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

by

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ABSTRACT

Studies on the development of infections produced by *Botrytis cinerea* or *B. fabae* in broad bean leaves were coupled with a systematic series of measurements of changes in wyerone acid concentrations in leaves as the fungi infect, and either fail or succeed in spreading through the leaf.

Wyerone acid could not be detected in healthy leaves but increased in tissues after fungal induced cell damage became macrosopically visible. The phytoalexin accumulated rapidly to antifungal concentrations after penetration of *B. cinerea* and was therefore probably responsible for the inhibition of growth of invading hyphae. However it was not responsible for the inhibition of the fungus on the leaf surface at many sites which developed little or no visible symptoms. Completely blackened tissues from rarely produced spreading *B. cinerea* infections contained very little wyerone acid. *B. fabae* was shown to be able to metabolize wyerone acid *in vivo* and prevent its accumulation to antifungal concentrations in infected tissue. *B. fabae* infections rapidly spread from inoculation sites. In studies on naturally infected leaves the phytoalexin was extracted from limited lesions but was present at very low levels, if at all, where *B. fabae* had advanced through tissue.

In vitro *B. fabae* appeared to be able to metabolize wyerone acid more rapidly than *B. cinerea*. A U.V. absorbing compound found to be produced from the interaction of *B. fabae* and wyerone acid *in vitro*

was subsequently isolated from B. fabae infected tissues and identified as reduced wyerone acid. This metabolite was not produced by B. cinerea. The reduction of wyerone acid appeared to occur in hyphae of B. fabae.

The addition of certain fungal stimulants including extracts of pollen grains stimulated the development of spreading B. cinerea infections. Experimental results showed that this activity could be explained in terms of modification of the interaction between fungus, plant and wyerone acid.

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INTRODUCTION

One of the most challenging problems in plant pathology concerns the physiologic basis of specific reactions between host and pathogen. We know little about the mechanisms by which a plant differentiates between invading organisms so that some succeed in multiplying within the host plant although others fail; or conversely, how the abilities of the pathogens differ in those properties involved in infection and establishment in the host. These problems are presented in their simplest form in the study of the processes causing the limitation of fungi to leaf spots, in particular the limitation of *Botrytis* to "chocolate spots" in the leaves of *Vicia faba*.

Both *B. cinerea* Sard. and *B. fabae* Pers. cause scattered dark brown spots primarily on the leaves in the "chocolate spot" disease of broad and field beans. The main difference between the fungi rests in the ability of *B. fabae* to cause lesions to spread so that the whole leaf blackens, and the fungus sporulates on the blackened areas. The purpose of this investigation is to study the mechanisms which underlie the success of *B. fabae* and the failure of *B. cinerea* to colonise and destroy leaves of the bean.

Epiphytotics of "chocolate spot" are associated with heavy rainfall during April, May, June and July. Water is essential for germination of conidia and infection, and factors which help to maintain a watery film on the foliage for several days (e.g. high humidity with little or no wind), plus a temperature between 15° and 20°C provide the optimum conditions for outbreaks of the disease. (Wilson 1937; Hogg 1956).

Although Wilson (1937) claimed that the principal cause of "chocolate

spot" was B. cinerea, Ogilvie and Munro (1947) found that B. fabae was associated with outbreaks of the disease in S.W. England. Leach (1955) found that B. cinerea caused "insignificant" restricted lesions principally confined to the epidermis compared with those caused by B. fabae, which spread through the leaf. Deverall and Wood (1961a), and Wastie (1962) confirmed the findings of Leach, that B. fabae was more pathogenic than B. cinerea.

Deverall and Wood (1961b) examined some properties of the two fungi, including the production of cell wall hydrolysing enzymes in vitro, and their interaction with phenolic systems of the host, but were unable to explain the higher pathogenicity of B. fabae.

The first studies specifically designed to investigate the mechanisms which discriminate between the two Botrytis species were carried out by Purkayastha and Deverall (1965a). They designed experiments to investigate the following hypotheses: 1) The cuticle presents a barrier which greatly slows the progress of B. cinerea into the leaf; 2) the leaf fails to supply essential nutrients to B. cinerea; 3) the germination or growth potential of B. fabae is inherently more favourable than that of B. cinerea for spread in the leaf; 4) B. cinerea, but not B. fabae is inhibited in its growth in the leaf tissues. They found that cuticle removal facilitated infection by either pathogen, but the cuticle did not have any role in discriminating between the two fungi. In the absence of the cuticle B. cinerea still remained limited to the infection site. They concluded that spread from the site of infection depends upon an interaction between host cells and invading hyphae.

Garber (1956) suggested that the failure of an invading organism to grow in host tissues may be attributed to the absence of an essential metabolite. Purkayastha and Deverall found that the addition of sugars and media, including extracts of healthy bean leaves, which supported excellent growth *in vitro* did not stimulate the development of spreading infection by *B. cinerea.* They concluded that there was no evidence that bean leaves are in any way deficient in nutrients essential for the growth of *B. cinerea.*

On all nutrient media tested *B. cinerea* grew more rapidly than *B. fabae*, but in water droplets on glass slides *B. fabae* had the higher linear growth rate. The average volume of a *B. fabae* conidium was calculated to be at least 5x that of *B. cinerea.* It was therefore considered likely that a conidium of *B. fabae* has a higher food reserve, and that this may permit a higher rate of germ tube extension in water. It was concluded that *B. fabae* undoubtedly has an advantage over *B. cinerea* in an infection droplet because of this greater inherent growth rate. However Purkayastha and Deverall emphasised that the growth rate of *B. cinerea* in water on glass only differed quantitatively from that of *B. fabae*.

Qualitatively different growth characteristics were manifested by *B. cinerea* only on the leaf surface, where germ tubes were shown to slow markedly in growth soon after germination (8h) and little further growth over the leaf surface took place 12 - 24h after inoculation.

These results were considered to indicate that there is "a particular result of the interaction between the germ tubes of B. cinerea and the leaf which causes the cessation of growth of B. cinerea". They concluded that their experimental results were consistent with the hypothesis that B. cinerea but not B. fabae is inhibited in its growth by substances arising from leaf cells.

In a subsequent paper Purkayastha and Deverall (1965b) described their attempts to detect antifungal substances before and after infection of Vicia faba. Three approaches were made to the problem of finding inhibitors of fungal invasion. First the leaf surface was extracted using a similar technique to that of Martin, Batt and Burchill (1957), to test for the presence, before infection, of substances which are inhibitory to B. cinerea, but not to B. fabae. Secondly they reexplored the discovery of Brown (1922), that drops of water incubated on the surface of bean leaves sometimes became inhibitory to conidia of B. cinerea. Thirdly, experiments were designed to test for the production of antifungal substances or phytoalexins, after invasion by B. cinerea. The term phytoalexin was proposed by Müller and Börger (1940) to mean a principle produced after infection which limited the growth of invading fungi.

No antifungal substances were detected in extracts of leaf surfaces. Drops of water incubated on leaves did become inhibitory 24h after inoculation, but no antifungal activity was observed in droplets incubated on leaves for 4h. This reinforced the conclusion that there were no preformed inhibitors on the leaf surface.

In their search for phytoalexins Purkayastha and Deverall used the drop diffusate technique developed by Müller (1958). They incubated droplets of water, or suspensions of B. cinerea or B. fabae conidia on the surface of leaves and seed cavities in pods for different intervals, in the dark. The diffusates collected were bioassayed against Botrytis conidia.

Quite different results were obtained from diffusates collected from leaves than from pods. Infection droplets containing conidia of B. cinerea or B. fabae both became inhibitory after incubation on leaf surfaces for 72h. Somewhat greater inhibitory activity was observed in diffusates from B. cinerea infected leaves. Droplets of water incubated under the same conditions became less inhibitory than drops of conidial suspension. In contrast water droplets incubated for 24h in the dark on pod seed cavities became highly stimulatory to germ tube growth by both fungi. The consequence of infection by B. fabae was the liberation of further stimulants. However, after infection by B. cinerea, antifungal substances could be detected. In the latter case it was presumed that the concentration of inhibitors was sufficiently high to mask any stimulants present. Preliminary attempts at solvent partition showed that some, but not all of the inhibitory material could be extracted into petroleum ether from water.

The presence of stimulatory activity in diffusates collected from B. fabae infected pods was considered to indicate that B. fabae caused no phytoalexin formation, or too little to be detected in the presence of stimulants. They proposed that B. fabae might actively suppress the

synthesis of the inhibitor. Inactivation by B. fabae was considered unlikely because both fungi were shown to be equally sensitive to a given dose of the inhibitory petroleum ether extract. It was concluded that, "...... metabolism in the lesion area is differently affected by the two fungi, and that infection of leaves and pods by B. cinerea leads to the accumulation of particularly high levels of antifungal substance."

Deverall (1967) carried out detailed investigations of the biochemical changes in infection droplets within the first 24h period after inoculation on to the seed cavities of bean pods. The antifungal component of diffusate from B. cinerea pod cavities was shown to be an ether soluble substance which was purified by paper chromatography. The compound was associated with strong UV absorbance at 360 nm in 50% ethanol. The water phase remaining after repeated extraction of an inhibitory diffusate with diethyl ether was highly stimulatory to germ tube growth by Botrytis. Diethyl ether was found to be much more efficient at extracting inhibitor from diffusates than petroleum ether. This was considered to explain the results on extraction with petroleum ether described by Purkayastha and Deverall (1965b). Contrary to the results obtained by Purkayastha and Deverall it was shown that over a series of concentrations B. fabae was less affected by a certain level of inhibitor than B. cinerea. The inhibitor appeared in infection droplets containing B. cinerea conidia within 18h. Droplets without conidia, or conidia of B. fabae were always found to be highly stimulatory.

Further progress in this research involving the induction of,

and sensitivity to the inhibitor by several isolates of Botrytis, and detection of inhibitor in pod tissues and leaves was reported by Deverall, Smith, and Makris (1968). Droplets of suspensions of conidia of each of four isolates of B. cinerea, but not those of four isolates of B. fabae, proved to contain an antifungal compound 24h after inoculation onto pod seed cavities. Partial inhibition of germ tube growth of three highly pathogenic isolates of B. fabae was caused at 2.5 times the concentration of inhibitor needed to cause similar inhibition of each isolate of B. cinerea, and a weakly pathogenic isolate of B. fabae. After extraction in 80% ethanol, solvent parition, concentration, and chromatographic separation, 5 - 10 times more inhibitor was obtained from lesions in pods caused by B. cinerea than those caused by B. fabae. However, it was of major interest that the amounts of inhibitor extracted from whole leaves bearing either large lesions caused by B. fabae, or small lesions caused by B. cinerea 24h after inoculation,were almost the same. No other components of the ether soluble fraction of infected tissues or diffusates possessed antifungal activity. The inhibitor was characterized by maximal absorbance at 347 nm in ethanol, and 360 nm in 50% ethanol. Because of the major difference between yields of inhibitor from degraded pod tissues and from leaves bearing scattered lesions amid healthy areas, it was suggested that the inhibitor was a product of cells affected, but not yet killed by the advancing pathogen and that germ-tubes and hyphae of Botrytis fabae may be able to metabolize the inhibitor to an inactive form, but the more sensitive germ-tubes of B. cinerea may be unable to

prevent the accumulation of fungistatic levels of the inhibitor.

In view of the repeated demonstration (Deverall, 1967; Deverall, Smith and Makris, 1968) that B. cinerea is more sensitive to the inhibitor than B. fabae, it would appear that the original conclusions of Purkayastha and Deverall (1965b) on the antifungal activity of the inhibitor in pod diffusates, are in doubt. It is possible that other inhibitors, with equal activity against both fungi, were formed in the infection droplets examined by Purkayastha and Deverall. However, the failure of subsequent workers to detect antifungal activity in other fractions of diffusates from pods or infected tissues is evidence against this possibility. It is more likely that in the early stages of research into the phytoalexin in Vicia technical difficulties contributed to the collection of contrary results.

The hypothesis that infection by either fungus induces inhibitor formation in leaves, but that B. fabae metabolizes the inhibitor to an inactive form was tested by Deverall and Vessey (1969).

They measured the amounts of phytoalexin in and around lesions in leaves, and studied some aspects of the removal of the inhibitor from aqueous nutrient solution by Botrytis. The phytoalexin was shown to behave as an ether soluble acid. This fraction of ethanolic extracts of leaves was bioassayed at fresh weight concentration against germination and germ tube growth by B. cinerea conidia. Measurements of antifungal activity were taken as an estimate of the concentration of phytoalexin within the tissues. The acids extracted from the B. cinerea infection site 21h after inoculation were shown to possess antifungal activity.

The highest yields of inhibitor were obtained from areas of leaf in which about half the tissue was discoloured; partly infected with either B. cinerea or B. fabae. Tissue completely blackened, and colonised by B. fabae was associated with low concentrations of inhibitor, but a peripheral ring of apparently healthy tissue surrounding the deep B. fabae lesion contained inhibitory amounts of the phytoalexin.

On the basis of these results Deverall and Vessey (1969) suggested that the phytoalexin was produced by apparently healthy cells, and perhaps by cells undergoing necrosis, in advance of the fungi. In vitro experiments on the ability of Botrytis to inactivate the phytoalexin during germ tube growth showed that both B. fabae and B. cinerea were capable of removing the inhibitor from aqueous nutrient solution, but per conidium, Botrytis fabae did so at a much faster rate. Deverall and Vessey suggested that the different sensitivity of the two fungi to the phytoalexin, and the subsequent ability of B. fabae to spread from the infection site might be explained by the apparently greater ability of B. fabae to metabolize the inhibitor.

It is convenient to summarise the hypothetical role of the phytoalexin in controlling lesion development in leaves of Vicia after infection by Botrytis. The principal hypothesis is that the degree of infection is largely dependent upon the outcome of the interaction between fungus, plant and phytoalexin. On the basis of experimental results this hypothesis can be extended to consider the possibility that B. fabae can metabolise the inhibitor at a faster rate than B. cinerea. Figure 1. illustrates the hypothesis of a balance between metabolism of the

Figure 1.

Phytoalexin degradation by the fungus

Phytoalexin production by the plant

Spreading lesion

Limited lesion

phytoalexin by the fungus, and phytoalexin production by the plant. Increase the rate of metabolism and the balance is tipped towards a breakdown of resistance, and the development of a spreading lesion (B. fabae infection). Decrease the rate of metabolism, and the phytoalexin accumulates. The balance is tipped in favour of lesion limitation (B. cinerea infection).

The phytoalexin from Vicia faba has been identified as wyerone acid, the trivial name for the acetylenic furanoid keto acid illustrated in Figure 2. The phytoalexin is closely related to the less antifungal wyerone, which was originally detected in healthy beans (Fawcett et al. 1968) and has recently been shown to increase after infection (Fawcett et al. 1971). Wyerone acid was not detected in healthy tissue (Letcher et al. 1970).

The theory that phytoalexins are the determinants of the disease reaction in plants was proposed by Müller and Borger (1940). The basic concepts of this theory have been discussed by Cruickshank (1963) and recently reviewed (Cruickshank, Biggs and Perrin 1971). Fungal-induced host metabolites with antifungal activity have been isolated and characterized from many genera distributed among several families of higher plants. The occurrence of phytoalexins can therefore no longer be questioned (Cruickshank, Biggs and Perrin 1971). There is however little direct evidence that a particular phytoalexin is responsible for controlling the disease reaction in any host-parasite combination. Correlations have been made between the presence of phytoalexin and the final disease reaction, but the chronological sequence of the

Figure 2.

$$CH_3.CH_2.CH\overset{c}{=}CH.C{\equiv}C.CO.C{=}CH.CH{=}C.CH\overset{t}{=}CH.COOR$$

(the two ring carbons $C{=}CH.CH{=}C$ are joined through O)

Wyerone, $R = CH_3$

Wyerone acid, $R = H$

production of inhibitors and the restriction of the pathogen is not always clear.

There is clearly no direct evidence that wyerone acid is causally related to the limitation of fungal growth in Vicia faba. Germ tube growth by B. cinerea was shown to practically cease on bean leaves 8h after inoculation, Purkayastha and Deverall (1965a), but the earliest indication of antifungal activity in leaf extracts was obtained after 21h, Deverall and Vessey (1969). Measurements of the antifungal activity of the phytoalexin from bean have been made against the development of conidia. Conclusions drawn from such experiments are of limited significance to the in vivo situation where the development of hyphae is inhibited. The differences between the biological activity of diffusates from leaves and pods are largely unexplained.

Circumstantial evidence that phytoalexins are the determinants of disease resistance has been obtained by demonstrating that experimental alteration in the concentration of phytoalexin within infected tissue can lead to changes in lesion development. Klarman and Gerdemann (1963a, 1963b) have demonstrated that removal of phytoalexin from limited lesions caused by Phytophthora sojae on resistant varieties of soybean, allows them to grow to sizes found in susceptible tissue. Restriction of lesion growth by adding phytoalexin has also been shown (Chamberlain and Paxton, 1968). Similarly the induction of susceptibility in resistant soybeans by a heat treatment Chamberlain and Gerdemann (1966), Chamberlain (1970), was shown to be related to the failure of treated hypocotyls to produce phytoalexin when challenged with P. sojae. This work has been confirmed

in part by Keen (1971) who followed changes in the concentration of hydroxyphaseollin in soybean after infection by Phytophthora.

It follows that if wyerone acid does have a major role in controlling the development of Botrytis in Vicia faba any change in the host response must affect the fungus, bean, wyerone acid interaction. Chou and Preece (1968) found that the addition of Vicia faba pollen grains to infection droplets enabled B. cinerea to spread from infection sites in bean leaves. An investigation of the pollen effect was therefore considered as a means of testing the interaction hypothesis.

The work reported in this thesis involves a further study of the development of lesions produced by Botrytis, to which has been coupled a systematic series of measurements of changes in wyerone acid concentration in leaves as the fungi infect, and either fail or succeed in spreading through the leaf. Deverall and Vessey (1969) measured antifungal activity during these events, but in this thesis attention has been devoted to wyerone acid as a component of this activity, using the methods of Letcher et al. (1970), for isolation of the previously unidentified phytoalexin. The effect of pollen extracts on the production of wyerone acid by the plant, and the sensitivity of the fungi to the substance have also been investigated. Finally the use of partially purified wyerone acid has permitted further study of fungal metabolism of wyerone acid, and has led to the isolation and identification of a breakdown product of the compound.

MATERIALS AND METHODS

1. Fungi

a) Origin and maintenance of stock cultures

Cultures of *Botrytis cinerea* Pers. (strain C_3) and *Botrytis fabae* Sard. (strain F_3) were obtained from Dr. B.J. Deverall. The culture of *Botrytis allii* was obtained from Dr. R.J. Threlfall (Botany Department, Imperial College). The cultures were maintained throughout on slants of Medium X (Last and Hamley 1958), and stored at -20°C and under sterile liquid paraffin at 5°C.

b) Production of conidia

"Pyrex" conical 250 ml flasks containing approximately 40 ml of Medium X were inoculated by spreading a suspension of conidia in sterile distilled water uniformly over the surface of the medium. Cultures of *Botrytis cinerea* and *B. allii* gave heavy sporulation when incubated on the laboratory bench (21 - 23°C). The isolate of *B. fabae* produced scanty aerial mycelium and many sclerotia when grown in this way. When grown at 21 - 23°C under "Philips, Black Light", fluorescent tubes with an emission spectrum range of 410 - 310 nm and maximum at 360 nm, with a 16h photoperiod; *B. fabae* produced a dense carpet of aerial mycelium and conidia.

c) Preparation of the conidial suspension

Ten to fifteen day old cultures of the fungi were selected to prepare suspensions. About 20 ml of sterile distilled water was added to the flask containing the sporulating culture. Mature conidia were

released into suspension by shaking the water on the culture surface. Mycelial debris was removed by filtering through four layers of muslin. The suspension was washed three times with sterile distilled water by centrifugation at 850 g for 1 min. The concentration of the conidial suspension was estimated by haemocytometer counts and a suspension of 5×10^5 conidia per ml made by dilution. Suspensions of this concentration were used in all experiments. Where conidia were to be suspended in a medium other than water, volumes of standard suspension were centrifuged and the conidia resuspended in the appropriate medium.

d) Isolation of fungi from infected beans

Excised leaf pieces from the edge of lesions were surface sterilized in 10^{-3}M Mercuric chloride solution for 1½ min., rinsed in sterile water and dissected out on Acid V8 agar. Sporulating mycelium was teased out without sterilization. Developing colonies were repeatedly subcultured on Acid V8, and pure cultures stored on Medium X.

2. Plant Material

a) Source of seeds and growth of plants for studies on detached leaves

A commercial variety of *Vicia faba* L. "The Sutton", was used throughout and was obtained from "Sutton and Sons Ltd", Reading. Seeds were wrapped in moist tissue paper and germinated in the dark at 20°C. After four days healthy seeds with an emergent radicle were sown in "John Innes No. 1" potting compost, in 5½" diameter plastic pots, two seeds per pot. The plants were grown in a greenhouse kept at a temperature of 20° - 25°C, with a 16h photoperiod. Additional light was provided by four 80-watt "White" fluorescent tubes suspended about two feet above

soil level. The minimum illumination at soil level was 300 lumens/ sq. ft. The pots were watered by hand daily until the first leaves emerged from the soil. Thereafter the greenhouse automatic watering system was considered adequate. The plants developed four expanded leaves 3 - 4 weeks after planting.

b) Source of naturally infected field beans

Healthy and infected plants of the field bean variety "Throw's bean", were collected on July 15th 1970 from a crop of beans grown at Bishop's Stortford, Herts. The roots, and adhering soil were enclosed in small plastic bags containing water. The whole plants were transported in large plastic bags to London and used the same day. The plants were about 1 m tall when harvested.

c) Source of pods

Pods of several varieties of broad bean were purchased from greengrocers on the day of their delivery. Pods of "The Sutton", and "Giant Windsor" broad bean, and "Minor" field bean were grown at the Imperial College Field Station, Silwood Park. Pods were harvested at different stages of maturity after the seeds had swollen. Washed pods were brought to London in moist plastic containers, stored at 5°C, and used on the following day at the latest.

d) Pollen diffusate

Flowers were collected from greenhouse grown beans already described. Mature anthers were dissected from the flowers and dipped into 10 ml aliquots of sterile distilled water in McCartney bottles. The dipped anthers immediately released their pollen grains. The pollen

from thirty flowers was collected in each bottle. The suspension was frozen at -20°C, thawed, and shaken for 15 min. at room temperature. This procedure was repeated. Several samples were bulked and the concentration of pollen grains was estimated from the average of ten haemocytometer counts. Pollen grains and anther debris were removed by centrifugation. The supernatant pollen diffusate was concentrated by rotary film evaporation (*in vacuo*) at 35°C to a volume calculated to contain the diffusate from 2×10^6 pollen grains/ml. This diffusate was stored at -20°C.

Dialysed pollen diffusate was prepared by dialysis of the dilute pollen diffusate for 12h against sterile distilled water at 5°C. "Visking" tubing of $\frac{8}{32}$" diameter obtained from the "Scientific Instrument Centre Ltd." England, was used for dialysis.

3. Chemicals

Anhydrous diethyl ether obtained from "British Drug Houses Ltd." was used for the preparation of tissue and diffusate extracts for chromatography. It was, however, found to contain certain U.V. absorbing, antifungal substances. Therefore where U.V. and visible absorption spectra or the biological activity of ether extracts were to be measured "Analar" diethyl ether from "B.D.H." was used. This contained no interfering impurities. Other chemicals used were "Analar" grade if available, and were obtained from "British Drug Houses Ltd." or "Koch Light Ltd."

4. Culture media

a) Medium X

Content

1.

Glucose	10g
Mycological peptone	2g
Casein hydrolysate (acid)	3g
KH_2PO_4	1.5g
$NaNO_3$	6g
KCl	0.5g
Yeast nucleic acids	0.5g
Distilled water	200 ml.

2.

$MgSO_4.7H_2O$	0.5g
Distilled water	100 ml.

3.

Oxoid agar	20g
Distilled water	700 ml.

