae is probably more able to kill epidermal cells of V. faba before phytoalexin accumulation takes place. Limitation of growth of B. fabae infection hyphae at the lowest spore concentrations tested may therefore reflect the failure of the few conidia within individual inoculum droplets to produce coalescing areas of necrosis, and thereby suppress phytoalexin production, as may occur at high spore concentrations.

III GENERAL CONCLUSIONS

Although results suggested that leaf surface inhibition may not be the main resistance mechanism under field conditions, the possibility that B. cinerea may lose inoculum potential by expending energy overcoming the effects cannot be overlooked.

Once leaf surface inhibition is surmounted however, phytoalexins, especially wyerone acid, appear likely to be involved in the differential pathogenicities of B. cinerea and B. fabae. Accumulation of

wyerone acid occurred at the appropriate place and time and to levels sufficiently high to inhibit and ultimately kill B. cinerea infection hyphae. B. fabae, on the other hand, is able to parasitize leaf tissue probably because it is able to reduce phytoalexins to non-antifungal derivatives by virtue of being insensitive to them. It is also possible the virulent species suppresses phytoalexin production by rapidly killing host cells.

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

BIBLIOGRAPHY

ANDERSON, W.P. & HIGINBOTHAM, N. (1975) A cautionary note on plant root electrophysiology. Journal of Experimental Botany, 26, 533-535

ALLEN, C.H. & THOMAS, C.A. (1971a) Trans-trans-3-11-tridecadiene-5,7,9-triyne 1,2-diol, on antifungal polyacetylene from diseased safflower (Carthamus tinctorius). Phytochemistry, 10, 1579-1582

ALLEN, C.H. & THOMAS, C.A. (1971b) A second antifungal polyacetylene compound from Phytophthora infected safflower. Phytopathology 61, 1107-1109

BAILEY, J.A. (1969) Phytoalexin production by leaves of Pisum sativum in relation to senescence. Annals of Applied Biology, 64, 315-324

BAILEY, J.A. (1971) Phytoalexins and the ability of leaf tissues to inhibition fungal growth. In Ecology of leaf surface micro-organisms. (eds. T.F. Preece & C.H. Dickinson) pp.519-528. Academic Press, London

BAILEY, J.A. (1974) The relationship between symptom expression and phytoalexin concentration in hypocotyls of Phaseolus vulgaris infected with Colletotrichum lindemuthianum Physiological Plant Pathology, 4, 477-488

BAILEY, J.A., BURDEN, R.S., MYNETT, A. & BROWN, C. (1977) Metabolism of phaseollin by Septoria nodorum and other non-pathogens of Phaseolus vulgaris. Phytochemistry, 16, 1541-1544

BAILEY, J.A., CARTER, G.A. & SKIPP, R.A. (1976) The use and interpretation of bioassays for fungitoxicity of phytoalexins in agar media. Physiological Plant Pathology, 8, 189-194

BAILEY, J.A. & DEVERALL, B.J. (1971) Formation and activity of phaseollin in the interaction between bean hypocotyls (Phaseolus vulgaris) and physiological races of Colletotrichum lindemuthianum. Physiological Plant Pathology, 1, 435-449

BALASUBRAMANI, K.A., DEVERALL, B.J. & MURPHY, J.V. (1971) Changes in respiratory rate, polyphenoloxidase and polygalacturonase activity in and around lesions caused by Botrytis in leaves of Vicia faba. Physiological Plant Pathology, 1, 105-113

BLAKEMAN, J.P. (1972) Effect of plant age on inhibition of Botrytis cinerea spores by bacteria on beetroot leaves. Physiological Plant Pathology 2, 143-152

BLAKEMAN, J.P. (1973) The chemical environment of leaf surfaces with special reference to germination of pathogenic fungi. Pesticide Science 4, 575-588

BLAKEMAN, J.P. (1975) Germination of Botrytis cinerea conidia in vitro in relation to nutrient conditions on leaf surfaces. Transactions of the British Mycological Society 65, 1-9

BLAKEMAN, J.P. & ATKINSON, P. (1976) Evidence for a spore germination inhibitor co-extracted with wax from leaves. In Microbiology of aerial plant surfaces (eds. C.H. Dickinson & T.F. Preece) pp.441-450

BLAKEMAN, J.P. & BRODIE, I.D.S. (1977) Competition for nutrients between epiphytic micro-organisms and germination of spores of plant pathogens on beetroot leaves. Physiological Plant Pathology 10, 29-42

BLAKEMAN, J.P. & FRASER, A.K. (1971) Inhibition of Botrytis cinerea spores by bacteria on the surface of chrysanthemum leaves. Physiological Plant Pathology 1, 45-54

BLAKEMAN, J.P. & SZTEJNBERG, A. (1973) Effect of surface wax on inhibition of germination of Botrytis cinerea spores on beetroot leaves. Physiological Plant Pathology 3, 269-278

BLAKEMAN, J.P. & SZETJNBERG, A. (1974) Germination of Botrytis cinerea spores on beetroot leaves treated with antibiotics. Transactions of the British Mycological Society 62, 537-545

BOSUND, I. (1962) The action of benzoic acid and salicyclic acids on the metabolism of micro-organisms. Advances in Food Research 11, 331-353

BRODIE, I.D.S. & BLAKEMAN, J.P. (1975) Competition for carbon compounds by a leaf surface bacterium and conidia of Botrytis cinerea. Physiological Plant Pathology 6, 125-135

BRODIE, I.D.S. & BLACKMEN, J.P. (1976) Competition for exogenous substrates in vitro by leaf surface micro-organisms and germination of conidia of Botrytis cinerea. Physiological Plant Pathology 9, 227-239

BROWN, A.E. & SWINBURNE, T.R. (1971) Benzoic acid: an antifungal compound formed in Bramley's seedling apple fruits following infection with Nectria galligena Bres. Physiological Plant Pathology 1, 469-475

BROWN, W. (1922) Studies on the physiology of parasitism. 8 On the exosmosis of nutrient substances from the host tissue into the infection drop. Annals of Botany London 36, 101-119

BURDEN, R.S. & BAILEY, J.D. (1975) Structure of the phytoalexin from soybean. Phytochemistry 14, 1389-1390

BYRDE, R.J.W. (1965) The chemical environment for fungal growth. 4. Chemical inhibition in: the fungi, an advanced treatise Vol.I (eds G.C. Ainsworth & A.S. Sussman) pp. 525-541 Academic Press, London and New York

CAIN, R.O. (1977) M.Sc. thesis, University of Stirling

CLARK, C.A. & LORBEER, J.W. (1976) The development of Botrytis squamosa and B. cinerea on onion leaves as affected by exogenous nutrients and epiphytic bacteria. In Microbiology of plant aerial surfaces (eds. C.H. Dickinson & T.F. Preece) pp.607-626

CLARK, C.A. & LORBEER, J.W. (1977) The role of phyllosphere bacteria on pathogenesis by Botrytis squamosa and B. cinerea on onion leaves. Phytopathology 67, 96-100

CROSSE, J.E. (1965) Bacterial canker of stone fruits. VI. Inhibition of leaf scar infection of cherry by a saprophytic bacterium from leaf surfaces. Annals of Applied Biology 56, 149-160

CRUICKSHANK, I.A.M. (1963) Phytoalexins. Annual Review of Phytopathology 1, 351-374

CRUICKSHANK, I.A.M., BIGGS, D.R. & PERRIN, D.R. (1971) Phytoalexins as determinants of disease resistance in plants. Journal of the Indian Botanical Society 50A, 1-11

CRUICKSHANK, I.A.M. & PERRIN, D.R. (1960) Isolation of a phytoalexin from Pisum sativum L. Nature, London 187, 799-800

CRUICKSHANK, I.A.M. & PERRIN, D.R. (1963a) Phytoalexins of the Leguminasae. Phaseollin from Phaseolus vulgaris L. Life Science 2, 680-682

CRUICKSHANK, I.A.M. & PERRIN, D.R. (1963b) Studies on phytoalexins. VI. Pisatin: The effect of some factors on its formation in Pisum sativum L, and the significance of pisatin in disease resistance. Australian Journal of Biological Sciences 16, 111-128

CRUICKSHANK, I.A.M. & PERRIN, D.R. (1971) Studies on phytoalexins. XI The induction, antimicrobial spectrum and chemical assay of phaseollin. Phytopathologie Zeitschrift 70, 209-229

CRUICKSHANK, I.A.M., PERRIN, D.R. & MANDRYK, M. (1977) Fungitoxicity of duvatrienediols associated with cuticular wax of tobacco leaves. Phytopathologie Zeitschrift 90, 243-249