Preparation

The solutions 1) and 2) were prepared while 3) was placed in a steamer. When the agar had dissolved 1), 2) and 3) were mixed. Aliquots (40 ml) were dispensed into 250 ml conical flasks and autoclaved 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	20g

Preparation

The mixture was steamed until the agar had dissolved then dispensed into medical flats and autoclaved for 20 min. at 1 kg/cm^2. Plates were poured when the bottles had cooled.

5. Spectral analysis

Ultravioltet (U.V.) spectra were obtained on a "Beckmann model DB" spectrophotometer, infrared (i.r.) spectra in $CHCl_3$ solution on a "Perkin Elmer 257" spectrometer, mass spectra on a "A.E.I., M59" spectrometer with direct insertion and proton magnetic resonance (p.m.r.) spectra on a "Varian HA 100" spectrometer. Dr. D.A. Widdowson, Imperial College, carried out i.r., mass and p.m.r. spectral analyses.

6. Measurement of pH

Measurements were made with "B.D.H." narrow range indicator papers.

7. Inoculation techniques

a) Detached leaves

Unless stated second and third bifoliate leaves without visible damage were cut from plants with four leaves expanded. The cut petioles were immediately wrapped in moist tissue paper. The leaves were washed thoroughly in a jet of distilled water and blotted dry with tissue paper. Detached leaves were placed inside transparent polystyrene sandwich boxes (11.5 x 17.5 x 3.0 cm.) with tight fitting lids. The boxes were lined with tissue paper moistened with tap water. The leaf laminae were supported by test tubes. The petiole tissue wicks were touching the moist box lining.(see plate 16) Three or four leaves were placed in each box.

Droplets (10µl) of conidial suspension were laid on the upper

surface of each leaflet by means of an "Agla", micrometer syringe fitted with an hypodermic needle. Conidia tended to settle down in the suspension, so homogeneity was maintained by introducing an air bubble into the barrel of the syringe when it was filled. 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 leaflet varied in individual experiments. There was always at least 5 mm. between the circumferences of each droplet. Leaves were incubated in a growth cabinet at 17°C ± 2°C and illuminated for 16h each day by a bank of "Philips Coolwhite", fluorescent tubes.

b) Pods

Pods were washed in tap water and opened to expose the seed cavities using a clean scalpel. The seeds were discarded and Broad bean half pods cut into pieces 2 - 4 seed cavities long. Larger segments sometimes tended to curl up on incubation. This segmentation was not necessary for the smaller field bean pods. Half pods were placed on moist tissue in sandwich boxes and inoculated with spore suspensions using a pipette (see Plate 32). As much suspension as possible was pipetted into each pod seed cavity without allowing the droplet to touch any cut pod surface. The boxes were incubated in the dark at 17°C for the times stated in individual experiments.

8. Measurement of infection

Three systems of measurement of *Botrytis* infections obtained in the laboratory were used. They were all based on an estimation of the browning, or blackening of the leaf tissue after inoculation.

System 1.

No lesions produced	-	No visible browning beneath the infection droplet.
Lesion	-	Visible browning on the upper leaf surface beneath the infection droplet.
Spreading lesion	-	Complete browning of the leaf surface beneath the infection droplet and at least a 1 mm. ring of brown tissue beyond this.

System 2.

Grade 1	-	No visible browning.
Grade 2	-	Some browning, up to almost complete browning of the droplet area.
Grade 3	-	Complete browning of the leaf surface beneath the infection droplet.
Grade 4	-	Some browning beyond the droplet but not greater than a 2 mm ring of brown tissue.
Grade 5	-	Lesion spreading further than 2 mm beyond the droplet area.

System 3.

The pictorial key used for this system is shown in Figure 3. Allocation to grades was made on the basis of the percentage browning/blackening of the droplet area, up to 100%. Lesions spreading beyond the tissue beneath the droplet were graded on the extent of spread beyond the infection site. This grading system was also used for the appearance of the lower surface opposite the infection droplet.

Figure 3. Measurement of infection : Grading system 3

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

The lesions found in naturally infected field beans were graded according to their size, and penetration to the opposite leaf surface (see plate Nos.28-31)in the following manner.

Limited lesion 1.	-	Red/brown lesion not greater than 2.5 mm in diameter (typically in the upper surface)
Limited lesion 2.	-	Lesions not greater than 2.5 mm diameter but clearly visible on the opposite leaf surface.
Spreading lesion	-	Brown/black lesion greater than 1 cm in diameter.

9. Preparation and examination of material for light microscopy

Excised leaf discs were stained and cleared by a modification of the technique of Shipton and Brown (1962). It was found that poor differentiation was obtained between fungal and plant cells by leaving the infected tissue in the stain for the prescribed 48h. Best results were obtained by examining the tissue immediately after the initial clearing in alcoholic lactophenol cotton blue, and without further clearing in chloral hydrate. This material mounted in glycerol could be stored without loss of differentiation. Numerous developing conidia were lost from the leaf surface by this staining method.

Quantitative studies on the development of B. cinerea conidia on the leaf surface were therefore carried out after using the chlorine clearing technique of Janes (1962). Leaf discs bearing infection droplets were bleached in chlorine gas generated by the oxidation of Conc. HCl by $KMnO_4$ crystals. Ammonia vapour was blown over the bleached discs to neutralize excess chlorine and a drop of phenol acetic aniline blue (Jones and Mollison, 1948), added to the infection droplet. In this

way the fungus could be fixed, and stained without displacement. The placement of a cover slip did not dislodge conidia.

Fresh, hand cut sections of infected leaves were mounted in water. The use of trypan, or cotton blue stain did not improve the recognition of fungal material. Numerous unsuccessful attempts were made to use sucrose solutions (0.5, 1.0 and 2.0 M) to detect differences in osmotic behaviour in cells in infected and healthy tissue. It was found that plasmolysed cells could not be easily distinguished even in sections of healthy tissue.

Fresh leaf discs or sections were examined for callose by the aniline blue fluorescence method (Jensen 1962).

Celloidin peels of the leaf surface were prepared using a 5% solution of "Gurr's celloidin", in absolute ethanol/ether (75/25).

10. Preparation of plant extracts

Precautions were taken throughout extractions to keep illumination to a minimum. Solvent evaporation was carried out by rotary evaporation at 35°C *in vacuo*.

a) Preparation and Extraction of leaf tissue

Infection droplets, where present, were either removed with tissue paper or collected (see extraction of diffusates). Tissue from inoculated leaves was cut out with cork borers. Excised pieces were collected at -20°C in a beaker of known weight. The various shapes of tissue beneath the infection droplet could be conveniently cut out with a 5 mm diameter cork borer. Rings of tissue surrounding this were collected using borers of larger diameter. At all times rings of tissue were cut in relation to

the actual shape of the infection site rather than a discrete 5 mm leaf disc. The beaker plus frozen tissue was weighed immediately before extraction, then filled with 80% ethanol at -20°C. Speed was essential in the collection of tissue at -20°C and weighing of frozen material. Delay resulted in the release of latent polyphenol oxidase (Kenten 1957, 1958) from damaged or thawed tissue, and the consequent blackening of leaf material.

The process of extraction of ether soluble acids is summarized in Figure 4. The most tissue extracted was 1.64g fresh material. The majority of extracts were made from less than 1g material. The volume of solvents required for the efficient extraction of 1g leaf tissue are recorded in the following description of the method. These volumes were dictated more by the minimum requirement for the use of the macerating chamber, and recovery of residues from the walls of flasks used for rotary evaporation than amount of tissue extracted.

Extraction of ether soluble acids.

1) Frozen tissue was homogenized in 100 ml of 80% ethanol at -20°C using a "sorval omnimixer" by three 15 sec. burst at half speed.

2) The homogenate was centrifuged in glass tubes for 10 min at 850g. The supernatant wascollected in a 500 ml rotary evaporating flask. The pellet was repeatedly washed and centrifuged with 40 ml aliquots of 80% ethanol, until the supernatant was colourless.

3) The 80% ethanol extract was taken to dryness.

4) The flask walls bearing the residue were washed successively with 15 ml. 5% Na_2CO_3 and 50 ml ether. This was repeated three times. Flask washings were collected in a 500 ml. separating funnel.

5) After shaking for 30 sec. the partition was allowed to stand for 10 min. The alkaline water phase was collected and the ether phase, containing basic and neutral ether soluble substances, discarded. Extraction of the alkaline water phase with ether was repeated until the ether phase was colourless.

6) The alkaline water phase was acidified by dropwise addition of orthophosphoric acid (specific gravity 1.75) to pH 4.0. It was then poured into the separating funnel and carefully agitated to release carbon dioxide.

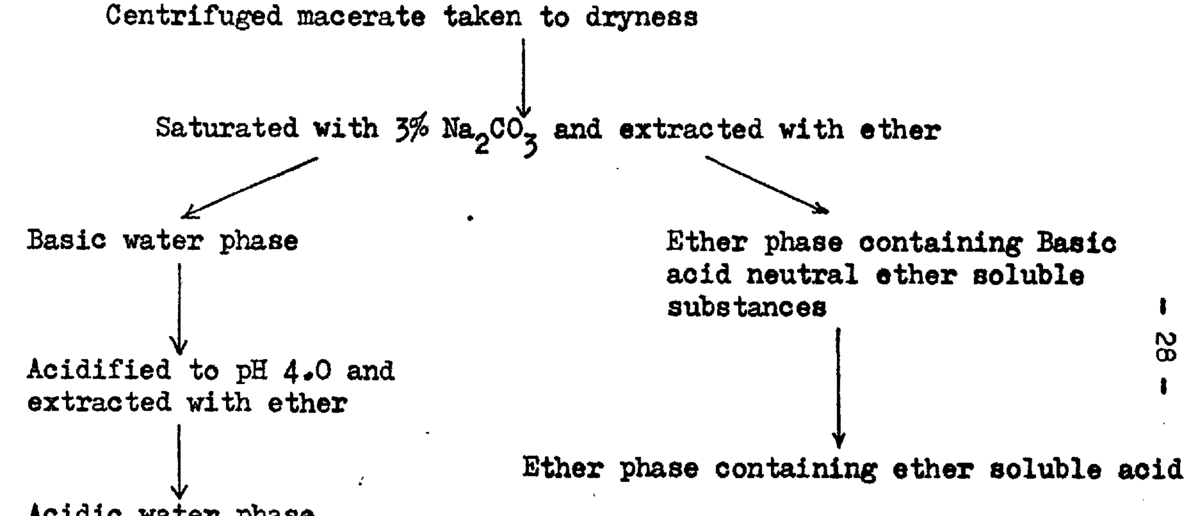
It was found to be more efficient to carry out up to three separate extractions at the same time. The time between washing the 80% ethanol soluble residue with 5% Na_2CO_3 solution, and acidification was therefore longer than one hour. This time of exposure to a high pH was sufficient to destroy a second antifungal compound found by Deverall (personal communication), to be generated by the extraction process, but very labile at pH 10 - 11.

7) The acidic water phase was extracted four times with twice its volume of ether.

8) The bulked ether phase was reduced to a smaller volume (about 20 ml) and decanted into a 50 ml rotary evaporating flask. The 500 ml flask was washed three times with ether. The careful transfer of the ether phase to a more practical size of flask had the additional benefit of removing droplets of water which passed the original partition. The ether phase was taken to dryness and the ether soluble acids suspended in ethanol for chromatography.

b) Preparation and extraction of diffusates

Figure 4. Extraction of ether soluble acids from tissues



i) Diffusates from pods.

Infection droplets, now collectively referred to as diffusate, were collected from pod seed cavities using a pasteur pipette. Large volumes were collected under suction. The test tube or conical flask in which the diffusates were collected was buried in crushed ice. The diffusate was immediately centrifuged at 5°C for 10 min at 850g to remove conidia and other debris, and either used at once or stored at -20°C.

Large volumes of diffusate collected for the preparation of substantial quantities of wyerone acid were extracted four times with equal volumes of ether in a separating funnel. The water phase of 24h *B. cinerea* diffusates, now referred to as pod nutrients, was divided into 10 ml aliquots and stored at -20°C. Pod nutrients were used as a nutrient source in bioassays. The bulked ether phase was taken to dryness in a 500 ml evaporating flask. The extraction of basic and neutral ether soluble substances and ether soluble acids from the residue was carried out as described for leaf tissues.

Volumes of less than 10 ml of diffusate were extracted three times, with three times their volume of ether in test tubes (125 mm x 15 mm). The two phases of the partition were mixed using a pasteur pipette. The pooled ether phase was collected in a 50 ml flask for rotary evaporation. The residue was further extracted into basic and acidic fractions by the techniques already described, except that appropriately smaller volumes of solvents were used to rinse some flasks. Three times 5 ml of 5% Na_2CO_3 and three times 5 ml ether were used to wash the flask wall.

ii) Diffusates from leaves

Infection droplets were collected from leaves by the method described for pod diffusate. The volumes of diffusate obtained from leaves were usually less than 3 ml. The diffusates were extracted three times with at least five times their volume of ether in a test tube. Further extraction was carried out as described for small volumes of pod diffusate.

Prepared extracts of diffusates were either used immediately or stored. Ether solubles were stored in ethanol at -20°C; water phases were frozen at -20°C.

11. Chromatography

a) Paper chromatography

Ascending paper chromatography was performed using "Whatman" papers held on polythene frames in "Shandon Universal Chromatanks". Unless stated 3MM grade papers were developed in n-propanol/water (12.5/87.5). The maximum loading rate for preparative chromatography of extracts from leaf tissues was the extract of 0.05g fresh material/cm. of origin. Extracts from diffusates were loaded on the basis of their wyerone acid content estimated from the absorption spectrum of the extract in ethanol. The maximum rate was 5 µg wyerone acid/cm. of origin.

b) Thin layer chromatography (T.L.C.)

Ascending T.L.C. was performed using freshly prepared "Merck" silica gel plates 0.25 mm thick or precoated "Merck T.L.C. plates silica gel F_{254}", 0.25 or 2.5 mm thick. Solvents used are described in the

experimental section.

Ethanolic extracts were applied to chromatograms using drawn out melting point tubes.

12. Detection and estimation of wyerone acid

Wyerone acid was routinely detected after paper chromatography of extracted ether soluble acids. Developed chromatograms were dried and examined under UV from a "Hanovia Chromatolite", with maximum emission at 254 nm. Wyerone acid was detected as a blue fluorescent band at R_f 0.9. The band was marked with a pencil, cut into small pieces and soaked overnight in 50% ethanol. The absorption spectrum of the supernatant was recorded. If the absorption spectrum indicated that wyerone acid was present, assuming that wyerone acid has a similar extinction coefficient to that of wyerone, (Fawcett et al., 1968) the amount in the eluate could be calculated on the basis of the optical density at 360 nm from the formula.

$$\mu g \text{ wyerone acid} = O.D. \times 9.04 \times \text{volume of solvent}$$

Extracts from certain tissues yielded fluorescent bands at R_f 0.9 which were not associated with the characteristic absorption spectrum of wyerone acid. The levels of wyerone acid in these eluates were estimated by their antifungal activity at concentrations related to the weight of fresh material extracted, or at a concentration of known absorbance at a selected wavelength. This technique may have over-estimated the amount of wyerone acid present, as other absorbing impurities may have possessed antifungal activity. The use of this

technique is recorded in the results.

Where small volumes of diffusates from leaves were extracted, and these contained only low levels of wyerone acid, further purification of an ether extract was impractical. An estimate of wyerone acid concentration was made from the O.D. of the extract in ethanol at 350 nm. Use of this technique is recorded in the results.

13. Bioassay techniques

The method used was based on that of Purkayastha and Deverall (1965b). The biological activity of solutions was assayed against germination and germ tube growth by Botrytis conidia. Soda glass slides (C.E. Payne and Co. Ltd.) were cleaned by rubbing with the hand in hot water and detergent (Stergene) and by repeated washing in tap water and then distilled water. They were soaked overnight in 5% acetic acid and then overnight in distilled water. They were then soaked for a few hours in acetone, and dried in an oven. This technique was found to give slides which had no deleterious effect on spore germination nor on the spread of test drops. This cleaning method was recommended by Deverall (1965).

Twenty µl drops of solutions to be bioassayed were pipetted on to separate slides using a 0.1 ml graduated pipette. Where possible three separate treatments were allocated to each slide. There were three replicates of each treatment unless stated. One µl of conidial suspension (5×10^5 conidia/ml) was added to the centre of each drop from an "Agla" syringe, so that there were about 500 conidia/drop. The slides were supported by test tubes in moist tissue paper, in sandwich boxes. Moist

tissue was also attached to the box lid. The bioassay was incubated at 20°C in the dark, usually for 21h.

At the end of the incubation period, the conidia were killed and stained by adding a small drop of cotton blue in lactophenol to each drop. Percentage germination was measured by examining every conidium in a number of high power fields (x 320). The criterion for inclusion in a high power field was the presence of the conidium (i.e. a germ tube with its conidium wholly or partly outside was excluded). Fields in each drop were counted until 150 non contacting conidia had been checked. Germination was considered as the production of a germ tube of any length.

Germ tube length was determined using a calibrated micrometer eyepiece. The longest germ tube produced by a conidium was measured. Twenty five germ tubes from separate conidia were selected for measurement in each droplet, by their coincidence, and parallel orientation with the micrometer eyepiece in separate microscope fields.

In experiments where differences between treatments were distinct, germ tube length was estimated to the nearest 10 μm for germ tubes less than 200 μm in length, and to the nearest 50 μm for germ tubes over 200 μm. All results are expressed as replicate means. Variation between replicate drops was invariably low.

Solutions of extracts to be bioassayed were prepared by the method of Deverall (1967). Ethanolic solutionsof extracts were brought to room temperature, and aliquots (<1.5 ml) were blown to dryness by an air stream in test tubes. Residues were resuspended in appropriate volumes of pod nutrients. Resuspension was aided by agitating the test tube on a

"Whirlimixer".

This bioassay system was modified to permit the detection of diffusible antifungal substances in segments of paper chromatograms. One cm x 0.5 cm segments were halved diagonally. The two halves were then added to a 20 µl drop of pod nutrients on a slide,conidial suspension was added to the soaking paper and the bioassays carried out in the usual way.

14. <u>Experiments with wyerone acid</u>

Wyerone acid was extracted from 24h <u>B. cinerea</u> pod diffusates by solvent partition and paper chromatography in all experiments, unless stated.

a) <u>The effect of daylight on an ethanolic solution of wyerone acid</u>

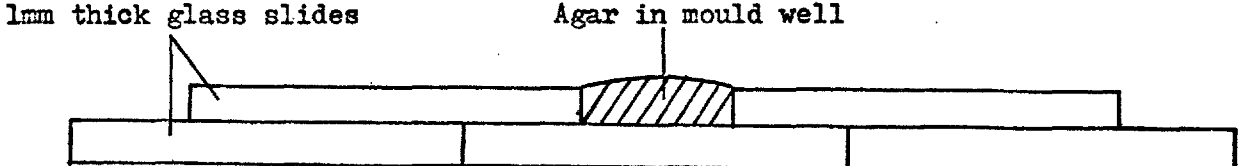
An ethanolic solution of wyerone acid (6.8 µg/ml) in a stoppered test tube was exposed to daylight. At appropriate times after exposure 0.5 ml aliquots were withdrawn and stored at -20°C for subsequent bioassay. At the same time a sample of the solution was examined in the "Beckman" UV spectrophotometer, and the absorption spectrum recorded. The sample was returned to the exposed tube after spectrophotometry. An earlier experiment had shown that five repeated measurements of absorption spectrum from 420 - 200 nm produced no change in the spectrum, while the wyerone acid was in a curvette in the spectrophotometer.

b) <u>The antifungal activity of wyerone acid against mycelial growth by Botrytis.</u>

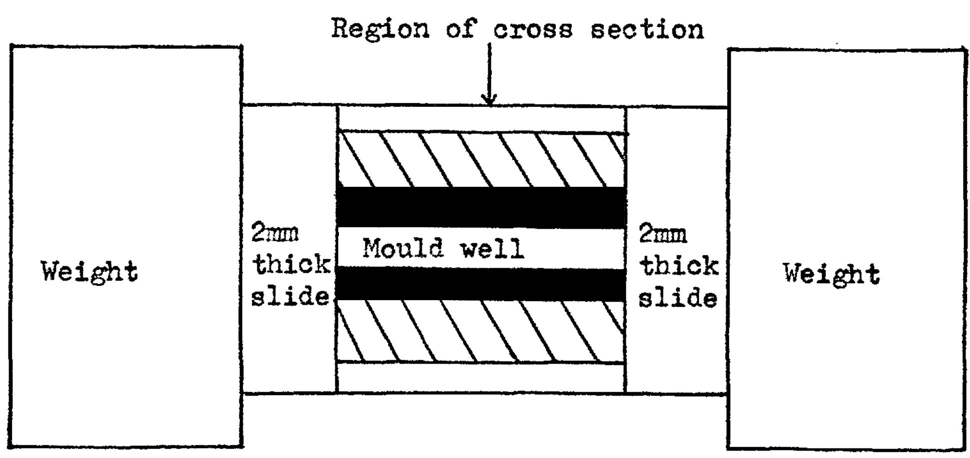
A small scale system was designed to operate with readily available quantities of wyerone acid. Sterile conditions were maintained throughout. Solutions of pod nutrients, 1% ion agar and various concentrations of

wyerone acid were moulded into agar blocks 75 x 10 x 1.15 mm. The blocks were prepared on cleaned microscope slides. The agar slides were kept on bent glass rods laid on moist filter paper in petri dishes. The petri dish lids were also lined with moist filter paper. Each agar block was inoculated at one end with a 1 mm thick, 5 mm diameter disc of pod nutrients in agar bearing Botrytis mycelium. B. cinerea mycelium was cut from the edge of a 3 day old colony on 1 mm thick pod nutrients agar. The F_3 strain of B. fabae does not produce discrete mycelial colonies. Inoculum discs were therefore prepared by seeding a pod nutrients agar plate with B. fabae conidia. After 3 days discs bearing conidia and developing hyphae were cut out and used as inocula. Four replicates of each wyerone acid concentration were prepared for each fungus. The slides were incubated at 20 - 22°C in the dark. Mycelial growth was measured at the centre of the agar strip, as it grew from the edge of the inoculum disc, with a pair of compasses. In preliminary experiments it was found that, where mycelium grew onto the glass slides, growth was more rapid than on the agar block. Advanced hyphae tended to re-inoculate the agar. It was therefore difficult to measure the actual growth of mycelium in the agar from the inoculum discs. Mycelium which grew onto the glass slides was therefore removed with a sterile scalpel. Filter papers were kept moist throughout the experiment by frequent addition of sterile distilled water. The agar blocks were prepared in the simple mould described in Figure 5. Eight ml aliquots of a solution of wyerone acid were prepared at the required concentrations in pod nutrients. "Oxoid ionagar"

Figure 5. Diagram of mould for preparation of agar blocks



a) Cross section of mould



b) Aerial view of mould

(.08g/aliquot) was added to give a final concentration of 1% agar. The aliquots were then autoclaved for 10 min at 10 lb p.s.i. The sterile solutions were kept at 47°C until required. Enough agar solution was introduced using a sterile pasteur pipette, to fill the mould at its edges. The block consequently took a domed "sandwich loaf" shape. The agar was allowed to solidify. The block was then transferred to a fresh slide with a spatula. It was found that up to five blocks could be prepared from each mould. Excess agar hampered further mouldings. Blocks were prepared from each solution until enough of similar dimensions had been produced for the experiment. A fresh mould was prepared for each concentration of wyerone acid.

c) The metabolism of wyerone acid and associated fungal growth in vitro

One ml of solution of appropriate concentration of wyerone acid were pipetted into test tubes (125 mm x 15 mm), held at an angle of about 1° from the horizontal in prepared plastic frames. A 0.05 ml drop of conidial suspension was added to the centre of the elliptical 1 ml droplet. The tubes were closed with aluminium caps and incubated at 20°C in the dark. After incubation the tubes were removed from the frames and held vertically in a test tube rack. Fungal growth adhered to the test tube wall. Eight ml of ether were added to the solution and fungus, and the ether phase extracted as described for leaf diffusate. The residual nutrient solution was discarded and fungal growth then examined through the test tube. Germination and germ tube growth by conidia were estimated as previously described for bioassays. The development of this system is described in detail in the experimental section.

d) Investigation of wyerone acid metabolising enzyme activity in *B. fabae* pod diffusate

Sterile glassware was used throughout. Where possible flasks containing preparations were buried in crushed ice until experimentation. Aliquots of diffusates were extracted with ether in the manner described for leaf diffusates.