CRUICKSHANK, I.A.M., VEERARAGHAVAN, J. & PERRIN, D.R. (1974) Some physical factors affecting the formation and/or net accumulation of medicarpin in infection droplets on white clover leaflets. Australian Journal of Plant Physiology 1, 149-156

DALY, J.M. (1972) The use of near-isogenic lines in biochemical studies of the resistance of wheat stem rust. Phytopathology 62, 392-400

DEVERALL, B.J. (1967) Biochemical changes in infection droplets contains spores of Botrytis spp. incubated in the seed cavities of pods of beans (Vicia faba). Annals of Applied Biology 59, 375-387

DEVERALL, B.J. (1972) Phytoalexins and disease resistance. Proceedings of the Royal Society of London B 81, 233-246

DEVERALL, B.J. (1977) Defence mechanisms of plants. Cambridge monographs in experimental Biology. Cambridge University Press

DEVERALL, B.J. & ROGERS, P.M. (1972) The effect of pH and composition of test solutions on the inhibitory activity of wyerone acid towards germination of fungal spores. Annals of Applied Biology 72, 301-305

DEVERALL, B.J. & VESSEY, J.C. (1969) Role of a phytoalexin in controlling lesion development in leaves of Vicia faba after infection by Botrytis spp. Annals of Applied Biology 65, 449-458

DIX, N.J. (1974) Identification of a water soluble fungal inhibitor in leaves of Acer platanoides L. Annals of Botany 38, 505-514

DUCZEK, L.J. & HIGGINS, V.J. (1976) Effect of treatment with the phytoalexins medicarpin and maackiain on fungal growth in vitro and in vivo. Canadian Journal of Botany 54, 2620-2629

FAWCETT, C.H., FIRN, R.D. & SPENCER, D.M. (1971) Wyerone increases in leaves of broad bean (Vicia faba L.) after infection by Botrytis fabae. Physiological Plant Pathology 1, 163-166

FAWCETT, C.H., SPENCER, D.M. & WAIN, R.L. (1969) The isolation and properties of a fungicidal compound present in seedlings of Vicia faba. Netherlands Journal of Plant Pathology 75, 72-81

FAWCETT, C.H., SPENCER, D.M., WAIN, R.L., FALLIS, A.G., JONES, E.R.H., LE QUAN, M., PAGE, C.B., THALLER, V., SCHUBROOK, D.C. & WHITMAN, P.M. (1968) Natural acetylenes: Part XXVII. An antifungal furanoid keto-ester (wyerone) from shoots of the broad bean, Vicia faba L.: Family Papilionaceae. Journal of the Chemical Society (C) 2455-2462

FERNANDES, A.M.S., BAKER, E.A. & MARTIN, J.T. (1964) Studies on the plant article VII. The isolation and fractionation of cuticular waxes. Annals of Applied Biology 53, 43-58

FOKKEMA, N.J. (1973) The role of saprophytic fungi in antagonism against Drechslera sorkiniana (Helminthosporium sativum) on agar plates and rye leaves with pollen. Physiological Plant Pathology 3, 195-205

FOKKEMA, N.J. & LORBEER, J.W. (1974) Interactions between Alternaria porri and the saprophytic mycoflora of onion leaves. Phytopathology 64, 1128-1133

FRASER, A.K. (1971) Growth restriction of pathogenic fungi on the leaf surface. In Ecology of leaf surface micro-organisms (eds. T.F. Preece & C.H. Dickinson) pp.529-535. Academic Press, London

FUKUDA, D.S. & BRANNON, D.R. (1971) Oxidation of alcohols by Botrytis cinerea. Applied Microbiology 21, 550-551

GARRETT, S.D. (1970) Pathogenic root-infecting fungi. Cambridge University Press

HAMILTON-MILLER, J.M.T. (1973) Chemistry and Biology of polyene macrolide antibiotics. Bacteriological Reviews 37, 160-196

HARGREAVES, J.A. (1976) The role of phytoalexins in the disease resistance of Vicia faba L. to infection by Botrytis. Ph.D. thesis, University of Stirling.