Pod diffusates were made "cell free" by centrifugation for 20 min at 16000 g at 5°C. The supernatant was pipetted into a conical flask. Five ml of a solution of 72 µg wyerone acid/ml of diffusate were prepared and added to 19 ml of "cell free" diffusate. Two 1.5 ml aliquots of the resultant solution were withdrawn for extraction. Four 5 ml aliquots were pipetted into four 25 ml "Pyrex" conical flasks with cotton wool bungs. Two were autoclaved at 0.35 kg/cm^2 for 10 min to serve as denatured controls. The flasks were incubated in the dark at 25°C on a shaking platform. Aliquots (1.5 ml) were withdrawn from the replicate flasks after 1, 5 and 25h incubation.

EXPERIMENTAL WORK AND RESULTS

Chapter 1. Some observations on the development of *Botrytis* infections in detached leaves.

The pathogenicity of the isolates of *B. fabae* and *B. cinerea* were compared in the same leaves. One leaflet of each of five leaves was inoculated with eight 10 µl drops of a suspension of *B. fabae* conidia in water. The other leaflet was similarly inoculated with *B. cinerea*. The development of infections was observed, and recorded using Grading System 1. The production of lesions recorded 1 and 5 days after inoculation is shown in Table 1. Plate 1 shows an infected leaf 5 days after inoculation.

Lesions had been produced at all *B. fabae* infection sites one day after inoculation. The upper leaf surface beneath the infection droplet was completely blackened after 40 h. By this time there was some black flecking in the lower leaf surface. This in turn became completely blackened, and hyphae emerged from the lower leaf surface during the third day after inoculation. Little spread of the lesion beyond the infection site was observed until the complete blackening of the leaf disc beneath the infection droplet. One this disc was black throughout the lesion rapidly spread from the infection site, through the leaf. The observed development of the *B. fabae* lesion is summarised in Figure 6.

Only 20% of *B. cinerea* lesions had produced lesions 1 day after inoculation. The lesions were brown, and not black. The browning of the leaf increased in some of these infections until the area beneath the

Table 1. The development of lesions produced by B. fabae and B. cinerea

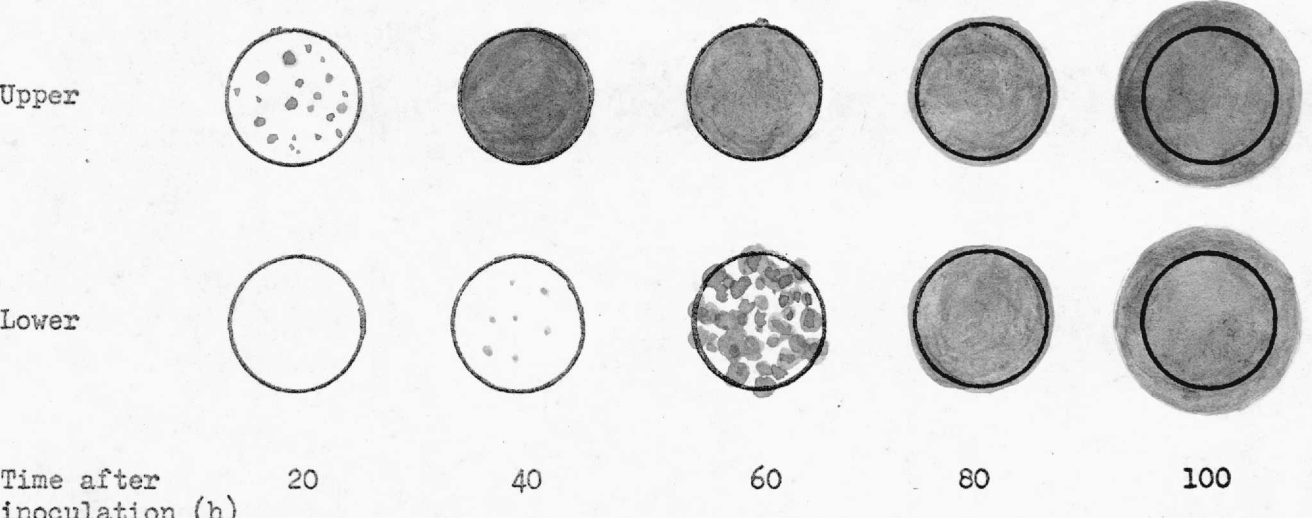
Fungus	Time after inoculation			
	1 day		5 days	
	% Lesions	% Spreading	% Lesions	% Spreading
B. fabae	100	0	100	100
B. cinerea	20	0	65	0

All percentages calculated from 40 inoculations

Plate 1. Infected leaves 5 days after inoculation

Note that the B. fabae lesions have spread from the infection site (circled). The B. cinerea infections are limited, and in one case (circled) no lesion was produced.

Figure 6. The development of lesions caused by *B. fabae*



infection droplet was completely discoloured. No infections spread from the site of inoculation. There was rarely any browning in the lower leaf surface. No marked development of the lesions was observed after 5 days.

A second batch of leaves were inoculated with a suspension of B. fabae or B. cinerea conidia in the same way. The B. fabae infections were examined microscopically 1, 2, 3 and 5 days after inoculation. The various types of B. cinerea infection produced were examined after 5 days. Free hand sections of infected tissues were prepared, and leaf pieces stained using the technique of Shipton and Brown (1962).

One day after inoculation, B. fabae had produced lesions with about 50% blackening of the infection site. Developing conidia could be seen on the leaf surface. Germ tubes frequently terminated in appressoria, defined as terminal swellings. In whole mounts, it was impossible to determine whether penetration of epidermal cells had taken place beneath the appressoria, however stomatal penetration could be recognised by this technique. (Plates 2a, b and c). Penetration through stomata was not observed in sectioned material but examples of direct penetration were common. Hyphae penetrating into the epidermis were frequently associated with the browning or blackening of the epidermal, and palisade mesophyll cells in advance of the fungus (Plate 3). Where B. fabae had invaded the mesophyll tissues they were typically

discoloured, and disorganised. The growth of these hyphae appeared to be intercellular. (Plate 4).

Two days after inoculation the upper leaf surface was covered with a mesh of branching B. fabae hyphae. (Plate 5). Sections showed the continued collapse of the upper epidermis, and palisade mesophyll. The spongy mesophyll in general remained intact, green and apparently healthy.

After 3 days hyphae had extended throughout the leaf disc beneath the infection site. The majority of spongy mesophyll cells were blackened. (Plate 6). Hyphae could be seen within the spongy mesophyll, and emerging from the underside of stained leaf pieces (Plates 7 and 8). They emerged through stomata, and the cells of the lower epidermis. No hyphae were seen extending into the healthy cells beyond the almost completely necrotic tissue beneath the infection droplet.

Five days after inoculation the lesions had spread from the infection site and were packed with hyphae, which were typically confined to the blackened, collapsed tissues. The discolouration was typically 2 or 3 mesophyll cells in advance of the invading fungal hyphae. In one infection this discolouration of the leaf extended some way ahead of the fungus, and was associated with a water soaked appearance. Both blackened, and apparently healthy cells could be seen within the water soaked tissue (Plates 9 and 10). Vascular browning was common at this stage of lesion development (Plate 11).

Plate 2 a b and c. Penetration by B. fabae through a stomatal pore one day after inoculation.

Three depths of focus x 1280.

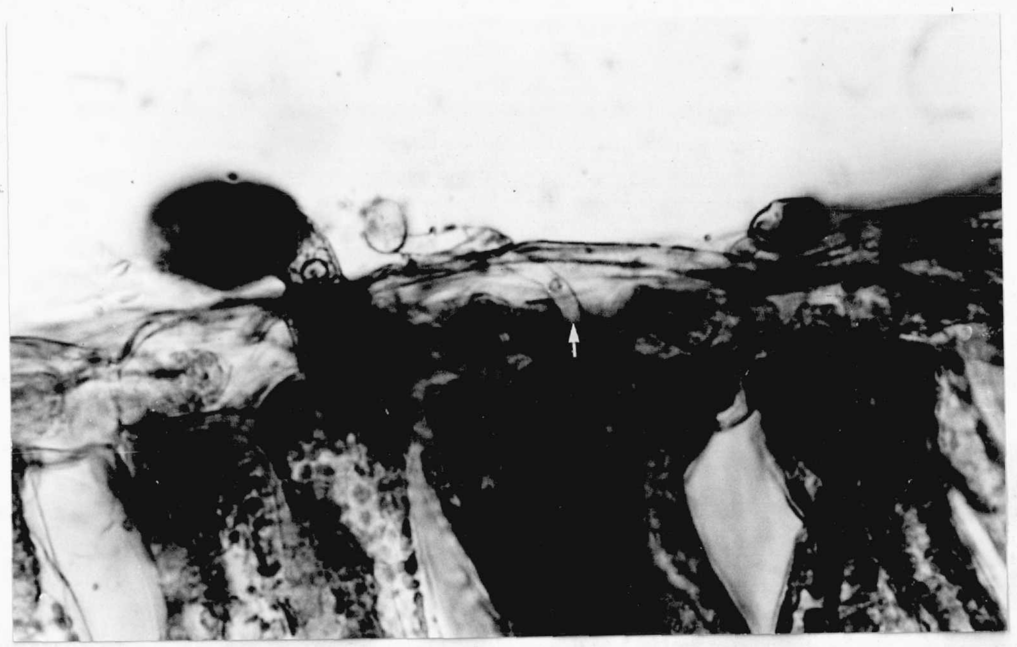
(a) (b) (c)

Note the penetration of the B. fabae hypha (arrowed) through the stomatal pore, and into the blackened palisade mesophyll tissues. Also compare the blackened (b), browning (r) and apparently healthy mesophyll cells (h).

Plate 3. Direct penetration by B. fabae one day after inoculation x 1280.

The conidium was dislodged from the leaf surface into the mesophyll tissues during sectioning. Note the constriction of the appressorium at the point of penetration of the cuticle and epidermal cell wall. The invading hypha can be seen within the disorganised, blackened palisade mesophyll.

Plate 4. Direct penetration by B. fabae one day after inoculation x 1280



Note that the hypha (arrowed) terminates within the epidermis, but the palisade mesophyll cells have blackened.

Plate 5. Development of B. fabae on the leaf surface two days after inoculation x 800.

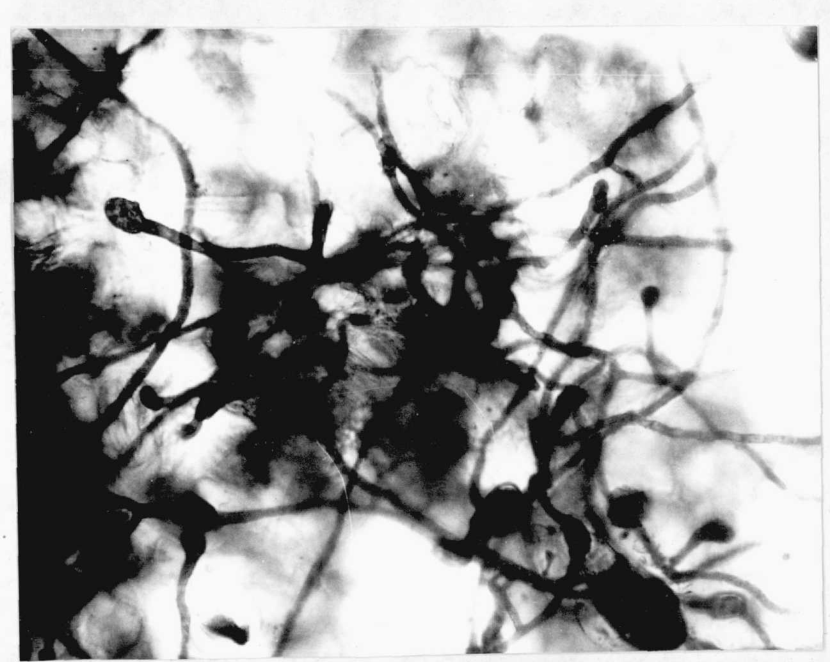


Plate 6. Intercellular growth of a B. fabae hypha (arrowed) in partially necrotic spongy mesophyll tissue three days after inoculation x 1280.

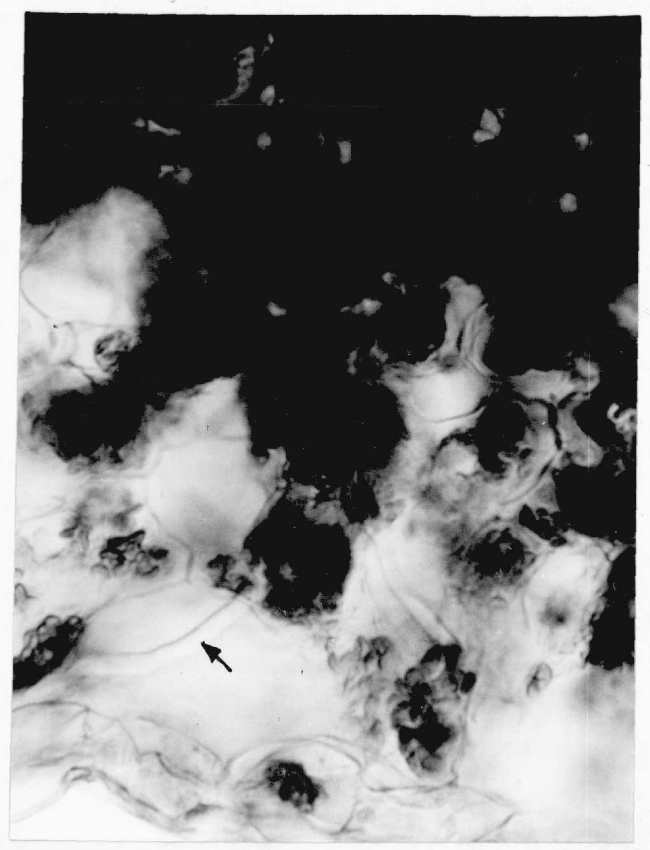


Plate 7. Hyphae (arrowed) in the spongy mesophyll and lower epidermis at the edge of a lesion, three days after inoculation x 1000.

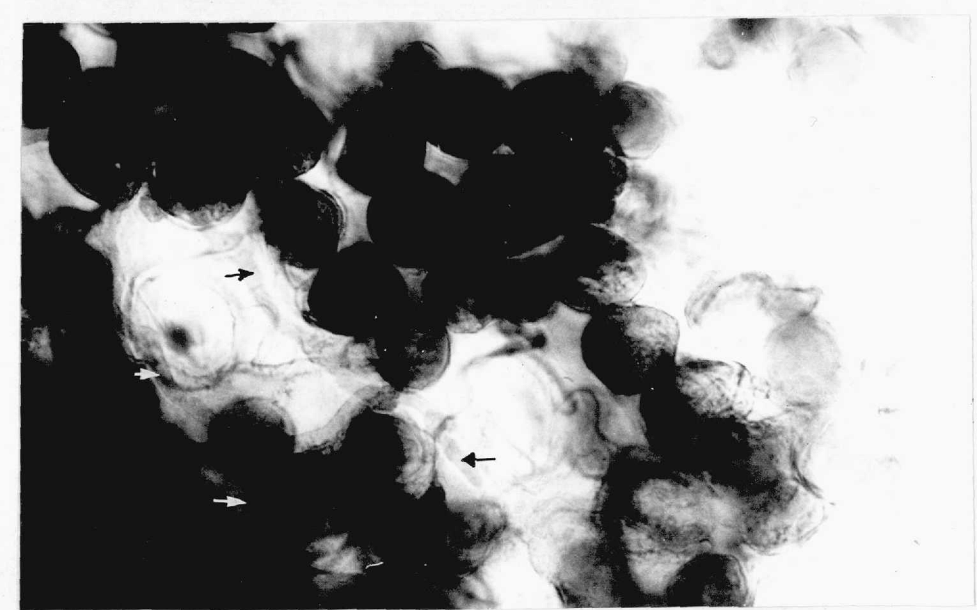
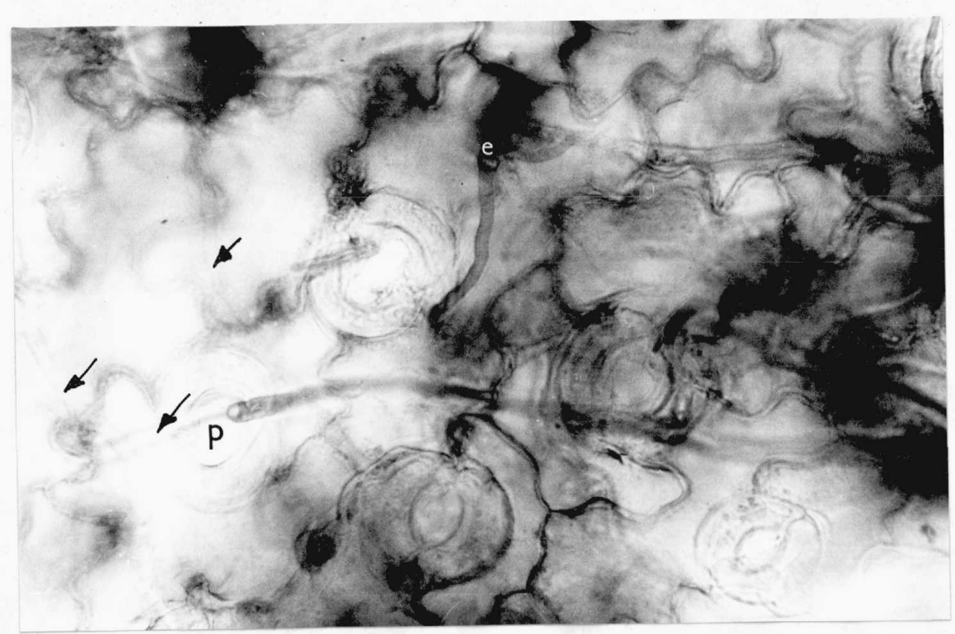
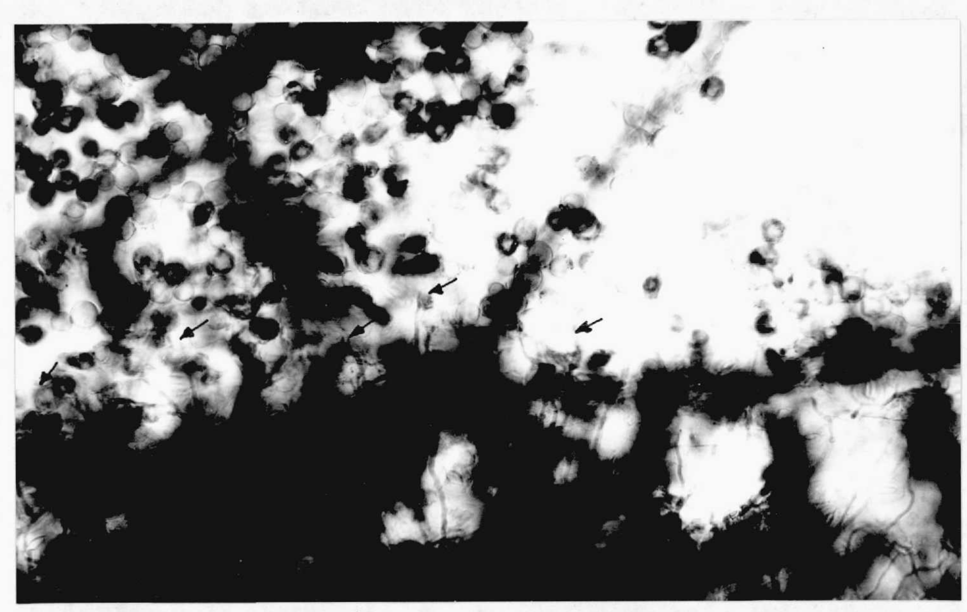


Plate 8. Hyphae emerging from the lower epidermis three days after inoculation x 1000.



Note emergence through a stomatal pore (p) and an epidermal cell (e), and the unstained hyphae (arrowed) within the leaf.

Plate 9. Cellular discolouration in advance of B. fabae hyphae five days after inoculation x 512.



Note the vascular browning, and apparently healthy cells amongst the blackened and browning palisade mesophyll, in advance of fungal hyphae (arrowed).

Plate 10. Cellular discolouration in advance of B. fabae hyphae (arrowed) five days after inoculation x 800.

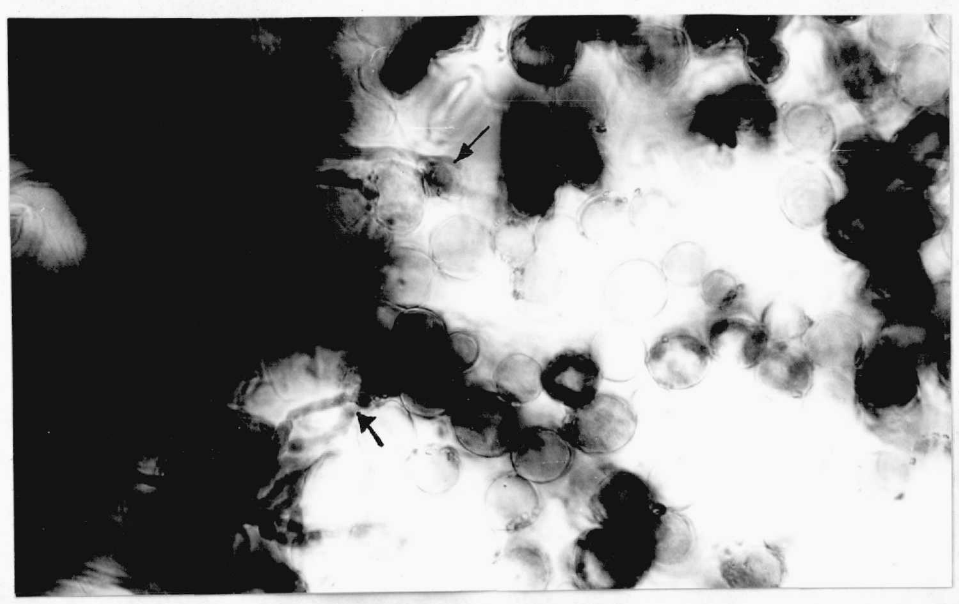
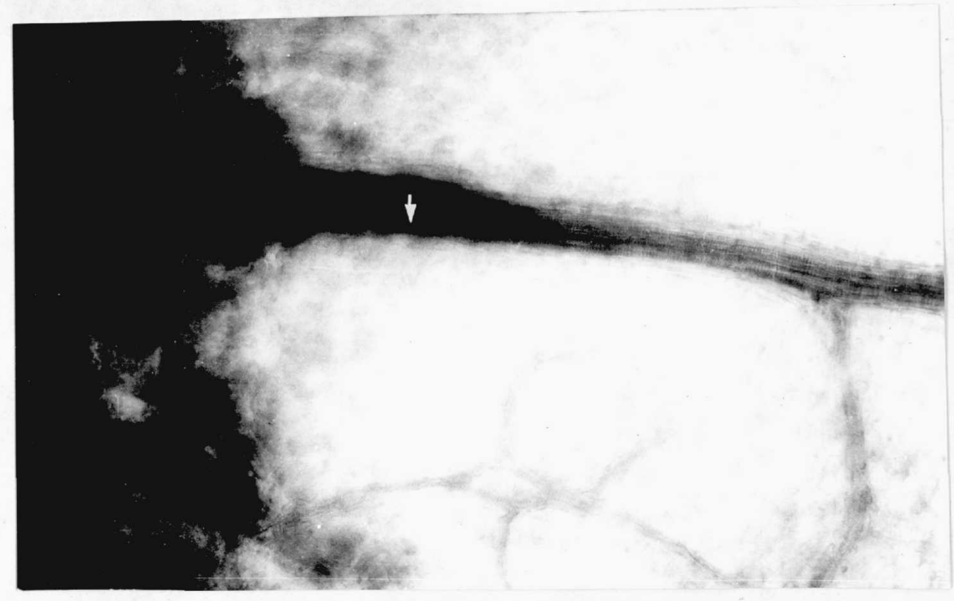


Plate 11. Vascular browning five days after inoculation x 632.