HARGREAVES, J.A. & BAILEY, J.A. (1978) Phytoalexin production by hypocotyls of Phaseolus vulgaris in response to constitutive metabolites released by damaged bean cells. Physiological Plant Pathology 13, 89-100

HARGREAVES, J.A. & MANSFIELD, J.W. (1975) Phytoalexin production by Vicia faba in response to infection by Botrytis: Annals of Applied Biology 81, 271-276

HARGREAVES, J.A., MANSFIELD, J.W. & COXON, D.T. (1976a) Conversion of wyerone to wyerol by Botrytis cinerea and B. fabae in vitro. Phytochemistry 15, 651-653

HARGREAVES, J.A., MANSFIELD, J.W. & COXON, D.R. (1976b) Identification of medicarpin as a phytoalexin in the broad bean plant (Vicia faba L.). Nature 262, 318-319

HARGREAVES, J.A., MANSFIELD, J.W., COXON, D.T. & PRICE, K.R. (1976) Wyerone epoxide as a phytoalexin in Vicia faba and its metabolism by Botrytis cinerea and B. fabae in vitro. Phytochemistry 15, 1119-1121.

HARGREAVES, J.A., MANSFIELD, J.W. & ROSSALL, S. (1977) Changes in phytoalexin concentrations in tissues of the broad bean plant (Vicia faba L.) following inoculation with species of Botrytis. Physiological Plant Pathology 11, 227-242

HEATHER, W.A. (1967) Leaf characteristics of Eucalyptus bicostata Maiden et al seedlings affecting the deposition and germination of spores of Phaeoseptoria eucalypti (Hansf.) Walker. Australian Journal of Biological Sciences 20, 1155-1160

HEWITT, B.R. (1958) Spectrophotometric determination of total carbohydrate. Nature, London 182, 246-247

HIGGINS, V.J. (1972) Role of the phytoalexin medicarpin in three leaf spot diseases of alfalfa. Physiological Plant Pathology 2, 289-300

HIGGINS, V.J. (1978) The effect of some pterocarpanoid phytoalexins on germ tube elongation of Stemphylium botryosum. Phytopathology 68, 339-345

HIGGINS, V.J. & MILLAR, R.J. (1968) Phytoalexin production by alfalfa in response to infection by Colletotrichum phomoides, Helminthosporium turcicum, Stemphilium loti and S. botryosum. Phytopathology 58, 1377-1383

HILTNER, N. (1904) Arb. dt. Landwiges 98, 59- From Last, F.T. & Warren R.C. Endeavour 31, 143-150

HOLLOWAY, P.J. & CHALLEN, S.B. (1966) Thin layer chromatography in the study of natural waxes and their constituents. Journal of Chromatography 25, 336-346

INGHAM, J.L. (1972) Phytoalexins and other natural products as factors in disease resistance. Botanical Review 38, 343-424

INGHAM, J.L. (1973) Disease resistance in higher plants: The concept of pre-infectional and post-infectional resistance. Phytopathologie Zeitschrift 78, 314-335

INGHAM, J.L. & HARBORNE, J.B. (1976) Phytoalexin production as a new dynamic approach to the study of systematic relationships among higher plants. Nature 260, 241-243

IRVINE, J.A., DIX, N.J. & WARREN, R.C. (1978) Inhibitory substances in Acer platanoides leaves: seasonal activity and effects on growth of phylloplane fungi. Transactions of the British Mycological Society, 70, 363-371

JANES, B.S. (1962) Leaf clearing technique to assist fungal spore germination counts. Nature, London 193, 1099-1100

JANSSEN, F.W. & RUELIUS, H.W. (1967) Alcohol oxidase, a flavo-protein from several basidiomycetes species. Crystallization and fractional precipitation with polyethylene glycol. Biochimica et Biophysica Acta 151, 330-342

JEFFREE, C.E., BAKER, E.A. & HOLLOWAY, P.J. (1975) Ultrastructure and recrystallization of plant epicuticular waxes. New Phytologist 75, 539-549

JOHNSTON, H.W. & SPRONSTON, T. (1965) The inhibition of fungus infection pegs in Ginkgo biloba. Phytopathology 55, 224-227

JONES, D.R., UNWIN, C.H. & WARD, E.W.B. (1975) The significance of capsidiol induction in pepper fruit during an incompatible reaction with Phytophthora infestans. Phytopathology 65, 1286-1288

KEEN, N.T. (1971) Hydroxyphaseollin production by soybeans resistant and susceptible to Phytophthora megasperma var. sojae Physiological Plant Pathology 1, 265-275

KEEN, N.T. (1972) Accumulation of wyerone in broad bean plants and dimethylhomopterocarpan in jack bean after inoculation with Phytophthora megasperma var sojae. Phytopathology 65, 91-92

KLARMANN, W.L. & STAMFORD, J.B. (1968) Isolation and purification of an antifungal principle from infected soybeans. Life Science 7, 1095-1103

KO, W.H., & LOCKWOOD, J.L. (1967) Soil fungistasis: relation to fungal spore nutrition. Phytopathology 57, 894-901

KUĆ, J. (1972) Phytoalexins. Annual Review of Phytopathology 9, 207-232

KUĆ, J. (1976) Phytoalexins. In Encyclopaedia of plant physiology new series Vol.4. Physiological plant pathology (eds. B. Hertefuss & P.H. Williams). Springer-Verlag; Berlin, Heidelberg & New York, pp.632-652

KUC, J., CURRIER, W.W. & SHIH, M.J. (1976) Terpenoid phytoalexins. In Biochemical aspects of plant-parasite relationships (eds. J. Friend & D.R. Threlfall) pp.225-237

LAMPARD, J.F. & CARTER, G.A. (1973) Chemical investigation of resistance of coffee berry disease in Coffea arabica: an antifungal compound in coffee cuticular wax. Annals of Applied Biology 73, 31-37

LAST, F.T. (1960) Longevity of conidia of Botrytis fabae Sardina. Transaction of the British Mycological Society 43, 165-177

LAST, F.T. & DEIGHTON, F.C. (1965) The non-parasitic microflora on the surfaces of living leaves. Transactions of the British Mycological Society 48, 83-99

LAST, F.T. & HAMLEY, R.E. (1956) A local lesion technique for measuring the infectivity of conidia of Botrytis fabae Sard. Annals of Applied Biology, 76, 77-89

LEACH, R. (1955) Recent observations of the Botrytis infection of beans. Transactions of the British Mycological Society 38, 171

LEBEN, C. (1964) Influence of bacteria isolated from healthy cucumber leaves on two diseases of cucumber. Phytopathology 54, 405-408

LEBEN, C. & DAFT, G.C. (1965) Influence of an epiphytic bacterium on cucumber anthracnose, early blight of tomato and Northern blight of corn. Phytopathology 55, 760-762

LETCHER, R.M., WIDDOWSON, D.A., DEVERALL, B.J. & MANSFIELD, J.W. (1970) Identification and activity of wyerone acid as a phytoalexin in broad bean (Vicia faba) after infection by Botrytis. Phytochemistry 9, 249-252

LINGAPPA, B.T. & LOCKWOOD, J.L. (1964) Activation of soil microflora by fungus spores in relation to soil fungistasis. Journal of General Microbiology 35, 215-227

MANNERS, J.G. & HOSSAIN, J.M.M. (1963) Effects of temperature and humidity on conidial germination of Erysiphe graminis Transactions of the British Mycological Society 46, 225-234

MANSFIELD, J.W. (1972) Studies on the process of resistance of Vicia faba L. to infection by Botrytis. Ph.D. thesis, University of London

MANSFIELD, J.W. & DEVERALL, B. (1974a) The rates of fungal development and lesion formation in leaves of Vicia faba during infection by Botrytis cinerea or B. fabae. Annals of Applied Biology 76, 77-89

MANSFIELD, J.W. & DEVERALL, B.J. (1974b) Changes in wyerone acid concentration in leaves of Vicia faba after infection by Botrytis cinerea or B. fabae. Annals of Applied Biology 77, 227-235

MANSFIELD, J.W., DIX, N.J. & PERKINS, A.M. (1975) Role of the phytoalexin pisatin in controlling saprophytic fungal growth on pea leaves. Transactions of the British Mycological Society 64, 507-511

MANSFIELD, J.W., HARGREAVES, J.A. & BOYLE, F.C. (1974) Phytoalexin production by live cells in broad bean leaves infected with Botrytis cinerea. Nature, 252, 316-317

MANSFIELD, J.W. & WIDDOWSON, D.A. (1973) The metabolism of wyerone acid (a phytoalexin from Vicia faba L.) by Botrytis fabae and B. cinerea. Physiological Plant Pathology 3, 393-404

MARTIN, J.T. (1964) Role of cuticle in the defence against plant disease. Annual Review of Phytopathology 2, 81-100

MARTIN, J.T., BATT, R.F. & BURCHILL, R.T. (1957) Defence mechanism of plants against fungi. Fungistatic properties of apple leaf wax. Nature, London 180, 796-799

MARTIN, J.T. & JUNIPER, B.E. (1970) The cuticles of plants. Edward Arnold, London

MERCK, E. (1971) Dyeing reagents for thin layer and paper chromatography. Darmstadt, W. Germany