The arrow indicates the position of the most advanced B. fabae hypha.

The development of B. cinerea conidia on the leaf surface was found to depend upon the type of infection produced.

In infections with little or no browning of the leaf, conidia had produced short, thin germ tubes (Plate 12). The sites with more extensive browning were associated with longer, thicker germ tubes which typically terminated in appresoria above discoloured cells (Plate 13). Germ tubes were often distorted. The occurrence of penetration beneath the appressoria could not be determined.

In sections developing conidia could be seen on the leaf surface but it was difficult to see invading hyphae. In a few sections of sites bearing brown cells, hyphae were seen to have penetrated the cuticle and epidermal cell wall (Plate 14). However hyphae of B. cinerea were confined to within discoloured epidermal cells. Despite this there was often browning of the palisade, and of the spongy mesophyll cells (Plate 15). This type of discolouration was considered to account for the browning which was occasionally visible in the lower leaf surface.

Plate 12. Developing conidia at an inoculation site without microscopically visible symptoms x 632

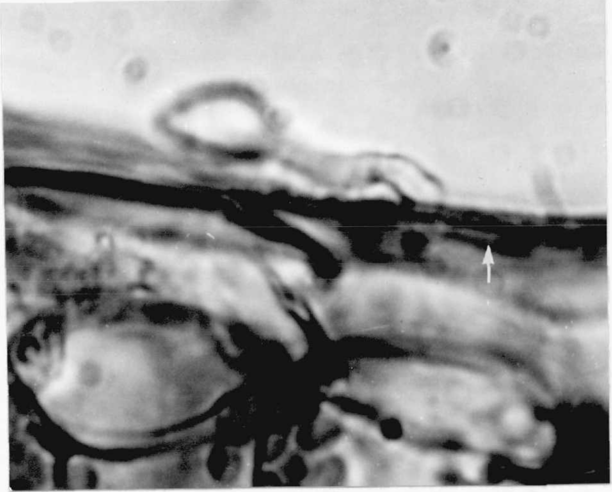
Note the poorly developed germ tubes without appressoria

Plate 13. Developing conidia at a site with extensive browning x 1000.

Note the well developed germ tubes and appressoria.

Plate 14. Penetration of the cuticle and epidermal cell wall by B.cinerea

x 3200



Note the restriction of the hypha (arrowed) within a discoloured epidermal cell.

Plate 15. Section of a lesion with extensive browning of the infection site

x 632.

Note the browning (b) of the upper epidermal, palisade, and spongy mesophyll cells, but the absence of fungal hyphae.

It was concluded that, though necrosis did occur in advance of B. fabae, the diagram of lesion development (Figure 6) could, in general, also be considered to represent the typical progress of fungal invasion of the leaf. Irrespective of the type of limited lesion produced, despite the browning of mesophyll cells, B. cinerea was confined to the epidermis in the infections examined 5 days after inoculation.

Chapter 2. Quantitative studies on the development in leaves of lesions produced by *B. cinerea*, or *B. fabae* conidia suspended in sterile distilled water

a) The development of lesions produced by *B. cinerea*

i) Progress of lesion development

Variation in type of lesion produced by *B. cinerea* in water was reported in Chapter 1. In experiments to be described in Chapter 7, it became apparent that after each inoculation there was a potential for infection to vary from no browning to the production of a spreading lesion six days after inoculation. Between these extremes there was a further range of lesion types. An example of the variation observed in this work is illustrated in Plate 16.

In order to investigate the development of different types of lesion it was decided to trace the fate of individual infection sites. Grading system 3 was developed to enable the classification of infection on the basis of the percentage browning of the droplet area. In this way, with frequent reference to the pictorial key (Figure 3) infections could be classified quantitatively as the mean of the grade boundaries. Four bifoliate leaves were detached from each of seven plants, and each half leaflet was inoculated with six 10µl droplets of conidial suspension. A separate record was kept of the development of the lesion beneath each droplet every day for 6 days. In this way the total numbers of lesions of each type, and the numbers per plant and leaf could be calculated. Calculations could also be made of the

Plate 16. Lesions produced in four leaves of one plant 4 days after inoculation with *B. cinerea* conidia in water

The infections range from Grade 0 - Grade 100.

number of lesions progressing from one grade to another.

Twenty three per cent of the infection droplets were absent 1 day after inoculation, due to run off or drying up; no visible lesions had formed at these sites. After 6 days visible lesions had developed at only 17.4% of these sites. The absence of droplets 1 day after inoculation had a marked effect on lesion development, as illustrated in Table 2. It was considered that sites at which the droplet was absent on day 1 should be regarded as a separate category from sites at which infection developed beneath the droplet. Therefore in the following results, where the percentage of infections in grades is recorded, the value was calculated from the infections in which the droplets were present 1 day after inoculation.

The percentage of infections in the different grades on successive days after inoculation are shown in Table 3. The distribution of infections calculated on days 1, 3 and 6 are expressed graphically in Figure 7. There was a gradual increase in browning after inoculation; however, even after 6 days the majority of lesions were graded only in the 6.5 category. The second highest number of lesions were graded 0, and the third 100. Lesions appeared to develop as two populations; the first large population with a modal value at 6.5, the second smaller population with a mode at 100. A very few lesions spread from the site of infection. There were few movements between grades 5 days after inoculation. All infections, graded S on the fifth day were limited to this condition. There was little browning in the lower leaf surface opposite these lesions.

Table 2. The production of lesions by *B. cinerea* in relation to the presence of the infection droplet 1 day after inoculation

Feature of inoculation site	Percentage of infections within the categories on successive days after inoculation					
	1 day	2 days	3 days	4 days	5 days	6 days
Droplets present on Day 1						
- lesions produced	26	43	46	52	54	56
- no lesions	51	34	31	25	23	21
Droplets absent on Day 1						
- lesions produced	0	0	1	2	3	4
- no lesions	23	23	22	21	20	19

Table 3. The progress of *B. cinerea* lesion development

Days after inoculation	Percentage of infections in grades										
	0	6.5*	19	38	63	87.5	100	S+	S_1	S_2	S_3
1	66	33	1	-	-	-	-	-	-	-	-
2	45	49	4	2	1	0.4	0.2	-	-	-	-
3	39	49	4	3	2	2	2	0.1	-	-	-
4	32	52	4	3	2	4	4	0.6	0.2	-	-
5	29	55	3	3	0.7	6	6	0.6	0.6	0.2	-
6	26	57	4	3	0.7	7	7	0.6	0.2	-	0.6

* Grade means

\+ Categories of lesion spread

Figure 7. The progress of *B. cinerea* lesion development

● = Day 1
○ = " 3
▲ = " 6

Percentage of infections: 10, 20, 30, 40, 50, 60, 70

Infection grade: 20, 40, 60, 80, 100, S, S_1, S_2, S_3

Complete analysis of the data obtained from this experiment would enable the progress of development of lesions of each grade to their condition on the sixth day to be determined. The required calculations were, however, only made for two lesion types, those that developed to grades 100, and 19, six days after inoculation. Grade 100 was selected as a population mode and the upper limit of lesion development beneath the inoculum droplet. Grade 19 is described here but was examined in detail only after it had been shown to be the possible link between the two lesion populations (i.e. with modes at 6.5 and 100) by experiments described in Chapter 3.

The development of lesions to grade 100 after 6 days is illustrated in Table 4. The numbers of these infections in each grade on the earlier days after inoculation are recorded. The majority of infections which developed to grade 100 had produced grade 6.5 lesions by the first day after inoculation. It is possible to calculate an average grade of infection for these lesions each day after inoculation. This figure was calculated by multiplying the grade (b) by the number of lesions at this grade (a), summing the values obtained for each grade (Σab) and then dividing by the total number of lesions (Σa). e.g. Five days after inoculation.

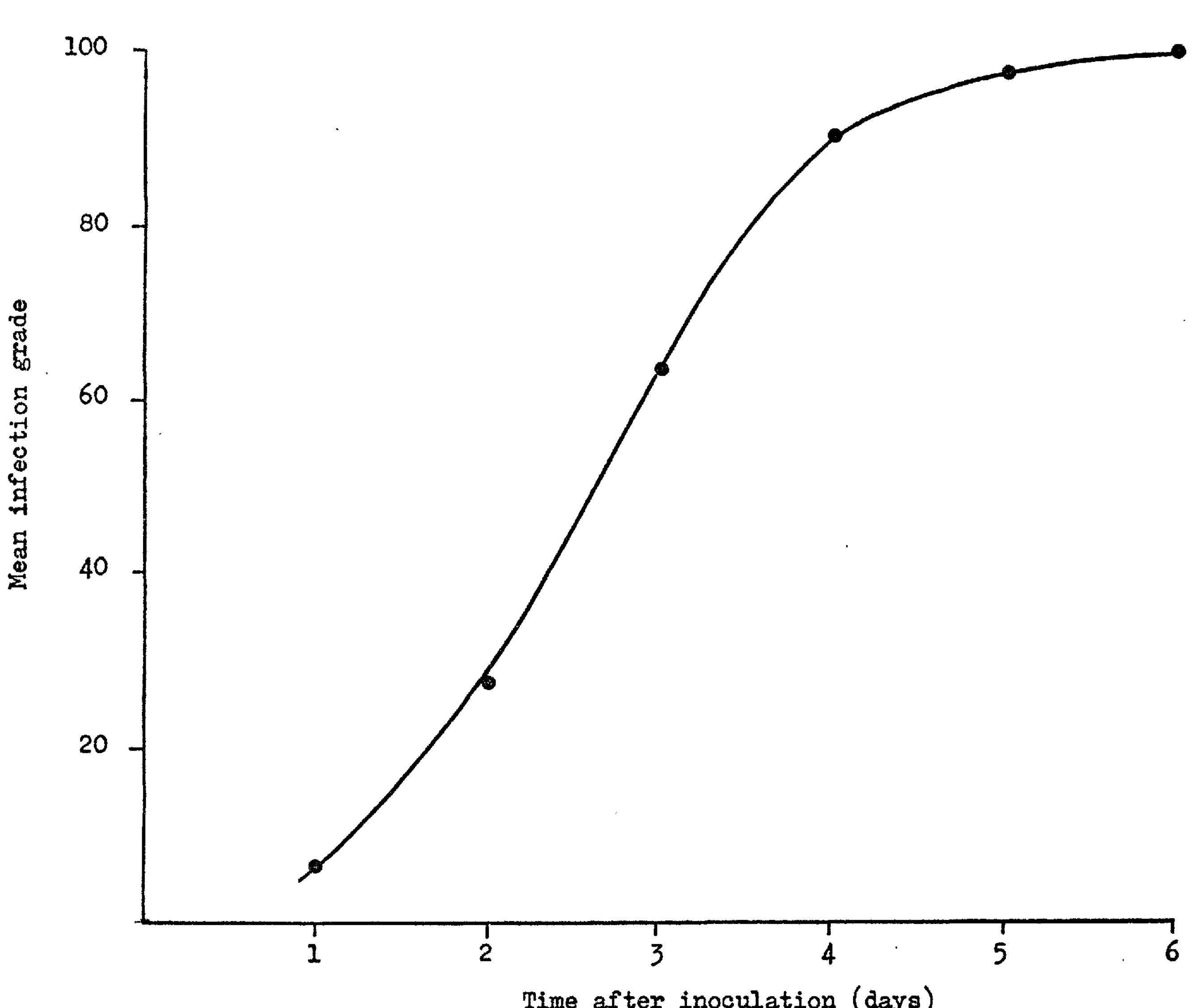
1 lesion (a) at grade 38 (b) ∴ ab = 38
2 lesions " " " 87.5" " " = 175
33 " " " " 100 " " " = 3300
a = 36 $\quad\quad$ Σab = 3513

∴ Mean grade of infection $\frac{\Sigma ab}{\Sigma a} = 97.6$

Table 4. The development of the 36 lesions in grade 100, six days after inoculation

Grade	Number of infections in each grade on successive days after inoculation					
	1	2	3	4	5	6
0	8	-	-	-	-	-
6.5	25	8	-	-	-	-
19	3	16	4	2	-	-
38	-	6	12	-	1	-
63	-	5	5	3	-	-
87.5	-	1	7	8	2	-
100	-	-	8	23	33	36

Figure 8. The development of grade 100 infections produced by *B. cinerea*



The mean grades of infection were calculated for each day, and plotted against time after inoculation in Figure 8. The development of browning in grade 100 lesions followed a sigmoidal pattern of increase.

The development of lesions to grade 19 infections six days after inoculation is shown in Table 5. The majority of infections which developed to grade 19 had produced grade 6.5 lesions one day after inoculation, but none of the infections had reached grade 19 after the first day. The mean grades of infection were calculated from the daily measurements, and are expressed graphically in Figure 9. The pattern of development of lesions to grade 19 was quite different from lesions developing to grade 100 infections.

The grades of infection recorded on the first day for lesions which developed to grade 6.5 six days after inoculation, are given in Table 6. The majority of these infections had not developed visible lesions 1 day after inoculation.

It was concluded that there was not a characteristic pattern of B. cinerea lesion development, but separate patterns for the development of lesions in different grades of infection. However, the majority of inoculations produced little or no visible symptoms (Grades 0 and 6.5) after 6 days.

ii) Between plant variation

The variation in the susceptibility of plants to infection by Botrytis has been investigated by several workers. The data obtained from the experiment described in section (i) was therefore processed to

Table 5. The development of the 21 lesions in grade 19, six days after inoculation

Grade	Number of infections in each grade on successive days after inoculation					
	1	2	3	4	5	6
0	4	1	-	-	-	-
6.5	17	14	14	7	2	-
19	-	6	7	14	19	21

Figure 9. The development of grade 19 infections produced by *B. cinerea*

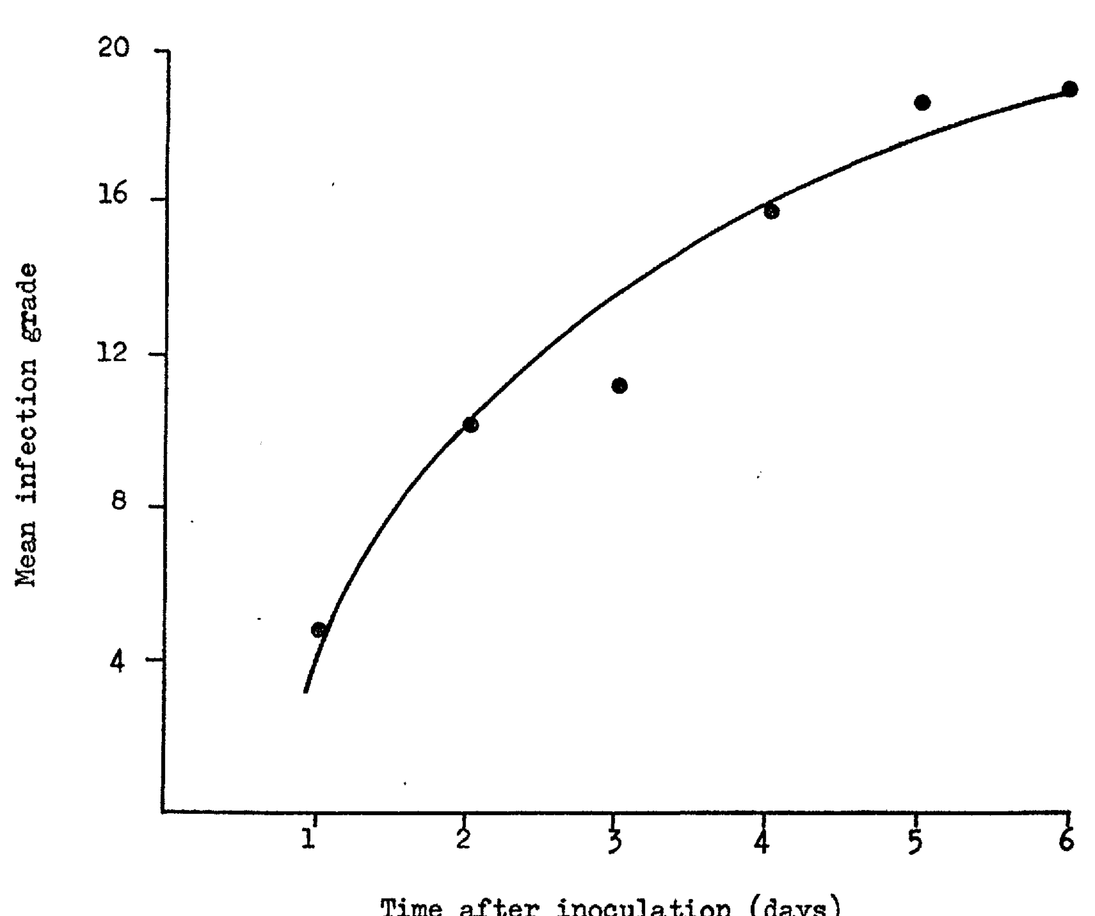


Table 6. **The grading of the 311 lesions one day after inoculation which were graded 6.5 five days later**

	Grade	
	0	6.5
Number of infections	221	90

enable a comparison between lesion development in different plants. The distribution of infections on each plant one and six days after inoculation is shown in Table 7. Though the distribution of lesion types in each plant had the common feature of division into two populations after six days, there were obvious differences between the plants in the proportion of infections in each grade. The plants were arranged in order in Table 7 (b) depending on their resistance measured on the basis of the percentage of infections which developed to grades higher than 6.5; a low percentage would correspond to high resistance. The same order would have been obtained by considering the percentage of grade 0 infections recorded; a high percentage would correspond to high resistance. The plant in which S_3 grade lesions developed was not classed as the most susceptible, because these lesions only developed in the oldest leaf, which was beginning to yellow when inoculated. The green leaves of all plants were resistant to the developmentof large spreading infections. The pattern of susceptibility was indicated 1 day after inoculation by the development of a greater percentage of visible lesions in the more susceptible plants. It was concluded that there was some feature of the more resistant plants which prevented the establishment of visible lesions by B. cinerea.

iii) Between leaf variation

The data obtained from the experiment described in section (i) was analysed to examine the effect of leaf age on the development of lesions by B. cinerea. Table 8 shows the distribution of infections calculated for leaves 1 - 4. Lesions graded S_3 developed only in the

Table 7. Infections produced by *B. cinerea* in leaves from different plants

a) One day after inoculation

Plant	Percentage of infections* in grades			Percentage of droplets absent on day 1
	0	6.5	19	
1	90	10		45
2	88	12		19
3	89	11		19
4	52	45	3	28
5	70	28	2	10
6	38	60	1	24
7	40	60		9

b) Six days after inoculation

Plant	Percentage of infections* in grades										
	0	6.5	19	38	63	87.5	100	S	S_1	S_2	S_3
1	61	32	-	3	-	-	4	-	-	-	-
2	36	57	1	1	-	-	5	-	-	-	-
3	29	62	1	1	-	-	7	-	-	-	-
4	28	57	4	4	1	1	4	-	-	-	-
5	22	64	4	1	1	-	7	1	-	-	-
6	18	60	1	6	2	1	6	2	-	-	4
7	1	61	14	7	-	1	14	1	1	-	-

* Calculated from infections with droplet present on Day 1.

Table 8. Infections produced by *B. cinerea* in leaves of different age

a) One day after inoculation

Leaf	Percentage of infections* in grades			Percentage of droplets absent on Day 1
	0	6.5	19	
4+	53	46	2	27
3	75	23	-	18
2	68	32	0.7	21
1	66	33	2	26

b) Six days after inoculation

Leaf	Percentage of infections* in grades										
	0	6.5	19	38	63	87.5	100	S	S_1	S_2	S_3
4+	27	42	8	8	2	-	14	-	-	-	-
3	28	57	4	0.7	-	-	9	0.7	-	-	-
2	26	64	1	3	-	1	3	0.7	0.7	-	-
1	24	64	2	3	0.8	0.8	2	0.8	-	-	2

* Calculated from infections with droplet present on Day 1

+ Youngest leaf

oldest leaf of plant 6. This leaf, as previously described, was showing obvious signs of senescence. Apart from this leaf there were no clear differences between the development of lesions on leaves of different age. The summation of the percentage of infections in Table 8 (b) graded greater than 6.5 for leaves 1, 2, 3 and 4 gives values of 11.4%, 9.4%, 14.4% and 32% respectively. On the basis of these figures it would appear that the youngest leaves (leaf 4) were the most susceptible, and that other leaves were of a similar susceptibility. However, no infections graded S or above developed on the youngest leaves. It was therefore concluded that while leaves showed no signs of senescence there were no significant differences between the development of B. cinerea lesions on leaves of different age.

iv) The effect of pre-treatment of the leaf surface, and variation between sites on the leaf

It was considered that the observed variation in the types of lesion produced by B. cinerea might have been caused by damage done to the leaves in their preparation for inoculation and/or the position of the droplet on the leaf surface. An experiment was therefore designed to examine these hypotheses.

The first four expanded leaves from five plants were selected for the experiment. The four half leaflets of each leaf were prepared for inoculation by one of the following four treatments. 1) No preparation, 2) The leaf quarter was washed in a jet of distilled water. The leaflet was then left to dry by evaporation, 3) As (2) but the

leaflet was dried with tissue paper (Normal leaf preparation),

4) A celloidin peel was made of the half leaflet. Stanbridge (personal communication) has shown that this latter treatment effectively removes the hydrophobic constituents of the leaf cuticle. None of these treatments had any visible effect on the leaf surface.

Last and Hamley (1958) concluded that there was no difference between the susceptibilities of each leaf quarter to infection by *Botrytis*, however in this experiment the treatments were randomized on each leaf by means of Fisher and Yates tables of random numbers. Seven 10 µl droplets of *B. cinerea* conidia suspended in water were placed on each half leaflet, in standard positions illustrated in Figure 10. The development of lesions was recorded for 6 days after inoculation using grading system 3.

The percentage of total droplets absent one day after inoculation are shown in Table 9. Substantially more droplets disappeared from leaves given treatment 2, than from leaves given the other treatments. Despite the apparent dryness of leaflets given treatment 2 before inoculation, the disappearance of droplets probably resulted from run off in a residual film of water particularly in depressions over leaf veins. The graphs of infection distribution 6 days after inoculation shown in Figure 11 were therefore drawn from values calculated from the lesions produced by infection droplets which were present after 1 day. The celloidin pre-treatment allowed the development of grade 100 lesions at most infection sites. In this respect it appeared to some extent to break the resistance of the leaves to infection by *B. cinerea*.

Figure 10. The position of droplets on pretreated leaves

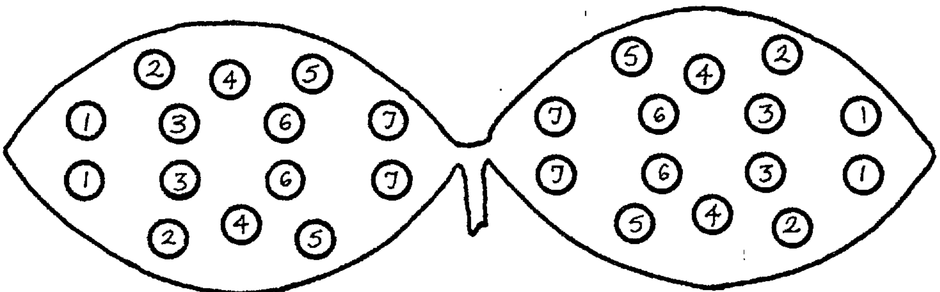
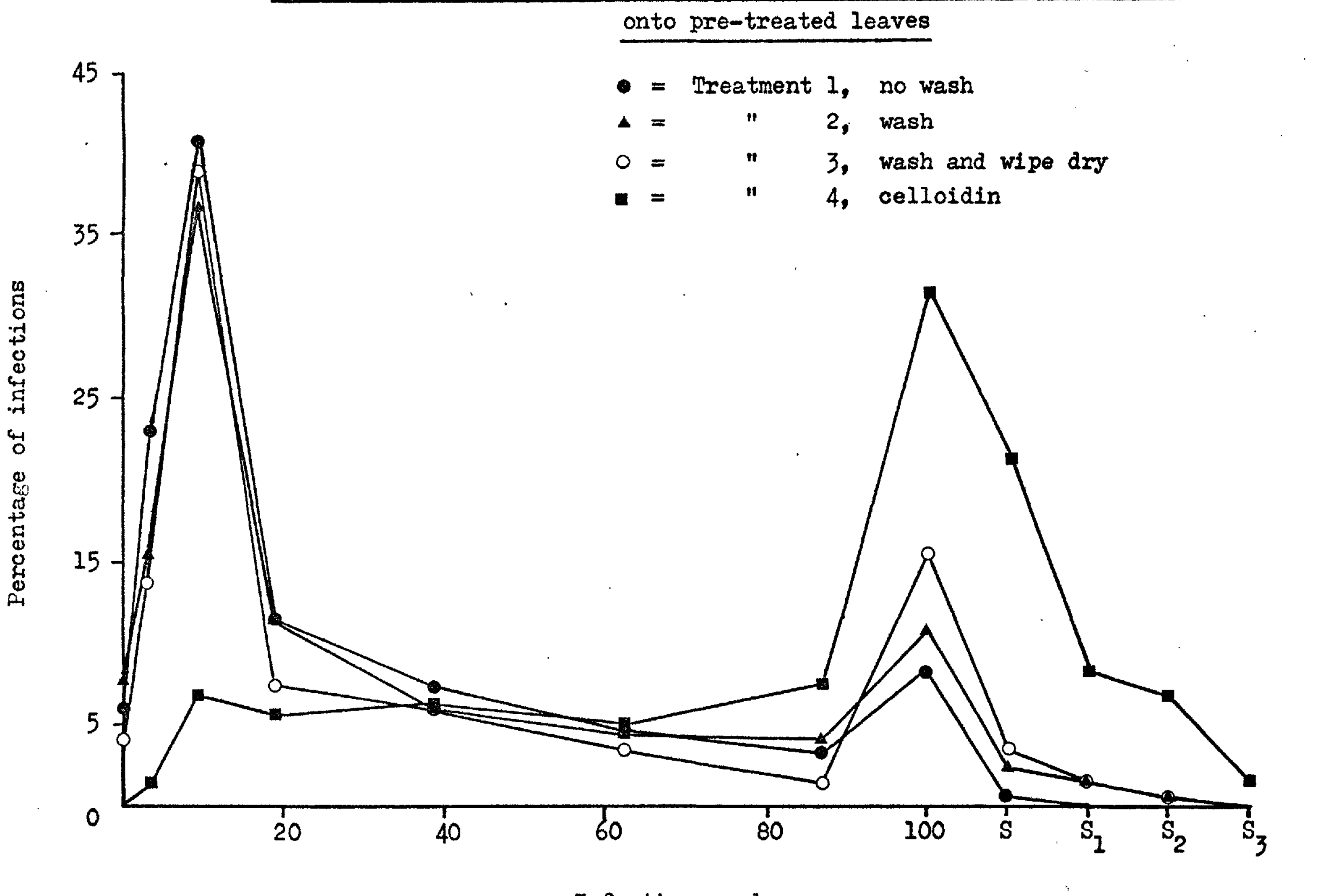


Table 9. The absence of droplets 1 day after inoculation onto pre-treated leaves

	Treatment	Percentage of droplets absent
1)	No wash	9
2)	Wash	24
3)	Wash and wipe dry	8
4)	Celloidin	4

Figure 11. The distribution of infections produced by *B. cinerea* 6 days after inoculation onto pre-treated leaves



Nevertheless the majority of lesions remained limited, only those in grades S_2 and S_3 had caused significant browning of the lower epidermis. The results from other treatments illustrated the division of infections into two populations as previously recorded. The differences between the distribution of infections in leaves given these treatments were not considered to be significant.

It was therefore concluded that the previously observed variation in the type of lesion produced by B. cinerea conidia suspended in water could not be related to damage done to detached leaves in their normal preparation for inoculation; and that the observed variation may reflect the composition of the cuticle at different sites on the leaf surface.

The effect of the position of the inoculum droplets on the leaf on the development of infection by B. cinerea conidia, 6 days after inoculation, is illustrated by the results in Table 10. More droplets were absent (OA) from position 7 one day after inoculation of leaflets given treatment 2, than from any other position. This was probably due to the convergence of numerous leaf veins, and in consequence the greater chance of the presence of a residual film of water at this site. The location of the inoculum droplets at other sites on leaves given treatment 2 had no obvious effect on lesion developments. Similarly there were no obvious differences between the grades of infection produced at any site of inoculation on leaflets given treatments 1, 3 or 4. It was therefore concluded that the potential to support lesion formation at any one site was based upon some factor

Table 10. The effect of droplet position on the development of infection by *B. cinerea* conidia suspended in water

Treatment	Droplet position	Numbers+ of infections in each grade												
		OA*	0	3.5	9.5	19	39	63	87.5	100	S	S_1	S_2	S_3
1	1	2	-	3	11	3	1	1	-	3	-	-	-	-
	2	1	-	4	11	-	1	-	1	3	2	1	-	-
	3	-	2	4	6	2	3	1	-	4	2	-	-	-
	4	5	1	2	6	3	-	1	-	4	2	-	-	-
	5	1	1	3	11	1	-	1	-	4	1	-	1	-
	6	1	1	3	6	5	2	1	1	3	-	1	-	-
	7	3	1	2	9	4	2	-	-	3	-	-	-	-
2	1	4	4	4	8	-	-	-	1	3	-	-	-	-
	2	6	3	2	6	5	-	-	-	2	-	-	-	-
	3	5	1	2	8	-	2	-	2	3	1	-	-	-
	4	6	1	3	6	5	-	-	-	2	1	-	-	-
	5	2	1	6	9	2	1	-	-	1	1	1	-	-
	6	6	-	2	6	-	3	3	-	2	-	-	2	-
	7	8	-	1	5	3	1	3	2	1	-	-	-	-
3	1	1	1	4	12	1	1	-	2	2	-	-	-	-
	2	1	3	7	10	-	2	-	1	-	-	-	-	-
	3	2	-	2	9	3	1	3	2	1	1	-	-	-
	4	2	1	8	8	2	1	-	-	2	-	-	-	-
	5	2	-	7	9	2	2	1	-	1	-	-	-	-
	6	-	3	2	10	2	2	2	-	3	-	-	-	-
	7	3	1	6	6	1	2	1	-	4	-	-	-	-
4	1	1	-	1	-	3	1	2	1	10	3	1	1	-
	2	1	-	-	3	-	3	-	1	9	4	1	1	1
	3	-	-	-	2	1	1	2	1	7	5	4	1	-
	4	2	-	-	2	2	-	1	1	4	8	2	2	-
	5	1	-	-	-	3	2	-	2	8	2	2	3	1
	6	-	-	1	2	-	-	3	4	6	5	2	1	-
	7	1	-	-	2	-	3	-	2	7	7	1	1	-

* Droplets absent one day after inoculation

\+ Total number possible = 24

randomly distributed upon or within the leaf.

b) The development of lesions produced by B. fabae

i) Progress of lesion development

In contrast to the variation in the type of infection produced by B. cinerea, inoculation with B. fabae conidia was reported in Chapter 1 to result in 100% development of visible lesions after 1 day, and of spreading lesions 5 days after inoculation. These observations took no account of any differences in the rate of spread of infections through the leaf. It was therefore decided to investigate any variations in lesion development by measuring the increase in diameter of lesions in leaves of different ages, from different plants.

Four bifoliate leaves were detached from each of seven plants, and each half leaflet was inoculated with three droplets of a suspension of B. fabae conidia in water. The percentages of lesions which developed into the various grades of infection 24 h after inoculation are given in Table 11. Forty hours after inoculation the majority of infections were grade 100. Three lesions of this type, beneath discrete infection droplets, were selected from each leaf, and their diameter measured. Subsequent measurements of the diameter of these lesions were made up to 163 h after inoculation.

Leaf age had a marked effect on infection. The development of lesions was most rapid in the youngest leaves (leaf 4). In this leaf lesion development was often associated with browning in the vascular tissues beyond the main circular body of the lesion. The edges of the lesions were frequently extended a short way along the sides of vascular

Table 11. Lesions produced by *B. fabae* 24h after inoculation

	Infection Grade						
	0	6.5	19	38	63	87.5	100
Percentage of infections	0	8	1	12	56	19	4

tissue, tapering to a point where only vascular browning could be clearly distinguished. This phenomenon gave the lesions a "star shaped" appearance. The diameter of the circular body of these lesions was measured, the vascular extensions were ignored. In two plants lesion development was so rapid in the youngest leaves that they had collapsed when examined 93 h after inoculation. Lesions in these leaves were ignored when the increase in lesion diameter in leaf 4 expressed graphically in Figure 12 was calculated. The majority of infections in other leaves developed as circular lesions. Vascular browning was common after 93 h but this was not associated with "stellate" lesion development. There were only slight increases in lesion diameter until about 80 h after inoculation after which time a linear increase in diameter was recorded in all leaves (Figure 12). The variation in the susceptibility of leaves of different ages was reflected by different rates of increase in lesion diameter.

The plants examined differed in their susceptibility to the spread of infections. The average increases in lesion diameter from 40 - 163 h after inoculation calculated for each leaf from 7 different plants are shown in Table 12. The plants are arranged in order in Table 12 according to the average increase in lesion diameter in each plant - collapsed leaves were ignored. Plant No. 1 supported lesions of the largest diameter. The lowest increase in diameter was recorded from plant 7, however despite the apparent resistance of leaves 3, 2 and 1 of this plant to lesion spread, the youngest leaf had collapsed

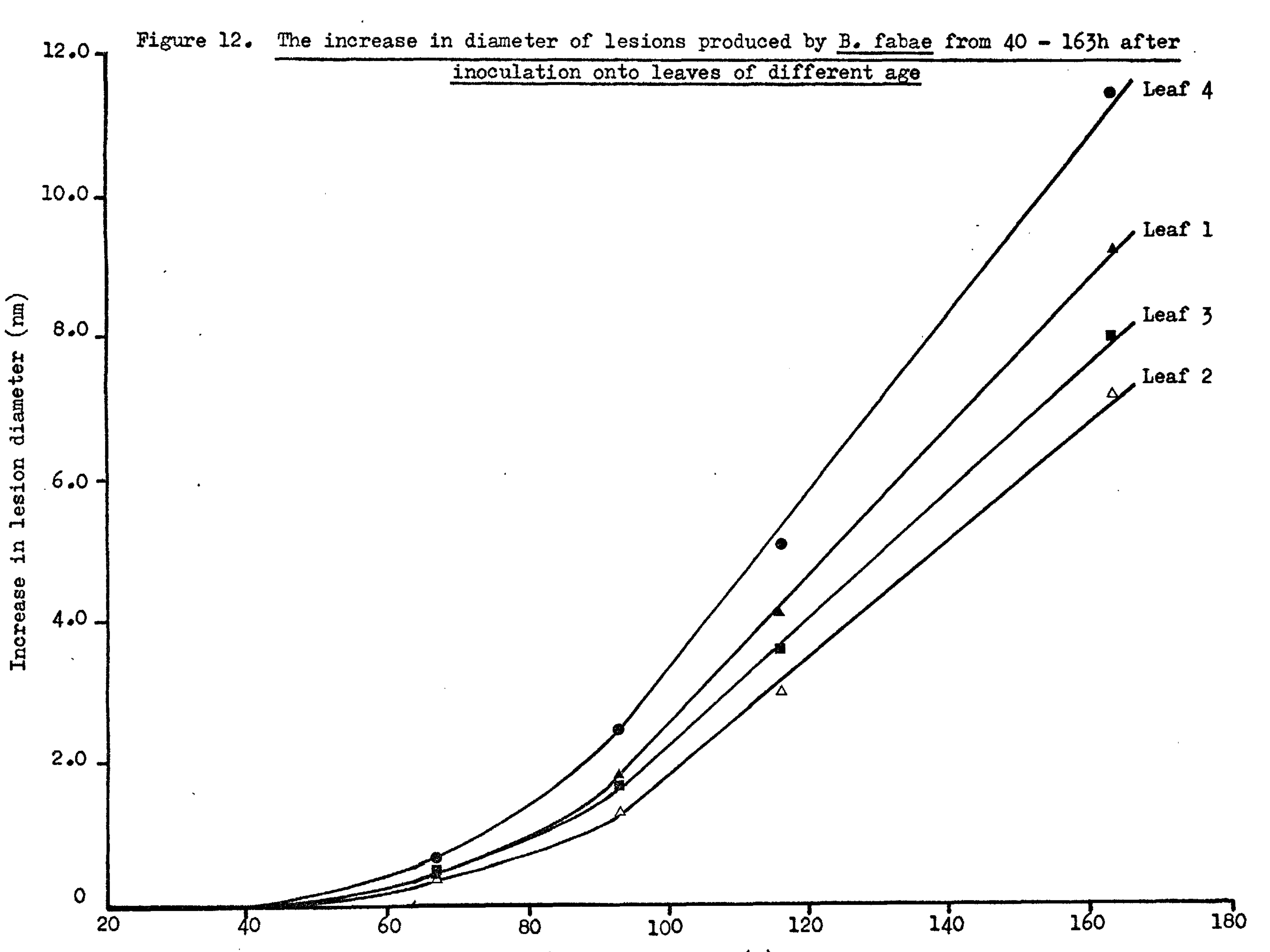


Figure 12. The increase in diameter of lesions produced by *B. fabae* from 40 - 163h after inoculation onto leaves of different age

Table 12. The effect of leaf age and plant/plant variation on the increase in diameter of the *B. fabae* lesion from 40 - 163h after inoculation

Leaf	Plant and average increase in lesion diameter (mm)						
	1	2	3	4	5	6	7
4	12.8	14.0	11.5	10.1	Collapsed	7.9	Collapsed
3	10.3	6.4	8.25	9.2	9.5	3.7	3.5
2	10.1	10.1	9.1	7.1	8.0	7.7	3.9
1	10.3	10.0	10.8	8.4	8.9	8.3	8.0

when examined 93 h after inoculation.

ii) The effect of the presence of the infection droplet

The inoculum droplets above the lesions measured in the previous experiment had not dried out when examined 116 h after inoculation. Other lesions had lost their droplets. In these lesions the rate of increase of lesion diameter appeared to be less than that of the measured droplets.

An experiment was therefore designed to examine the effect of the presence of infection droplet water on the development of the *B. fabae* lesion. Four treatments were devised to examine the effect of droplet water : 1) Water was added daily to make good any loss from the droplet 2) Water was added daily until 97 h after inoculation when the droplet was carefully dried off with a piece of tissue paper 3) Droplets were dried off after 24 h 4) As 3) but 97 h after inoculation a 10 µl water droplet was placed at the infection site and subsequently the presence of droplet water maintained daily. The second and third bifoliate leaves from 5 plants were inoculated with three droplets for each treatment on separate half leaflets. Therefore, each leaf was in effect a replicate of the experiment.

Several droplets were absent when examined 24 h after inoculation. These infections were treated in the same way as others on the same half leaflets. It was found that the disappearance of infection droplets within 24 h from inoculation had a profound effect on the development of the *B. fabae* lesion. Measurements of infections in which the droplet was absent after 1 day were therefore omitted when the increases

Figure 13. Effect of the presence of the infection droplet on the increase in diameter of lesions caused by *B. fabae*

(The values for L.S.D. (P=0.001, 0.01, 0.05) were calculated after analysis of variance of the results obtained 116 and 171 h after inoculation)

● = Treatment 1 water added daily

○ = " 2 droplet removed after 24h

△ = " 3 water added, then removed after 97h

▲ = " 4 droplet removed after 24h, then water added after 97h

Increase in lesion diameter (mm)

10.0 8.0 6.0 4.0 2.0

40 60 80 100 120 140 160 180 200

Time after inoculation (h)

Table 13. The effect of droplet absence on Day 1, on the development of lesions caused by *B. fabae*

Treatment*	Replicates	Time after inoculation and average lesion diameter (mm)					
		48h	72h	97h	116h	147h	171h
1. Water added daily	2	4.8	5.3	5.8	5.9	6.0	6.0
2. Droplet removed after 24h	4	4.9	5.0	5.1	5.1	5.1	5.1
4. Droplet removed after 24h then water added after 97h	2	4.9	5.1	5.1	5.1	5.1	5.1

* No droplets were absent from treatment 3 on Day 1

in lesion diameter expressed graphically in Figure 13 were calculated. The presence of water droplets at the infection site promoted the rate of increase in lesion diameter.

The measurements taken from lesions in which droplets were absent after 24 h are recorded in Table 13. The effect of the disappearance of droplets before 24 h was, essentially, to inhibit the production of a spreading lesion no matter what subsequent treatment was applied. Because grade 100 lesions were produced in all infections it was considered unlikely that the failure to develop spreading lesions was caused by loss of inoculum.

The results of these experiments suggest that there are two distinct phases in the development of lesions caused by _B. fabae_ governed by the presence of the infection droplet. The first phase was less than 24 h after inoculation, when the water droplet must be present to permit the subsequent establishment of a spreading infection. The second phase had begun 24 h after inoculation, when the infection was established, and the presence or absence of droplets changed the rate of spread of the lesion through the leaf.

Chapter 3. Development of *Botrytis cinerea* conidia on the leaf surface in relation to symptoms produced

The following experiments were carried out in an attempt to determine the relationship between the development of *B. cinerea* conidia on the leaf surface, and the various symptoms produced after infection. The primary aim of the experiments was to obtain quantitative data on fungal development in different grades of infection. Where appropriate a description of qualitative observations follows the quantitative results of each experiment.

a) Comparison between fungal growth on the leaf surface and on glass slides

This experiment was designed to compare the rate of development of *B. cinerea* conidia on leaves and glass slides. The development of conidia on slides was arrested by addition of a drop of lactophenol/ cotton blue. Leaf discs bearing infection droplets were bleached with chlorine and stained with phenol acetic aniline blue. Conidia and germ tubes were selected for measurement by the techniques described for bioassays.

Seventy-two leaves of varying age but without signs of senescence were inoculated with a maximum of six droplets containing *B. cinerea* on each half leaflet. At the same time ten clean glass slides were each inoculated with two 10 µl droplets, and incubated like the detached leaves. Included amongst the leaves were the first four expanded bifoliates from five plants. Twelve hours after

inoculation one leaf disc bearing an infection droplet was cut from each leaf of these plants and conidial development was examined. In this way an infection was examined in leaves 1 - 4 from five plants. The droplets on two slides were stained at the same time after inoculation. The remaining leaves, in thirteen sandwich boxes, were grouped for examination at the subsequent times after inoculation; three boxes each for 24, 36 and 48 h, and four for the 96 h measurements. Examples of the different grades of infection present were cleared and stained, and the development of conidia was measured. Only sites where the infection droplet was present were examined.

Development of conidia on leaves of different age, and on glass 12 h after inoculation is shown in Table 14. There was little difference between the behaviour of conidia on leaves of different age. The major differences between leaf and glass surfaces were the much higher mean germination in 12 h on glass, and the much wider range of percentage germination and germ tube length at different sites on leaves. There was little variation within individual droplets. Clearly conidia at different infection sites were affected in different ways by leaf surfaces, some being inhibited, and others stimulated in their growth.

When the results of fungal growth after longer periods were assessed, (Table 15 and Figures 14 - 18), the most interesting phenomena concerned conidial behaviour at sites bearing grade 0, and 100 infections. At many of the sites where no symptoms had developed (grade 0), percentage germination was much lower than at all other sites (Figure 14).

Table 14. The development of *B. cinerea* conidia on leaves of different age, and on glass, 12h after inoculation

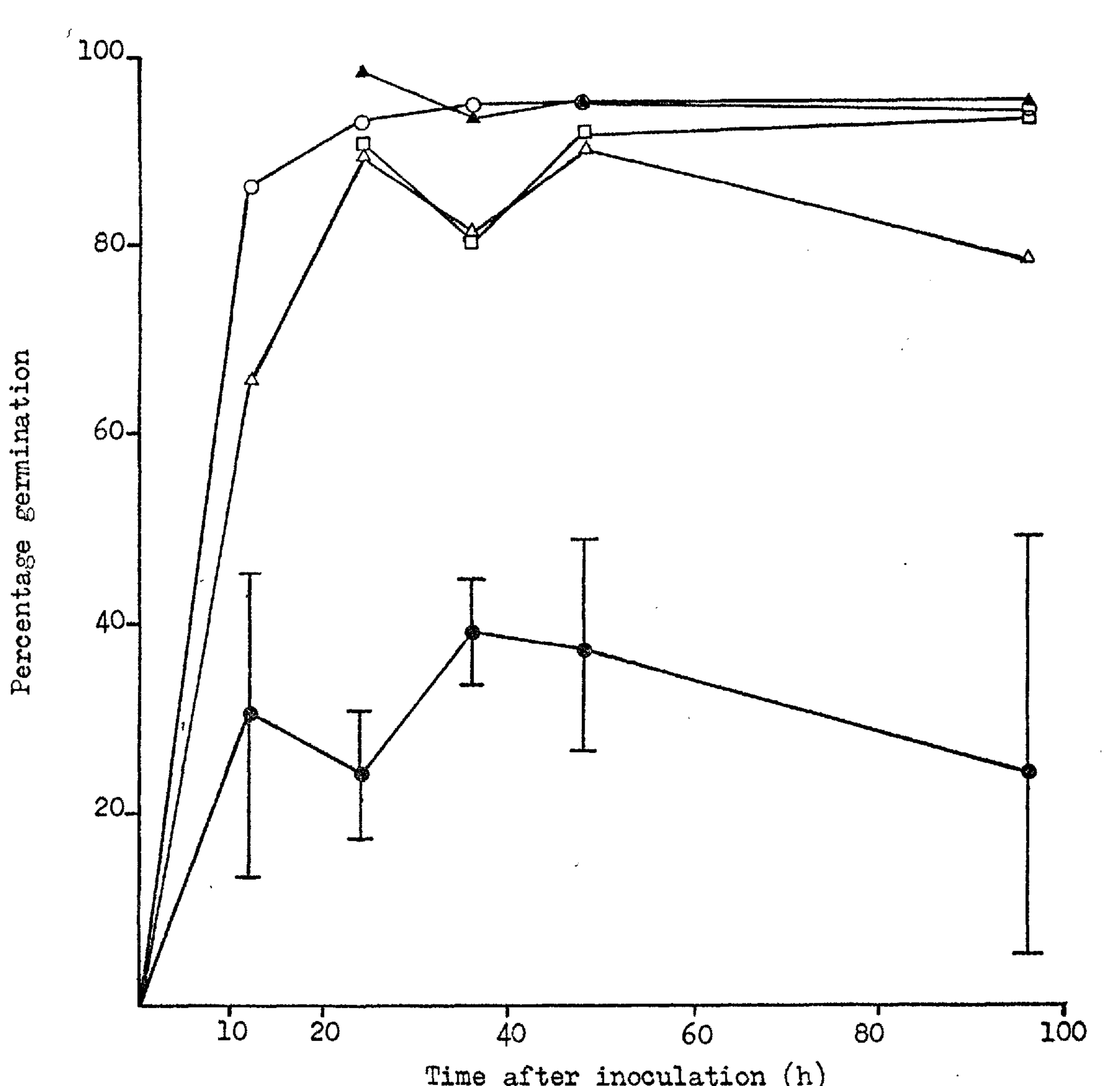
Leaf	Percentage germination+	Range*	Germ tube length (µm)△	Range*
4	40.0	21.4 - 54.7	11.9	5.3 - 17.8
3	57.3	31.0 - 97.5	17.8	9.9 - 34.0
2	37.9	20.0 - 84.0	12.0	10.0 - 15.4
1	42.3	13.3 - 61.0	13.9	6.4 - 17.6
On glass	86.0	82.0 - 91.2	14.1	13.5 - 14.7

* Maximum range between replicate droplet mean values

\+ Mean calculated after the examination of at least 150 conidia in each replicate droplet

△ Mean calculated after the measurement of 30 germ tubes in each replicate droplet

Figure 14. Germination of *B. cinerea* conidia on the leaf surface and on glass



▲ = Grade 87.5 infection
□ = " 6.5 "
△ = " 0(a) "
● = " 0(b) "
○ = Germination on glass

Maximum range between replicate values

The grade 0 infections examined were therefore categorized on the basis of the percentage germination of conidia being above or below 50%. These two divisions are described as grade 0(a) and 0(b) respectively. After 4 days three of the twelve grade 100 infections examined, and those sites where lesions had spread, supported much greater germ tube growth than any other site where symptoms were apparent. Accurate measurement of germ tube length in the mesh of hyphae in these infections was not attempted, but germ tubes were estimated to be >250 μm in length. Those grade 100 lesions which were associated with a mesh of hyphae were considered as a separate category (termed grade 100 mesh) from the other grade 100 infections in which only short germ tubes had developed.