MULLER, K.O. (1956) Einige einfache Versuche zum Nachweis von Phytoalexinen. Phytopathologie Zeitschrift 27, 237-254

MULLER, K.O. & BORGER (1974) Experimentelle Untersuchungen uber die Phytophthora - Resisteng der Kartoffel. Arb. Biol. Anst. (Reidsowst) Berlin 23, 189-231

OGILVIE, L. & MUNRO, M.D. (1947) Occurrence of Botrytis faba Sard. in England. Nature London 160, 96

PERRIN, D.R. (1964) The structure of phaseollin. Tetrahedron Letters 1, 29-35

PERRIN, D.R. & BOTTOMLEY, W. (1962) Studies on phytoalexins V. The structure of pisatin from Pisum sativum L. Journal of the American Chemical Society 84, 1919-1922

PRICE, N.K. & DWEK, R.A. (1974) Principles and problems in physical chemistry for biochemists. Oxford: Clarendon Press

PUEPPKE, S.G. & VAN ETTEN, H.D. (1975) Identification of three new pterocarpans (6a, 11a-dihydro-6H-benzofuro (3,2-c) (1) benzopyrons) from Pisum sativum infected with Fusarium solani F. sp. pisi. Journal of the Chemical Society (Perkin Transactions) 1, 946-948

PUEPPKE, S.G. & VAN ETTEN, H.D. (1976) Accumulation of pisatin and three additional antifungal pterocarpans in Fusarium solani infected tissue of Pisum sativum. Physiological Plant Pathology 8, 51-61

PURKAYASTHA, R.P. (1968) Investigations on the phytotoxicity of metabolic by-products in the culture filtrates of Botrytis spp. Science and Culture 35, 385-398

PURKAYASTHA, R.P. & DEVERALL, B.J. (1965a) The growth of Botrytis fabae and B. cinerea into leaves of bean (Vicia faba L.) Annals of Applied Biology 56, 139-147

PURKAYASTHA, R.P. & DEVERALL, B.J. (1965b) The detection of antifungal substances before and after infection of beans (Vicia faba L.) by Botrytis spp. Annals of Applied Biology 56, 269-277

ROSEN, H. (1957) A modified ninhydrin colourometric analysis for amino-acids. Archives of Biochemistry and Biophysics 67, 10-15

ROSSALL, S. (1974) The inhibition of Botrytis cinerea on leaves of Vicia faba. B.A. thesis, Stirling University

ROSSALL, S., MANSFIELD, J.W. & PRICE, N.K. (1977) The effect of reduced wyerone acid on the antifungal activity of the phytoalexin wyerone acid against Botrytis fabae. Journal of General Microbiology 102, 203-205

ROTHSTEIN, A. (1965) Uptake and translocation I Uptake in: The fungi an advanced treatise Vol.I (Eds. G.C. Ainsworth & A.S. Sussman) pp.429-455. Academic Press, London & New York

ROTMAN, B. & PAPERMASTER, B.W. (1966) Membrane properties of living mammalian cells as studied by enzymic hydrolysis of fluorogenic esters. Proceeding of the National Academy of Science 55, 134-141

RUINEN, J. (1966) The phyllosphere in cuticle decomposition by micro-organisms in the phyllosphere. Annale de l'Institute Pasteur 3, 342-346

SCHNATHORST, W.C. (1965) Environmental relationships in the powdery mildews. Annual Review of Phytopathology 3, 343-366

SCHÜTT, P. (1971) Untersuchungen uber den Einfluss von Cuticular-wachsen auf die Infektionsfahrgbeit pathogener Pilze. European Journal of Forestry Pathology 1, 32-50

SINHA, A.K. & TRIVEDI, N. (1969) Immunization of rice plants against Helminthosporium infection. Nature London 223, 963-964

SKIPP, R.A. & BAILEY, J.A. (1976) The effect of phaseollin on the growth of Colletotrichum lindemuthianum in bioassays designed to measure fungitoxicity. Physiological Plant Pathology 9, 253-264

SKIPP, R.A. & BAILEY, J.A. (1977) The fungitoxicity of isoflavanoid phytoalexins measured using different types of bioassay. Physiological Plant Pathology 11, 101-112

SMITH, D.A. (1976) Some effects of the phytoalexin, kievitone, on the vegetative growth of Aphanomyces euteiches, Rhizoctonia solani and Fusarium solani F. sp. phaseoli, Physiological Plant Pathology 9, 45-55

SMITH, D.A. (1978) Observations on the fungitoxicity of the phytoalexin kievitone. Phytopathology 68, 81-88

SMITH, D.A., VAN ETTEN, H.D. & BATEMAN, D.F. (1975) Accumulation of phytoalexins in Phaseolus vulgaris hypocotyls following inoculation with Rhizoctonia solani. Physiological Plant Pathology 5, 51-64

SMITH, D.G., McINNES, A.G., HIGGINS, V.J. & MILLAR, R.J. (1971) Nature of the phytoalexin produced by alfalfa in response to fungal infection. Physiological Plant Pathology 1, 41-44

SMITH, I.M. (1973) The diversity of phytoalexin like responses in the Leguminosae. In Fungal pathogenicity and the plants response (Eds. R.J.W. Byrde & C.V. Cutting) pp. 355-364. London and New York, Academic Press

STOESSEL A., STRUTHERS, J.B. & WARD, E.W.B. (1976) Sesquiterpenoid stress compounds of the solanaceae. Phytochemistry 15, 855-872

STOESSEL, A., UNWIN, C.H. & WARD, E.W.B. (1973) Post-infectional fungal inhibitors from plants: fungal oxidation of capsidiol in pepper fruit. Phytopathology 63, 1225-1231

SZTEJNBERG, A. & BLAKEMAN, J.P. (1973a) Studies on leaching of Botrytis cinerea conidia and dye absorption by bacteria in relation to competition for nutrients on leaves. Journal of General Microbiology 78, 15-22

SZTEJNBERG, A. & BLAKEMAN, J.P. (1973b) Ultraviolet-induced changes in population of epiphytic bacteria on beetroot leaves and their effect on germination of Botrytis cinerea spores. Physiological Plant Pathology 3, 443-451

TUKEY, H.B. (Jr.) (1971) Leaching of substances from plants. In Ecology of leaf surface micro-organisms (eds. T.F. Preece & C.H. Dickinson) pp. 67-80 Academic Press, London

VAN ETTEN, H.D. (1973) Differential sensitivity of fungi to pisatin and to phaseollin. Phytopathology 61, 1363-1372

VAN ETTEN, H.D. (1976) Antifungal activity of pterocarpans and other selected isoflavanoids. Phytochemistry 15, 655-659

VAN ETTEN, H.D. & BATEMAN, D.F. (1971) Studies on the mode of action of the phytoalexin phaseollin. Phytopathology 61, 1363-1372

VAN ETTEN, H.D. & PUPPKE, S.G. (1976) Isoflavanoid phytoalexins. In Biochemical aspects of plant parasite relationships (eds. J. Friend & D.R. Threlfall) pp.239-289

VAN ETTEN, H.D. & SMITH, D.A. (1975) Accumulation of antifungal isoflavanoids and 1 -hydroxy-phaseollone, a phaseollin metabolite, in bean tissue infected with Fusorium solani F. sp. phaseoli. Physiological Plant Pathology 5, 225-237

VARNS, J. & KUĆ, J. (1971) Suppression of rishitin and phytuberin accumulation and hypersensitive response in potato by compatible races of Phytophthora inrestans. Phytopathology 61, 178-181

VENKATA RAM, T.S. (1962) Tea leaf wax as a stimulant and fungistat of spore germination. Current Science 31, 428-429

WIDHOLM, J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 17, 189-194

WILSON, A.R. (1937) The chocolate spot disease of beans (Vicia faba L.) caused by Botrytis cinerea Pers. Annals of Applied Biology 24, 258

WOOD, R.K.S. (1967) Physiological Plant Pathology. Blackwell, Oxford & Edinburgh

WOOD, R.K.S. (1974) Disease in higher plants. Oxford Biology Reader No.57 Ed. J.J. Head, Oxford University Press

YODER, O.C. & WHALEN, M.L. (1975) Factors affecting post-harvest infection of stored cabbage tissue by Botrytis cinerea. Canadian Journal of Botany 53, 691-699

YOSHIKAWA, M., YAMEUCHI, K. & MASAGO, H. (1978) Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with Phytophthora megasperma var. sojae Physiological Plant Pathology 12, 73-82