Figure 15 describes the germ tube lengths measured on the leaf surface at different intervals after inoculation. The mean germ tube length was greatest in lesions with the highest percentage of browning in the droplet area. The time after which no further increase in germ tube length was observed depended on the grade of infection examined. The measurement of germ tubes of similar length in lesions ranging from grade 19 to 100, 96 h after inoculation indicated that symptom expression within this range was not directly proportional to germ tube length on the leaf surface.

This is further illustrated in Figure 16 in which the relationship between germ tube length and infection grade after 96 h is examined in detail. The infections could be clearly divided into two groups, with less or more than grade 19 infection.

Table 15. The development of *B. cinerea* conidia on the leaf surface in relation to symptom development, and on glass

Time after inoculation	Infection grade	Percentage germination+	Range*	Germ tube length (μm)△	Range*
24	0 (b)	24.3	17.8 - 31.1	8.6	5.3 - 17.6
	0 (a)	89.4	87.9 - 90.9	24.4	14.4 - 34.4
	6.5	91.3	87.0 - 95.7	26.3	14.3 - 50.4
	19	97.1	96.0 - 98.3	33.0	22.9 - 46.6
	38	98.1	95.6 - 99.0	42.5	34.9 - 85.0
	63	97.3	-	36.1	-
	87.5	98.3	-	38.2	-
	On glass	93.2	93.0 - 93.3	38.3	30.9 - 43.1
36	0 (b)	39.7	33.8 - 44.6	16.1	14.5 - 17.6
	0 (a)	81.2	-	21.3	-
	6.5	80.7	63.6 - 92.6	20.7	17.5 - 25.1
	19	96.4	95.0 - 97.1	46.5	22.2 - 89.0
	38	91.9	89.1 - 94.7	44.4	41.6 - 47.2
	63	86.8	82.9 - 90.6	61.8	36.7 - 86.8
	87.5	94.7	93.0 - 96.4	58.9	54.4 - 63.4
	100	96.4	95.7 - 97.1	80.4	77.3 - 83.6
	On glass	94.8	93.7 - 95.9	61.7	53.7 - 69.9

* Maximum range between replicate droplet mean values

\+ Mean calculated after the examination of at least 150 conidia in each replicate droplet

△ Mean calculated after the measurement of 30 germ tubes in each replicate droplet

Table 15. (contd...) The development of *B. cinerea* conidia on the leaf surface in relation to symptom development, and on glass

Time after inoculation	Infection grade	Percentage germination+	Range*	Germ tube△ length (μm)	Range*
48	0 (b)	37.6	26.3 - 48.9	11.8	9.5 - 14.2
	0 (a)	90.2	86.2 - 92.5	19.3	17.6 - 21.3
	6.5	92.2	73.3 - 98.2	34.2	13.5 - 67.2
	19	93.3	86.6 - 97.4	47.9	28.2 - 74.6
	38	94.6	91.4 - 96.9	53.8	40.5 - 68.5
	63	95.1	92.4 - 98.0	59.9	45.8 - 86.6
	87.5	95.5	93.4 - 97.6	69.3	63.3 - 75.4
	100	95.1	93.3 - 97.9	85.8	56.9 -129.1
	On glass	94.9	94.0 - 96.1	74.4	64.4 - 83.8
96	0 (b)	24.3	5.3 - 49.7	11.2	5.8 - 14.7
	0 (a)	78.7	-	17.4	-
	6.5	93.7	92.0 - 95.5	30.0	13.5 - 44.7
	19	94.6	91.4 - 98.7	48.4	31.6 - 63.9
	38	94.2	90.7 - 98.7	53.3	35.8 - 82.0
	63	93.9	90.7 - 95.5	59.2	34.9 -108.6
	87.5	97.8	96.9 - 98.7	48.0	30.3 - 74.4
	100	95.2	90.1 - 98.7	81.3	44.4 -121.4
	100 (mesh)	-	-	>250	-
	S, S_1, S_2	-	-	>250	-
	On glass	94.2	90.9 - 95.7	109.3	95.4 -120.3

* Maximum range between replicate droplet mean values

\+ Mean calculated after the examination of at least 150 conidia in each replicate droplet

△ Mean calculated after the measurement of 30 germ tubes in each replicate droplet

Figure 15. Length of germ tubes produced by *B. cinerea* conidia in relation to infection grade

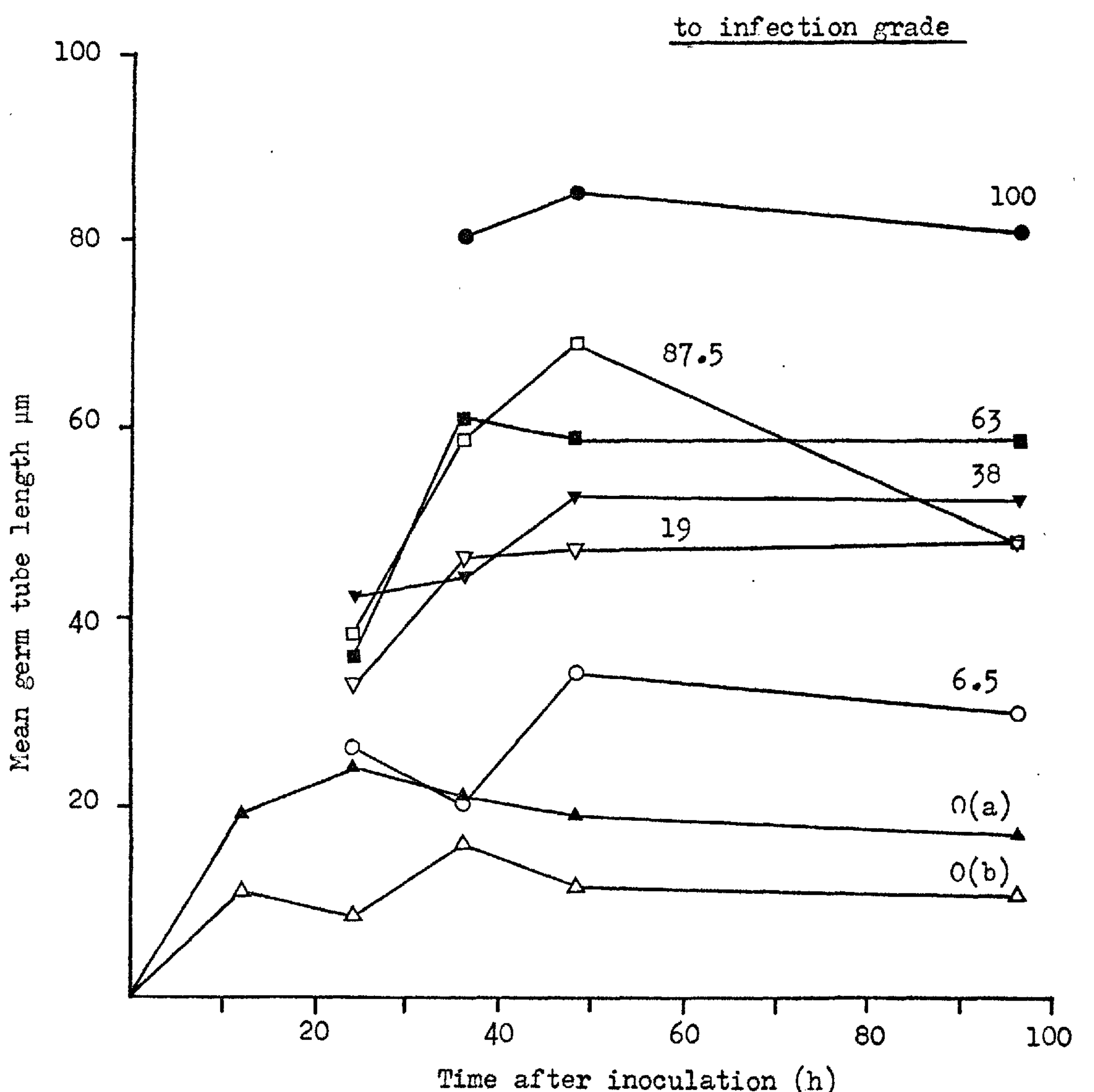
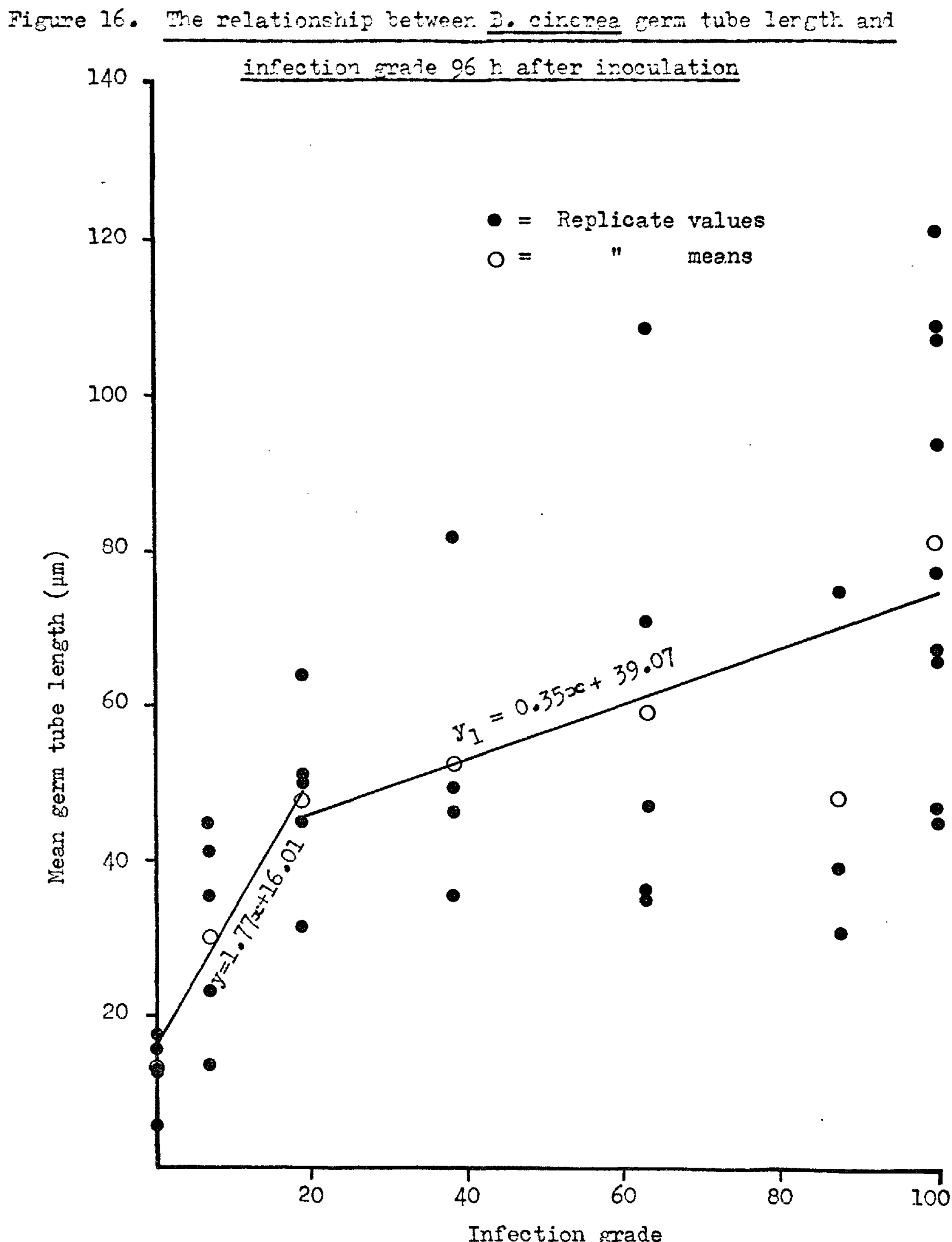
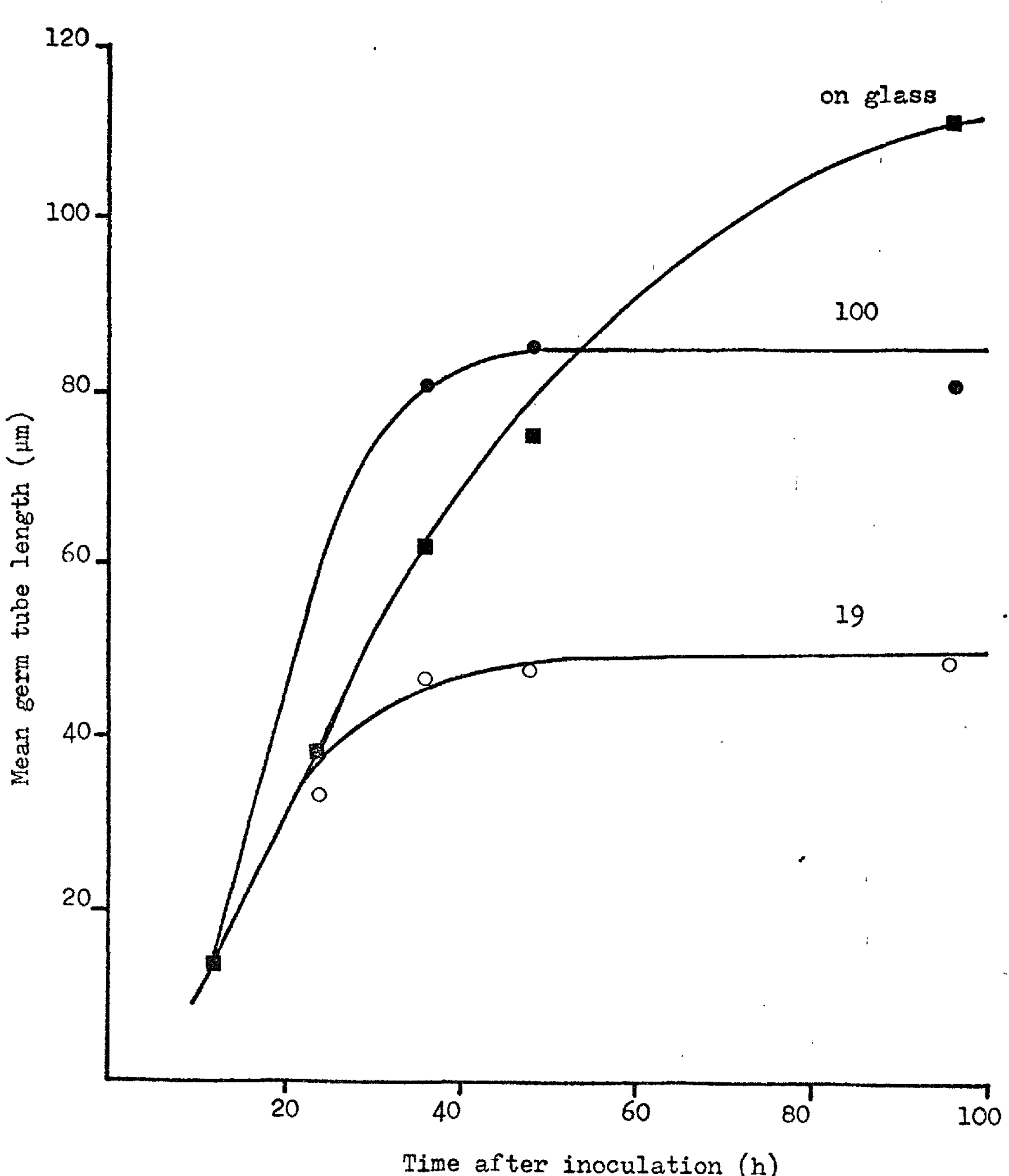


Figure 16. The relationship between *B. cinerea* germ tube length and infection grade 96 h after inoculation



(Regressions y and y_1 were calculated from the values of mean germ tube length at sites in infection grades 0, 6.5 and 19; and 19, 38, 63, 87.5 and 100, respectively)

Figure 17. Length of germ tubes produced by *B. cinerea* conidia on glass, and above grade 19 and 100 infections



The mean germ tube lengths measured in grade 19, and 100 infections which were not associated with a mesh of hyphae, are compared with germ tube length on glass in Figure 17. The higher value of germ tube length recorded in grade 100 lesions than on glass 36 h after inoculation indicates that there was an initial stimulation of germ tube growth on the leaf surface. There was little increase in germ tube length in grade 19, and 100 lesions after 36 h; by this time the majority of germ tubes in these infections were terminated by appressoria. In contrast, on glass, germ tubes continued to increase in length, though the growth rate decreased with time. This effect was probably due to the absence of nutrients. After 96 h incubation the germ tubes produced on glass were characteristically thin, and irregularly stained compared with those in 100 and 19 grade infections.

i) Development of germ tubes in a particular grade of infection

It must be emphasized that the development of conidia on the leaf surface measured in infections of the same grade at different times after inoculation does not illustrate the progress of fungal development in any single lesion. This is because the majority of lesions themselves progressed in time from one infection grade to another. It was, however, possible to calculate the length of germ tubes associated with the production of grade 19 infections six days after inoculation, by referring to the quantitative measurements of the development of infections caused by B. cinerea conidia, reported in Chapter 2.

The calculation of germ tube length at different times after

inoculation was basically the same as that applied to the estimation of mean grades of infection of developing lesions described in Chapter 2, but instead of multiplying the infection grade by the number of lesions in the grade; in this case, the number of lesions was multiplied by the mean germ tube length measured for the grade of infection. For example, the germ tube length in potential sixth day grade 19 infections 24 h after inoculation was calculated as follows:

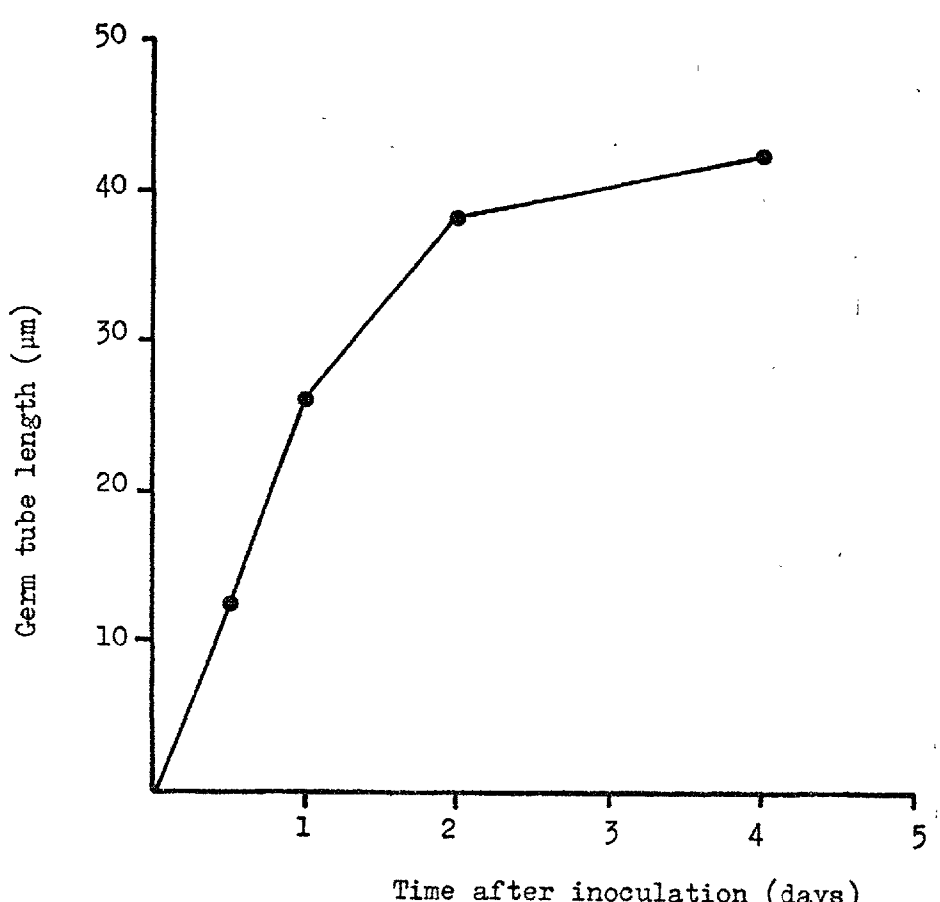
Infection grade	Number of Infections (a)	Mean germ tube length (b)	(ab)
0*	4	24.4 μm	97.6
6.5	17	26.3 μm	447.1
	$\Sigma a = 21$		$\Sigma ab = 544.7$

$\therefore$ Length of germ tubes associated with a developing grade 19 infection $= \frac{\Sigma ab}{\Sigma a} = 25.9\,\mu m$

* (It was assumed that only those grade 0 infections in which >50% of conidia had germinated would develop to grade 19 infections.)

The calculations made for 24, 48, and 96 h after inoculation are expressed graphically in Figure 18. The calculated values indicate that germ tube growth ceased between 24 - 48 h after inoculation. In Chapter 2 it was shown that lesions developing to grade 19 infections after 6 days had a mean infection grade of 10.2, 2 days after inoculation. Germ tube growth had ceased in these infections within 2 days. Therefore the percentage browning of the leaf at the infection site was almost doubled between 2 and 6 days after inoculation without any further fungal growth.

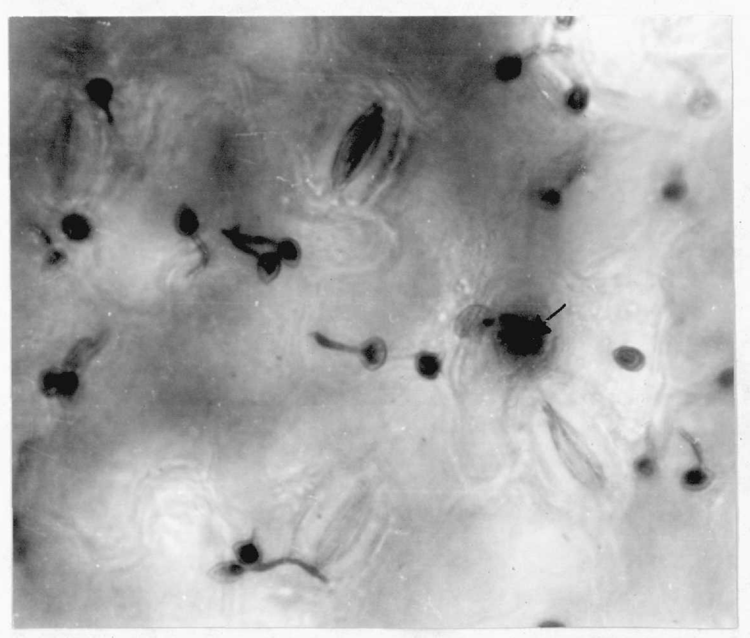
Figure 18. Calculated germ tube length produced by *B. cinerea* developing Grade 19 infections



ii) Observations on infections 96 h after inoculation

Plates 17 - 21 are photomicrographs of developing conidia in various types of infection 96 h after inoculation.

Plate 17. Developing conidia in an infection without macroscopically visible symptoms x 1000.



Note the browning (arrowed) in an epidermal cell associated with the tip of a germ tube. Other germ tubes are not associated with this brown localized reaction material.

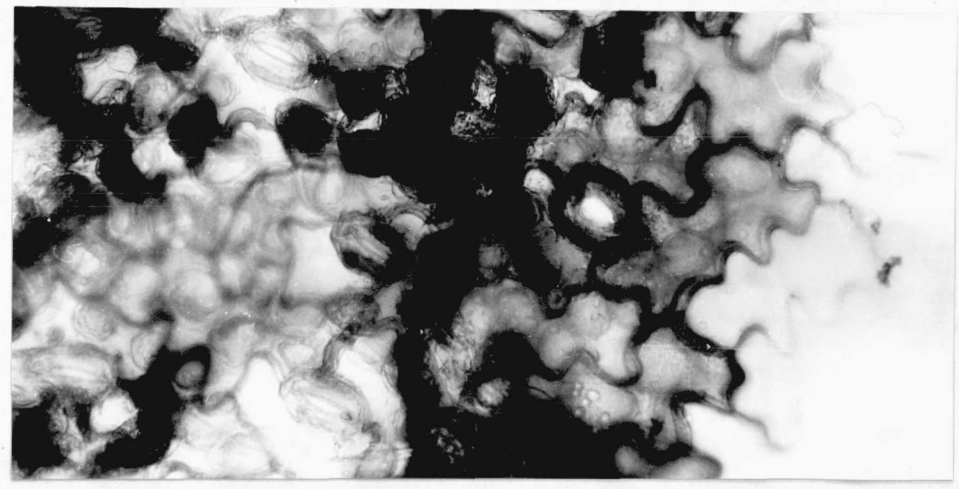
Plate 18. Developing conidia in a grade 100 infection x 632.

Note the appressoria and localized staining in distorted germ tubes.

Plate 19. A mesh of hyphae in an S grade infection x 632.

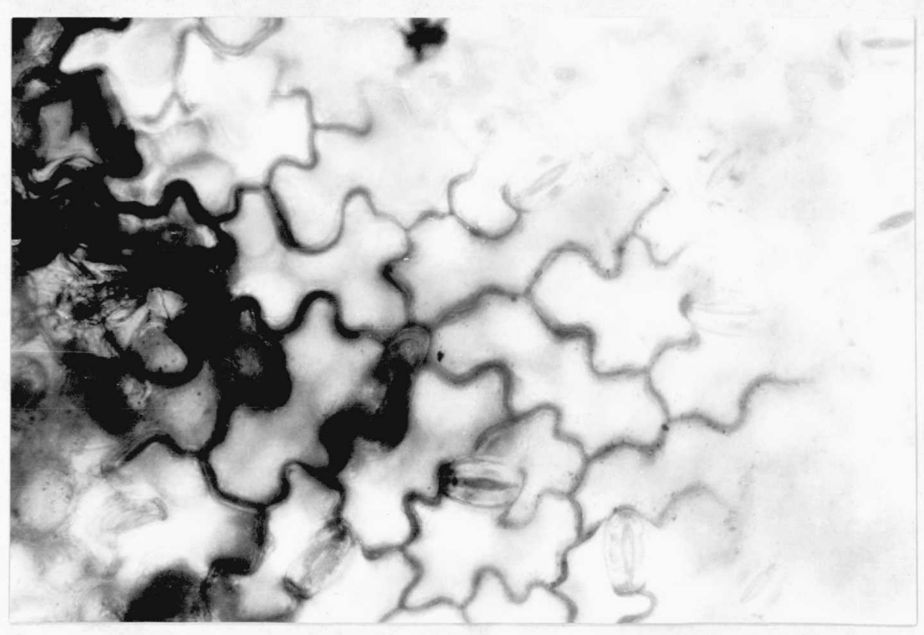
The hyphae extended only to the edge of the infection droplet.

Plate 20. Browning in epidermal and palisade mesophyll cells beyond the edge of the infection droplet in an S grade infection x 632.



Note the absence of superficial hyphae. The darker browning of the epidermal cell ealls gives a "crazed" appearance. There is evidence of granulation in several epidermal cells.

Plate 21. The edge of the brown halo around an S grade infection x 632.



Note the cessation of "crazing" of the epidermis at the edge of the halo in contrast to the healthy epidermal cells. The "crazing" is most obvious where adjacent palisade mesophyll cells are not necrotic.

The three S grade infections examined were characterized by a halo of light brown tissue beyond the area of the infection droplet to which fungal hyphae were confined (Plates 19 - 21). In one lesion the halo extended 700 μm beyond the nearest fungal hypha. The underside of the discs bearing S grade lesions was examined. Despite the apparent spread of the lesion in the upper leaf surface there was little browning in the lower leaf surface. Free hand sections were prepared of one of these lesions. Browning was predominantly in the upper epidermis and palisade mesophyll tissues, and the fungus appeared to be limited to brown cells in the epidermis in the S grade infections 4 days after inoculation. The browning, but not the fungus had extended beyond the infection site.

Cell browning was observed in other grades of infection in advance of fungal invasion, but it had not developed to form a distinct halo. In contrast, the localized browning within some epidermal cells in certain 0 grade infections (Plate 17) appeared to be adjacent to the fungal germ tube. The microscopic observation of this brown reaction material was evidence that despite the absence of symptoms visible with the naked eye a browning reaction had occurred at the infection site

b) <u>The significance of the development of a mesh of hyphae on the leaf surface</u>

One of the most interesting features of the previous experiment was the development of a mesh of hyphae at certain sites on the leaf surface 4 days after inoculation. It was impossible to determine

whether fungal growth had ceased in these infections. In order to study the significance of the development of a mesh of hyphae an experiment was designed to investigate the development of infections between 4 - 8 days after inoculation.

Thirty-six leaves were inoculated with a suspension of B. cinerea in water. After 4 days the infections were graded using system 3. After 8 days the grade of infection in the lower leaf surface, below the 31 lesions which had developed to grade 100 or beyond, was also recorded. Fungal growth in these infections was then measured as described in the previous experiment, and the presence of a halo of brown cells in advance of hyphae was noted. The results obtained from each lesion are arranged in order, depending on the grade of infection in the lower leaf surface, in Tables 16 - 18.

The observations made on the 11 grade S lesions after 4 days are recorded in Table 16. Only two lesions had developed to grade S_2 after 8 days, and they bore meshes of hyphae which had extended from the infection site to the edge of the lesions. Hyphae could be seen emerging from the blackened leaf surface beneath these lesions. The fungus had overcome the resistance of the leaf and spread from the infection site. At the other nine sites which remained grade S lesions after 8 days, there was little symptom development in the lower leaf surface, and hyphae were restricted to the infection site. It was concluded that any growth that had occurred at these sites, between 4 and 8 days after inoculation, had taken place within the droplet; and that the underlying leaf tissues were resistant to fungal infection.

Table 16. Germ tube length of *B. cinerea* and lesion grade 8 days after inoculation where lesions had been graded S, 4 days earlier

Grade of infection of leaf surface		Mean Germ tube*	Halo
Upper	Lower	Length (μm)	
S	6.5	43.3	+
S	6.5	62.1	+
S	6.5	>250	+
S	6.5	>250	+
S	6.5	>250	+
S	19	38.4	+
S	19	>250	+
S	38	>250	+
S	63	>250	+
S_2	S_2	>250	-
S_2	S_2	>250	-

* Calculated from 30 germ tubes

Table 17. Germ tube length of *B. cinerea* and lesion grade 8 days after inoculation where lesions had been graded 100, 4 days earlier

Grade of infection of leaf surface Upper	Lower	Mean germ tube* Length (µm)	Halo
S_1	0	>250	+
S	6.5	44.0	+
100	6.5	66.1	-
100	6.5	68.5	-
100	6.5	83.8	-
100	6.5	95.0	-
S	6.5	>250	+
S	6.5	>250	+
100	38	44.2	-
100	63	>250	-
100	100	>250	-

* Calculated from 30 germ tubes

Table 18. <u>Germ tube length of *B. cinerea* 8 days after inoculation, and symptoms at various sites</u>

Grade of infection of leaf surface			Germ tube*	Halo
4 days	8 days		length (µm)	
Upper	Upper	Lower		
63	100	0	35.7	-
87.5	100	0	50.2	-
63	100	6.5	34.9	-
87.5	100	6.5	48.0	-
87.5	S	6.5	54.7	+
87.5	100	19	27.5	-
63	100	19	54.6	-
63	S	38	46.3	+
87.5	S	38	>250	+

* Calculated from 30 germ tubes

The observations made on the grade 100 lesions at 4 days after inoculation are recorded in Table 17. Meshes of hyphae occurred over more grade 100 lesions after 8 days ($\frac{4}{11}$) than after 4 days in the previous experiment ($\frac{3}{12}$), which suggested that germ tube growth continued within some infection droplets after 4 days. Furthermore, four of the infection sites were now surrounded by wide haloes of brown cells, but the hyphae were confined to the infection site on the leaf surface. Therefore, the development of a mesh of hyphae at these sites was not related to the development of a spreading infection but was a reflection of continued fungal growth only in infection droplets.

The remainder of infections examined had developed to grade S or 100 from lesions in grades below 100, 4 days after inoculation (Table 18). No lesions in an infection grade <63 after 4 days had developed to grade 100 or S, although a mesh of hyphae had developed in one lesion. As no infections in grades below 100 were associated with germ tubes above 250 μm in length when examined 4 days after inoculation this indicated that hyphal growth may have continued after this time. Because the fungus was confined to the infection site it was concluded that any growth would have taken place only in the infection droplet.

At most of the sites examined after 8 days where many germ tubes were over 250 μm in length some germ tubes were short and terminated in appressoria. In contrast long germ tubes were not usually terminated by appressoria. Some long germ tubes were produced directly from conidia, but others had grown from appressoria produced

from short primary germ tubes. Thus, although germ tube growth by B. cinerea above brown lesions typically ceased after production of an appressorium, growth could continue from the appressorium in the infection droplet under certain conditions.

It was concluded that the development of a mesh of hyphae on the leaf surface at the infection site was typically associated with production of limited grade S infections characterized by a halo of brown cells surrounding the infection site, and little symptom development in the lower leaf surface. Apart from very limited invasion of the leaf, fungal growth was confined to the infection droplet.

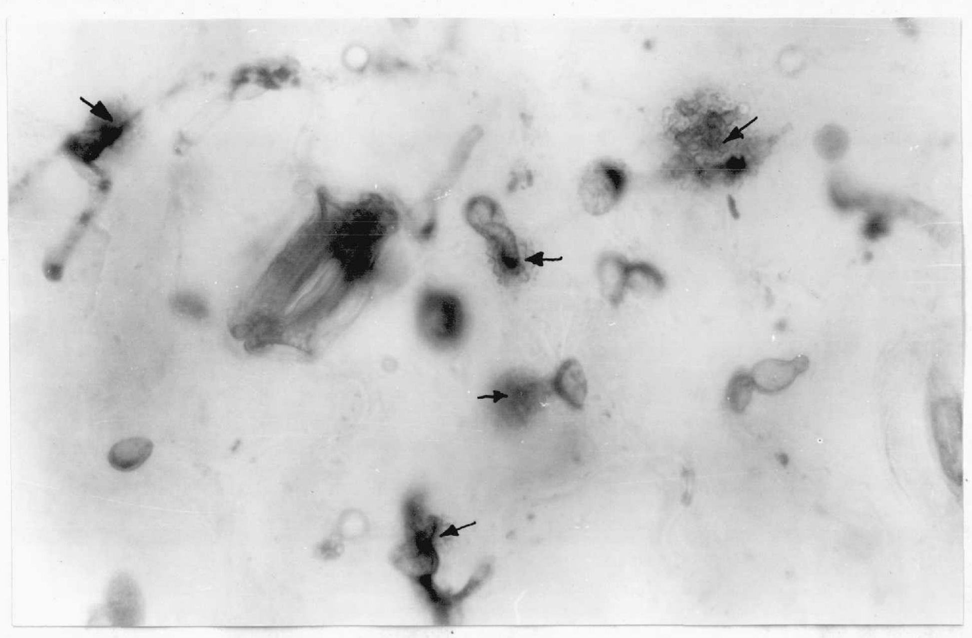
i) Observations on "reaction material"

Several leaves which bore only grade 0 infections 4 days after inoculation, and which had therefore not been used for the measurements of fungal development, were examined 15 days after inoculation. The leaves had not senesced, but numerous minute light brown flecks had developed within the area of the dried infection droplets. This browning was different from that previously observed, and recorded in the measurement of infection grade. Discs bearing brown flecks were bleached, stained with phenol acetic aniline blue, and examined microscopically.

The minute brown flecks were found to be related to the presence within epidermal cells of brown granular reaction material typically around the ends of germ tubes (Plate 22). The localization of reaction material within some cells contrasted with other cells in which all the

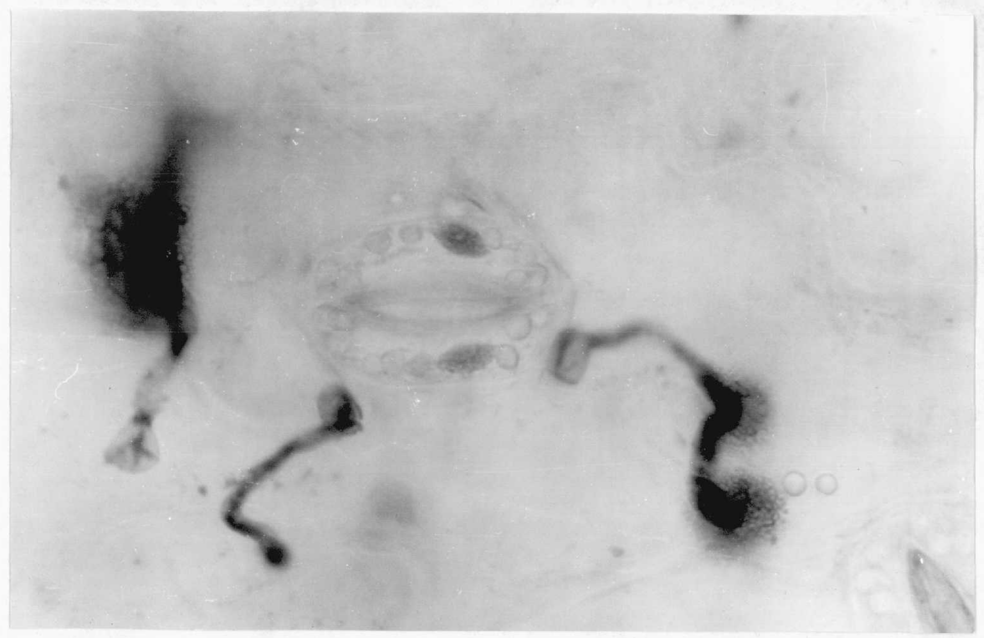
cell contents were brown (Plates 26 and 27). In some cells single germ tubes were associated with more than one patch of reaction material (Plate 23). Optical sections indicated that the material was within the cell rather than on the leaf surface (Plates 24 a,b,c.) Often the material was closely associated with the nucleus of the epidermal cell (Plate 25).

Plate 22. Reaction material in an infection 15 days after inoculation x 1280.



Note reaction material (arrowed) frequently associated with the end of germ tubes, and that all the contents of the stomatal guard cells are brown.

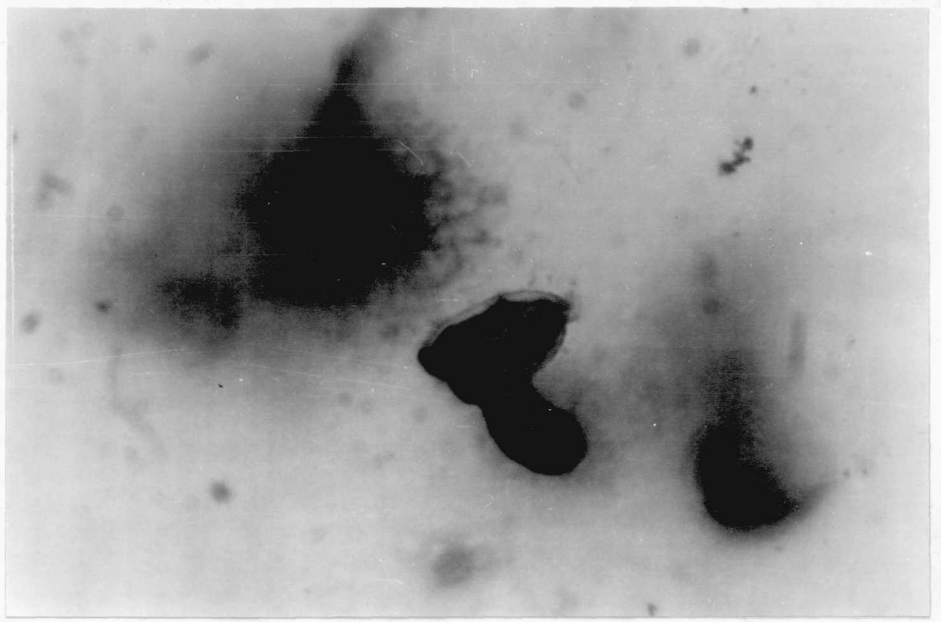
Plate 23. Reaction material in an infection 15 days after inoculation x 1600.



Note the absence of reaction material from the end of one germ tube, and two patches associated with a single germ tube.

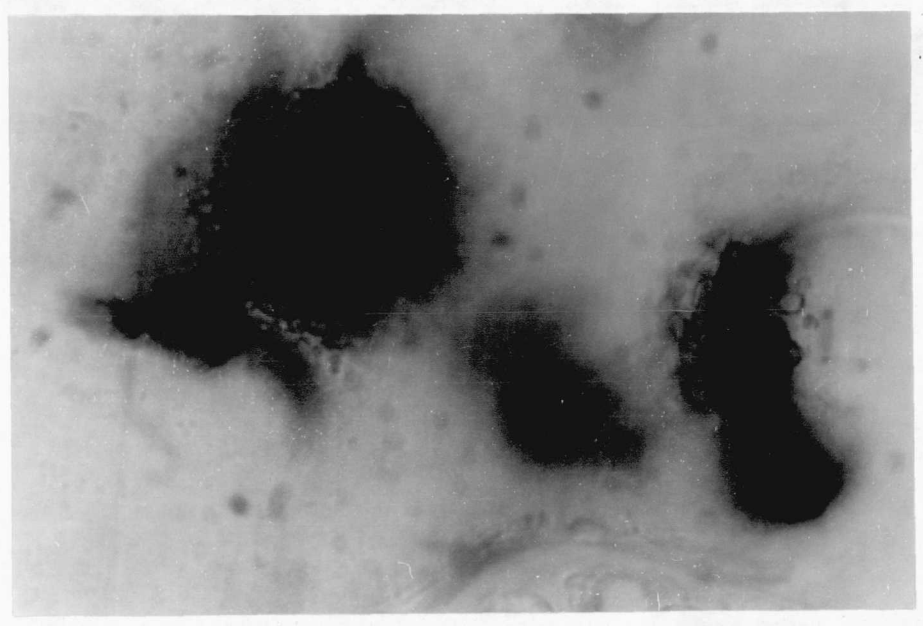
Plate 24 a b and c. Optical sections of an infection site with reaction material. x 4000

a) Germinated conidium on the leaf surface in plane 1.



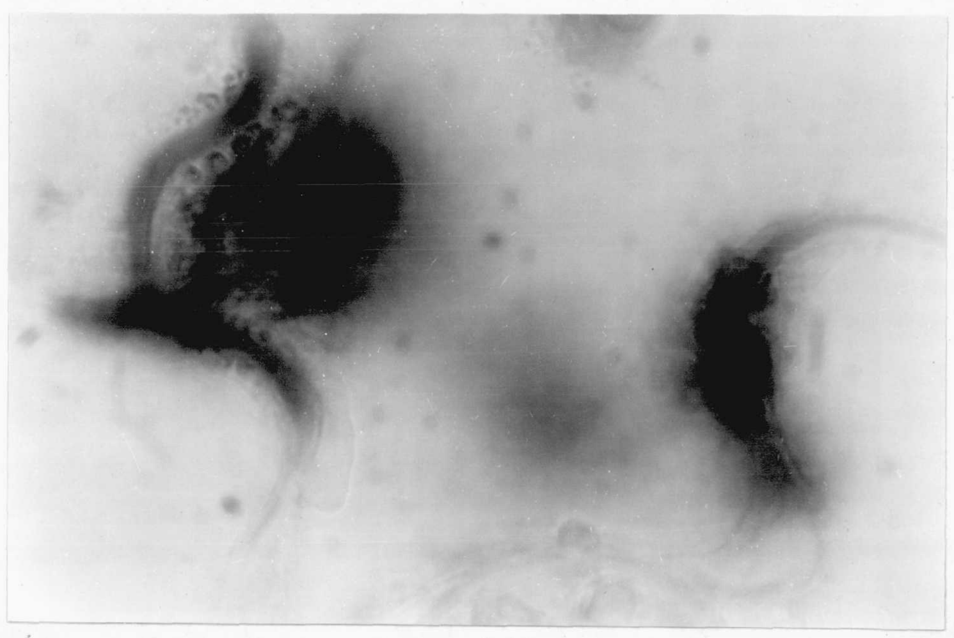
Note that reaction material is not clearly visible beneath the germ tube.

b) Reaction material in the epidermal cell in plane 1 - 3.5 μm.



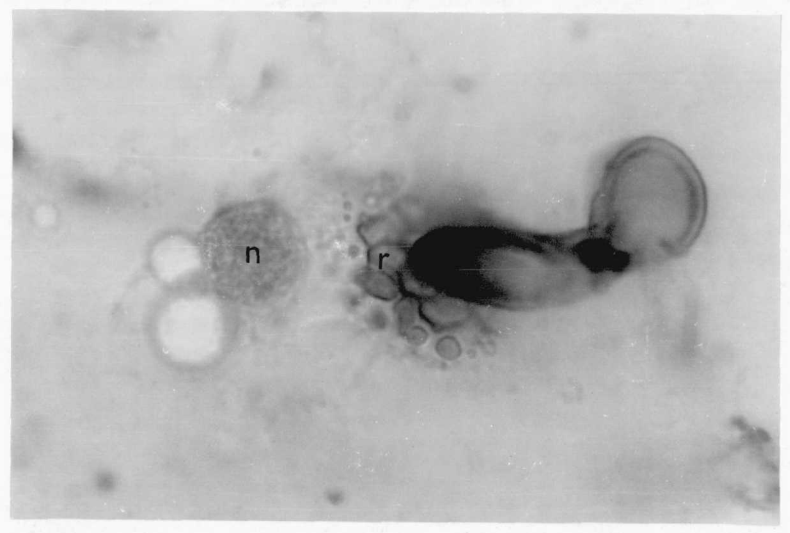
Note granular reaction material (arrowed) within the cell beneath the end of the germ tube, and other patches of reaction material within the same cell associated with other infections.

c) Reaction material in an epidermal cell in plane 1 - 7.75 μm.



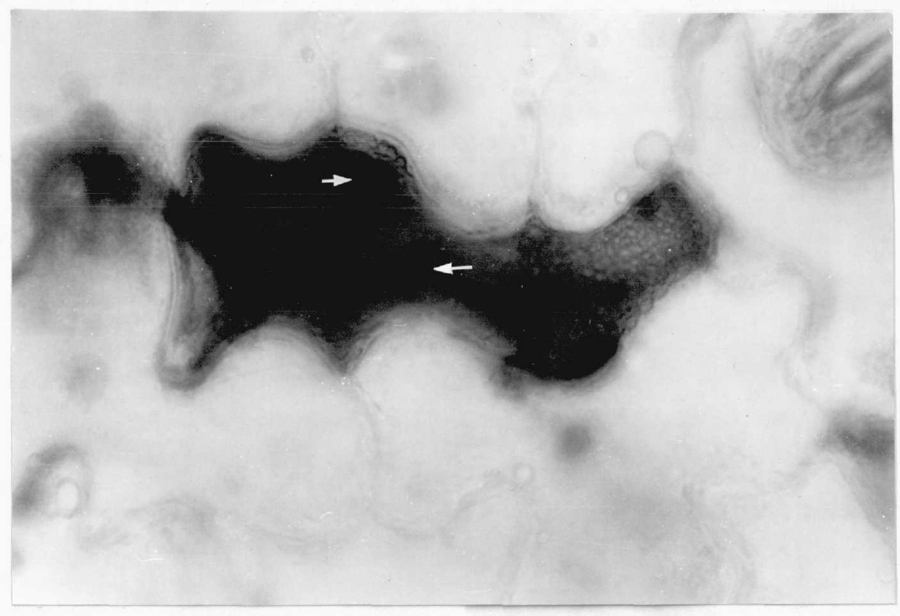
Note the close association of reaction material with localized browning in the cell wall, and granulation in the adjacent cell.