APPENDIX

STUDIES ON THE ROLE OF LEAF SURFACE BACTERIA IN INHIBITING GERMINATION OF CONIDIA OF *B. CINEREA* *IN SITU*

(a) *Elimination of bacteria from infection droplets using antibiotics*

Preliminary attempts to assess the significance of phyllophane bacteria in inhibiting conidial germination on the leaf surface of broad beans *in situ* were made by endeavouring to reduce or eliminate numbers of bacteria with inoculum droplets using antibiotics. This technique was reported by Blakeman and Sztejnberg (1974a), who used streptomycin sulphate, sodium benzylpenicillin and chloramphenicol incorporated within droplets of *B. cinerea* conidial suspension inoculated onto beetroot leaves. The former two antibiotics are specific against gram-negative and gram-positive respectively and chloramphenicol is active against both. As no information concerning the composition of the bacterial flora of broad bean leaves was available chloramphenicol was adopted in the studies reported.

The effect of incorporation of the antibiotic within inoculum droplets of *B. cinerea* on lesion development was first examined. Bifoliate leaves were inoculated with droplets (10 µl) of a conidial suspension of *B. cinerea* containing 0, 25, 50 and 100 µg/ml chloramphenicol on separate half leaflets. After incubation for 24h lesion development on the leaves was recorded in the usual way and the results obtained are given in Table 47. The results clearly show that increasing concentrations of chloramphenicol caused an increase in lesion formation on leaves 24h after inoculation with *B. cinerea*.

TABLE 47 The effect of chloramphenical on lesion formation on growth room grown bean leaves 24h after inoculation with B. cinerea

Lesion grade	Percentage of inoculation sites in lesion grades in presence of chloramphenicol			
	Control[a]	25 µg/ml	50 µg/ml	100 µg/ml
0	94	86	80	69
3.5	2	0	0	0
6.5	2	4	0	2
9.5	2	4	6	10
19		3	8	5
38		3	2	6
63			4	4
87.5				4
100				

a - Control lesion development of conidial suspension in SDW

The effect of the antibiotic on germination and germ tube growth of conidia of B. cinerea on leaves and glass slides was then examined. Bifoliate leaves and glass slides were inoculated with droplets (10 µl) of B. cinerea conidial suspension containing 0, 25, 50 and 100 µg/ml chloramphenicol on separate half leaflets and quarter slides respectively. Development of conidia was recorded 24h after inoculation, selecting symptomless sites on leaves. Results obtained (Table 48) suggest that although chloramphenicol caused an increase in fungal germination and germ tube growth at lesion free inoculation sites on leaves, it also stimulated growth on glass. It was therefore not possible to ascertain whether the effects of the antibiotic previously described in Table 47 were a result of reduction in bacterial numbers or merely caused by direct stimulation of conidial growth. Further studies using chloramphenicol to suppress bacterial numbers on leaves were therefore abandoned, although it is possible that other antibacterial compounds would yield results more easily interpreted.

N.B. Further attempts to elucidate the role of phylloplane bacteria in the inhibition of B. cinerea on broad bean leaves in situ were based on the growth of axenic plants. Although an aseptic environment suitable for plant growth was developed, it proved impossible to sterilize bean seeds without exposing them to treatments which also proved phytotoxic. The work was, therefore, also abandoned, and the question of the involvement of broad bean leaf surface bacteria inhibiting B. cinerea conidial germination in situ remains unanswered.

TABLE 48 The growth of B. cinerea conidia on leaves and glass slides in the presence of absence of increasing concentrations of chloramphenicol

Substrate inoculated	Concentration of chloramphenicol (µg/ml)	Growth of B. cinerea conidia		
		Percentage germination[a]		Germ tube length[b]
Bean leaves	Control[c]	12.4	(8-15)	19.2
	25	17.0	(13-20)	21.0
	50	24.2	(20-27)[+]	25.8*
	100	30.6	(26-33)[+]	30.2**
Glass slides	Control[c]	83.2	(80-87)	36.1
	25	94.8	(91-98)[Δ]	69.4[†]
	50	100[Δ]		83.0[†]
	100	100[Δ]		92.6[†]

a - Mean of 5 replicate counts of 100 conidia; figures in parentheses refer to range

b - Mean length of 35 germ tubes (µm)

c - Control growth on leaves or glass in absence of chloramphenicol

+ - Range of replicates ouside range of leaf control growth

Δ - Range of replicates outside range of glass slide control growth

* - Significantly different from leaf control (p = 0.002)

** - Significantly different from leaf control (p = 0.001)

† - Significantly different from glass slide control (p = 0.001)

Attention is drawn to the fact that the copyright of this thesis rests with its author.

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior written consent.