Plate 25. Reaction material in an infection 15 days after inoculation x 4000



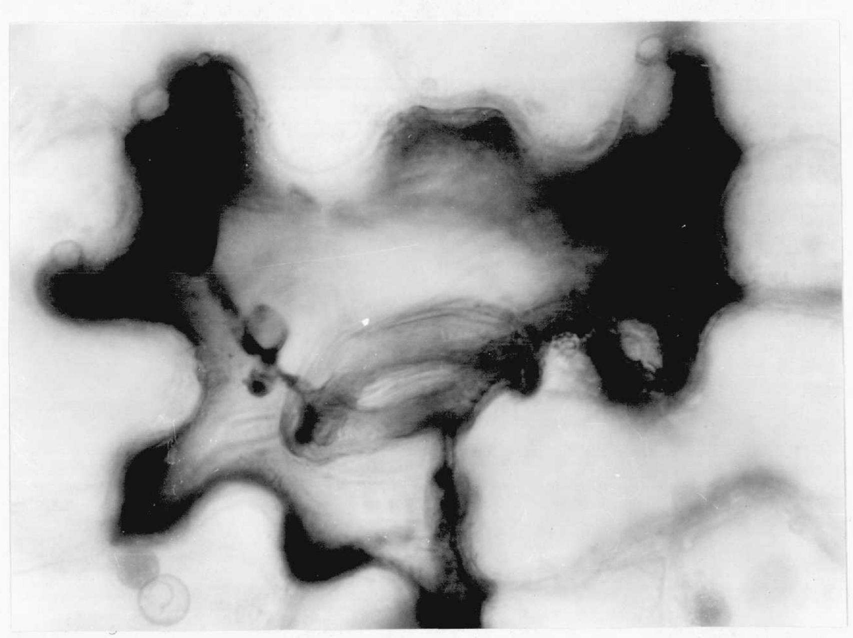
Note the association of granular reaction material (r) with the epidermal cell nucleus (n).

Plate 26. Necrosis of an isolated cell x 1600.



Note the associated developing conidia (arrowed) and granulation within the cell.

Plate 27. B. cinerea above a necrotic epidermal cell and adjacent stomatal guard cells x 1600.



Fresh discs and sections bearing reaction material were examined for the presence of callose by the aniline blue fluorescence method. Reaction material was not identified as callose.

c) Conclusions on the relationship between fungal growth on the leaf surface and lesion development

In general, the higher the infection grade, the longer were the associated germ tubes, but there was considerable variation in germ tube lengths measured in different infections of the same grade. It was concluded that the growth of B. cinerea in limited lesions stopped at one of three stages, and that the type of lesion produced depended on the stage of fungal limitation. The three stages were:

i) Before 50% germination of conidia on the leaf surface. This resulted in the development of infections without visible symptoms after 4 days. The time of cessation of fungal growth was within 12 h from inoculation.

ii) After development of germ tubes shorter than 250 μm. The type of lesion which developed in general, depended on the length of germ tubes produced, and there was probably a related variation in the time at which fungal growth ceased. At most sites which developed little or no symptoms, grades 0 and 6.5, few appressoria were formed, and fungal growth on the leaf surface had ceased by 12 h and 24 h after inoculation respectively. Grade 19 - 100 infections which were not associated with a mesh of hyphae, all supported germ tubes of similar length. In these infections fungal growth ceased between 24 - 48 h after inoculation and germ tubes typically terminated in well developed

appressoria above discoloured cells.

iii) After the production of germ tubes $>250\,\mu m$ in length. This growth was observed as a mesh of hyphae and the time of limitation of fungal growth could not be determined. The fungus only grew in the infection droplet and invading hyphae were confined to the epidermis. This type of growth was typically associated with limited S grade infections, though some grade 100 lesions were also produced by a superficial mesh of hyphae.

Chapter 4. The preparation, and some properties of wyerone acid from B. cinerea pod diffusate

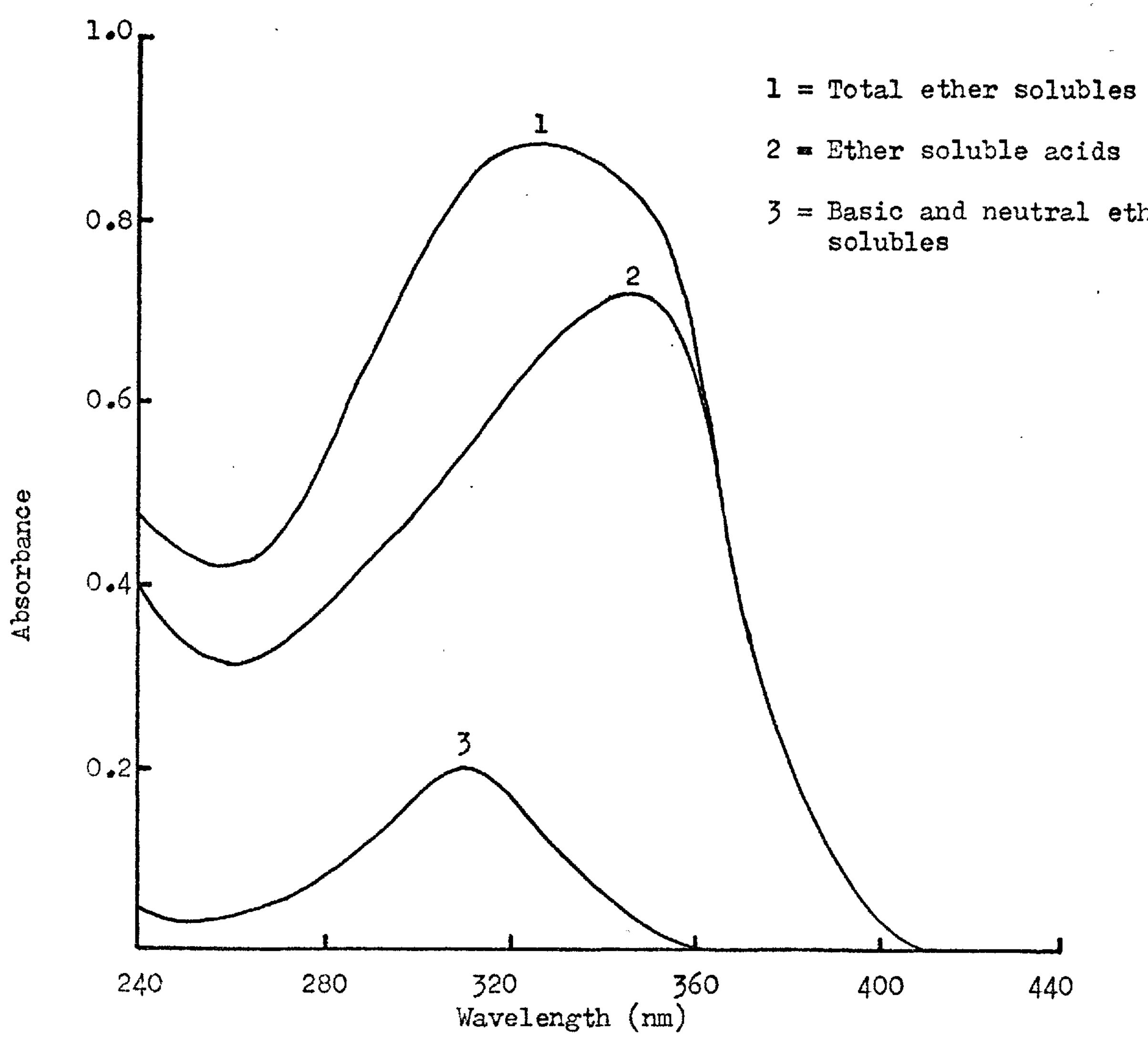
a) Extraction of wyerone acid from B. cinerea pod diffusate

The absorption spectra of various fractions of a diffusate collected 24 h after inoculation of pod seed cavities with a suspension of B. cinerea conidia in sterile distilled water are illustrated in Figure 12.

Paper chromatography of the basic and neutral ether solubles in n propanol/water (12.5/87.5) yielded a band at R_F 0.9 with very pale blue fluorescence under U.V. light. The eluate from this R_F had a peak of U.V. absorbance at 310 nm in both 50%, and absolute ethanol.

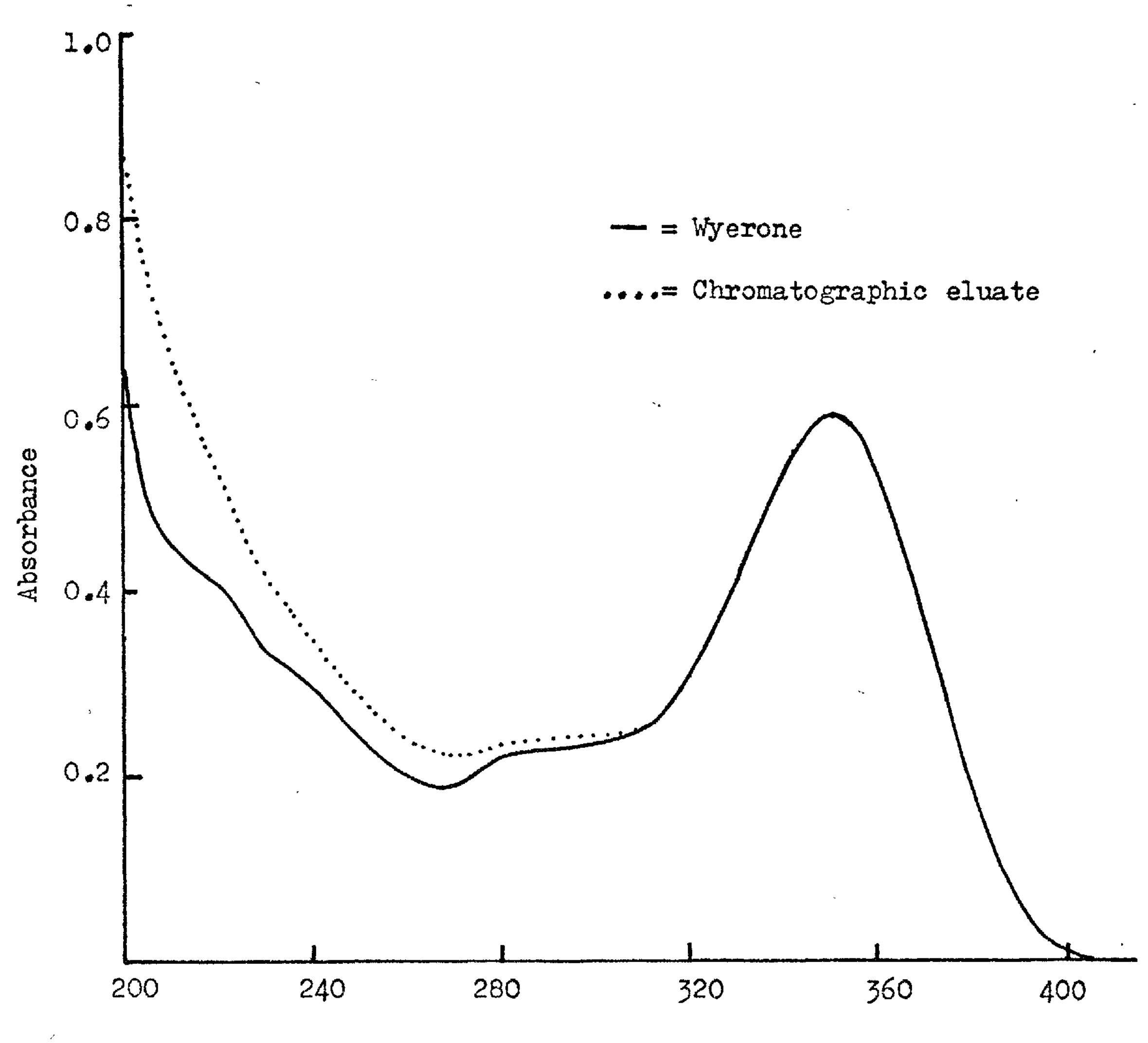
After paper chromatography of the ether soluble acids in the same solvent system a narrow blue fluorescent band was detected under U.V. light at R_F 0.9. This band was eluted. The eluate had a peak of light absorbance at 360 nm in 50% ethanol, and 350 nm in absolute ethanol. This shift in λmax was not associated with any change in absorbance at the respective wavelengths. The 350 nm absorbing substance ran as a single spot to R_F 0.3 on "Whatman 1" chromatography paper developed in 3% aqueous sodium chloride. Similar R_Fs and properties of light absorbance were reported by Deverall (1967) and Deverall and Vessey (1969) for the phytoalexin from broad bean, which also behaved as an ether soluble acid. Wyerone acid was shown to run to R_F 0.9 in the n-propanol/water solvent, and was reported to have an identical U.V. absorption spectrum to that of wyerone by Letcher et al. (1970).

Figure 19. U.V. absorption spectra in ethanol of ether soluble fractions of 24h B. cinerea pod diffusate



(All fractions in a volume of ethanol twice that of the diffusate)

Figure 20. U.V. absorption spectra in ethanol of wyerone, and the eluate from R_f 0.9 in a chromatogram of the ether-soluble acids extracted from 24h B. cinerea pod diffusate developed in n. propanol/water.



The U.V. absorption spectrum in ethanol of the chromatographic eluate of the extract of pod diffusate, was compared with the spectrum of a sample of wyerone kindly provided by Dr. C. H. Fawcett (Figure 20). The absorbance of the two spectra with the same λmax at 350 nm was identical over the range 410 - 310 nm. The extract from diffusate had slightly greater absorbance at wavelengths less than 310 nm. However it was concluded from the close similarity observed between the two spectra, that wyerone acid was the principal light absorbing constituent of the eluate. The yield of wyerone acid from the illustrated diffusate, calculated on the basis of O.D. of the separated compound in ethanol at 350 nm was 10.6 μg/ml.

B. cinerea pod diffusates collected 24 h after inoculation were used as a source of large quantities of wyerone acid. The wyerone acid prepared from the diffusates by solvent partition and paper chromatography, and used in the following experiments was at a similar state of purity as the preparation described in this section.

The absorption spectra of extracts before chromatography and yield of wyerone acid varied with each batch of diffusate. However the principal features of diffusate composition were shown after each extraction; namely: 1. The absorption spectra of the total ether solubles in ethanol had maximum absorbance between 310 - 350 nm. 2. Basic and neutral ether solubles always had a characteristic absorbance maximum at 310 nm. 3. Ether soluble acids had an absorbance maximum between 340 nm and 350 nm. 4. Wyerone acid could be extracted from the ether soluble acids by paper chromatography in n-propanol/water.

i) Extraction of wyerone acid by thin layer chromatography (T.L.C.) from *B. cinerea* pod diffusate

Wyerone acid was prepared for the repeat of an experiment (mentioned in Chapter 8) on the metabolism of wyerone acid by *B. fabae*, by T.L.C. of the ether soluble components of a *B. cinerea* pod diffusate collected 24 h after inoculation.

A sample of the ether extract was applied to a T.L.C. plate prepared in the laboratory, and developed in dichloromethane : methanol (5:1) solvent described by Letcher et al. (1970). The 50% ethanol eluate from a broad band at R_F 0.1 - 0.25 which fluoresced blue under U.V. light was examined in the U.V. spectrophotometer. The eluate had λmax at 360 nm characteristic of wyerone acid, but also a secondary peak of absorbance at 285 nm. No other eluted bands had λmax at 360 nm. It was therefore concluded that wyerone acid, and another U.V. absorbing substance λ max 285 nm were present in the eluate from R_F 0.18. A sample of this eluate was chromatographed on "Whatman 3 MM" paper in n propanol/water. Wyerone acid was successfully separated from the other U.V. absorbing substance by paper chromatography. Samples of the eluate from T.L.C. containing wyerone acid were spotted onto Merck T.L.C. 2.5 mm thick plates, and developed in a range of solvents. It was found that wyerone acid could be separated from the 285 nm absorbing substance using a solvent system of ether : methanol (2:1). Wyerone acid ran to R_F 0.28 - 0.56 and the impurity to R_F 0.65 - 0.83. The R_F of wyerone acid in other solvent systems assayed, but not found to afford separation of the impurity

were as follows: dichloromethane : methanol (2:1), R_F 0.39 - 0.55; chloroform : methanol (1:1) R_F 0.34 - 0.55; and methanol : chloroform (3:1) R_F 0.67 - 0.74. Ether alone separated wyerone acid from the impurity, but the acid ran only to R_F .06 - 0.1.

The bulk of wyerone acid was purified by T.L.C. on Merck 2.5 mm plates in ether : methanol (2:1). On the basis of the U.V. absorption spectrum of the eluate containing wyerone acid it was concluded that the acid was present at a similar state of purity to that recovered from diffusate ether soluble acids by paper chromatography.

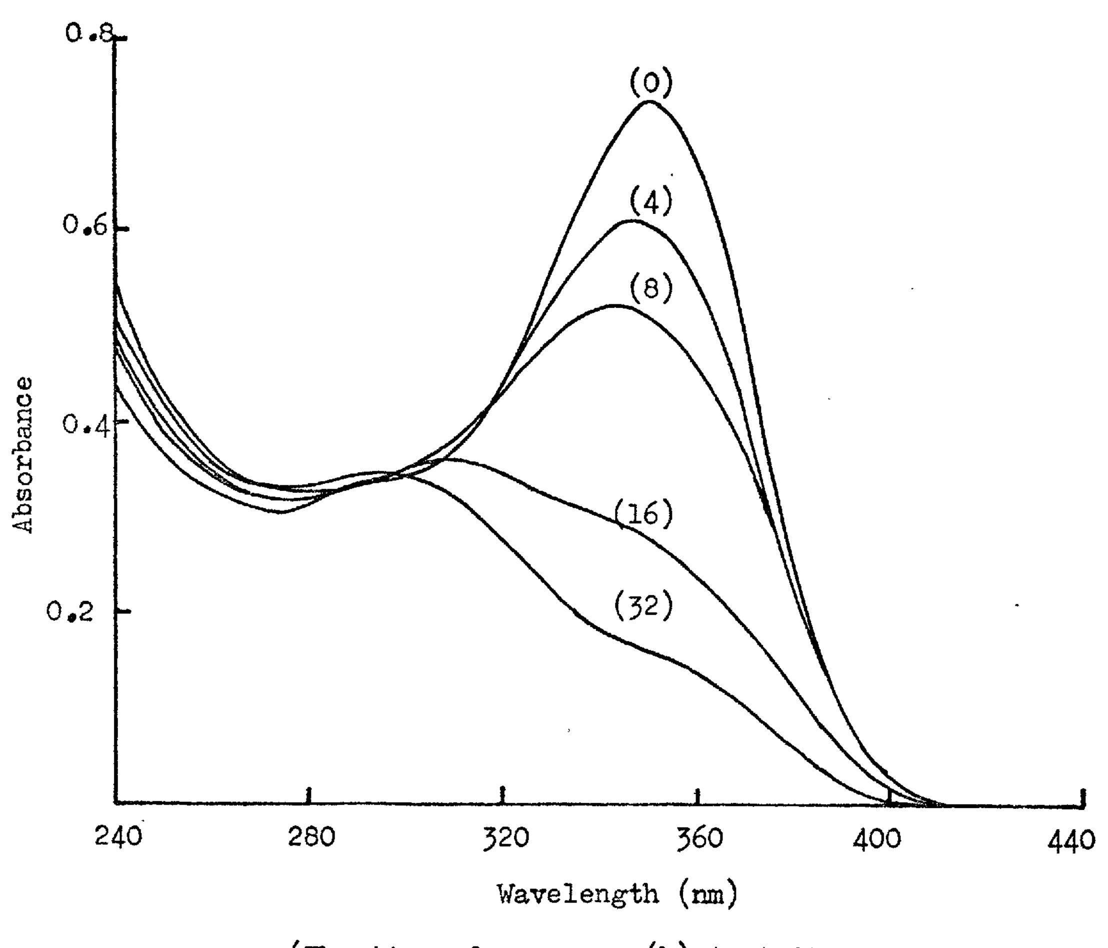
b) <u>The effect of daylight on an ethanolic solution of wyerone acid</u>

Deverall (1967) showed that the exposure of diffusate to daylight resulted in a loss of antifungal activity. Fawcett et al.(1969) reported that there was a considerable loss of wyerone during extraction due to polymerisation caused by the U.V. component of daylight. The effect of daylight on an ethanolic solution of wyerone acid was therefore examined. The change in absorption spectra after exposure is illustrated in Fig. 21. The bioassay results in Table 19 indicate that the observed change of the wyerone acid spectrum was associated with a parallel loss of antifungal activity.

c) <u>Sterilization of a solution of wyerone acid in pod nutrients</u>

Deverall (1967) showed that, despite the loss of antifungal activity from diffusates by exposure to daylight, boiling in the dark caused no such loss. The possibility of sterilizing wyerone acid solutions by autoclaving was therefore examined.

Figure 21. The effect of daylight on the U.V. absorption spectrum of an ethanolic solution of wyerone acid after its exposure to daylight.



(The time of exposure (h) is indicated in parentheses)

Table 19. The effect of the exposure of an ethanolic solution of wyerone acid to daylight on the antifungal activity of the acid against *B. cinerea* conidia

Exposure (h)	Bioassay results + (Mean of 3 replicates)
0	71% *
1	81% *
3	43µm
4	44µm
8	58µm
16	130µm
32	270µm
Control bioassay without wyerone acid	433µm

* Percentage germination unless 98-100%

\+ Germ tube length unless <20µm

A solution of wyerone acid in pod nutrients was bioassayed and extracted with ether before, and after autoclaving for 10 min at 0.7 kg/cm^2. There was no loss of antifungal activity during sterilization and the U.V. absorption spectra of the extracts were identical. It was concluded that solutions of wyerone acid in pod nutrients could be sterilized by autoclaving.

d) Extraction of an added known quantity of wyerone acid from excised healthy leaf discs

It was thought that the characteristic instability of wyerone acid might lead to considerable losses during the extraction of leaf tissues. To examine the extent of such losses a known quantity of the acid prepared from pod diffusate was put through the procedure of extraction from leaf tissues. Forty-five μg of wyerone acid was added to the 80% ethanol homogenate of 200 excised healthy 5 mm diameter leaf discs. The acid was then extracted as described in materials and methods. After chromatography the U.V. absorption spectrum of the processed wyerone acid was qualitatively identical to the original sample but only 30 μg were recovered; a 67% recovery. The recorded yields of wyerone acid in the following results may therefore be an under-estimate of the actual levels within the tissues.

e) The antifungal activity of wyerone acid against *Botrytis*

i) Antifungal activity against conidia

A series of bioassays were carried out with different concentrations of wyerone acid in pod nutrients. The growth of *Botrytis*

in pod nutrients alone is recorded in Table 20. The mean percentage of control germination and length of germ tubes produced by conidia of B. allii, B. cinerea and B. fabae in wyerone acid solutions was calcualted from three replicates and plotted against log. wyerone acid concentration. From the resultant graphs shown in Figure 22 it was possible to calculate the E.D.50 of wyerone acid against germination and measured germ tube growth by conidia. The calculated E.D.50s and the lowest concentration of wyerone acid which prevented all germination are shown in Table 21.

ii) Antifungal activity against mycelial growth

In order to measure mycelial growth, fungi had to be incubated for much longer periods of time than for studies of development by conidia. This long term incubation necessitated sterile techniques. The solutions of wyerone acid in pod nutrients used in the mycelial growth tests were therefore sterilized by autoclaving for 10 min. at 0.7 kg/cm^2.

Figure 23 shows the growth of B. cinerea mycelium estimated as the mean of four replicates for each wyerone acid concentration examined. No growth was observed on agar containing 54 μg wyerone acid/ml.

The E.D.50 for the activity of wyerone acid against mycelial growth by B. cinerea was estimated from the graph of growth rate against $\log_{10}$ wyerone acid concentration (Figure 24). The calculated growth rate on pod nutrient agar alone was 0.357 mm/h. The log. of the E.D.50 could therefore be read off as the log. of the concentration

Table 20. The development of *Botrytis* conidia in pod nutrients

Fungus	% Germination *	Germ tube growth μm*
B. allii	100	283
B. cinerea	100	522
B. fabae	99	479

* Mean of three replicate bioassay droplets

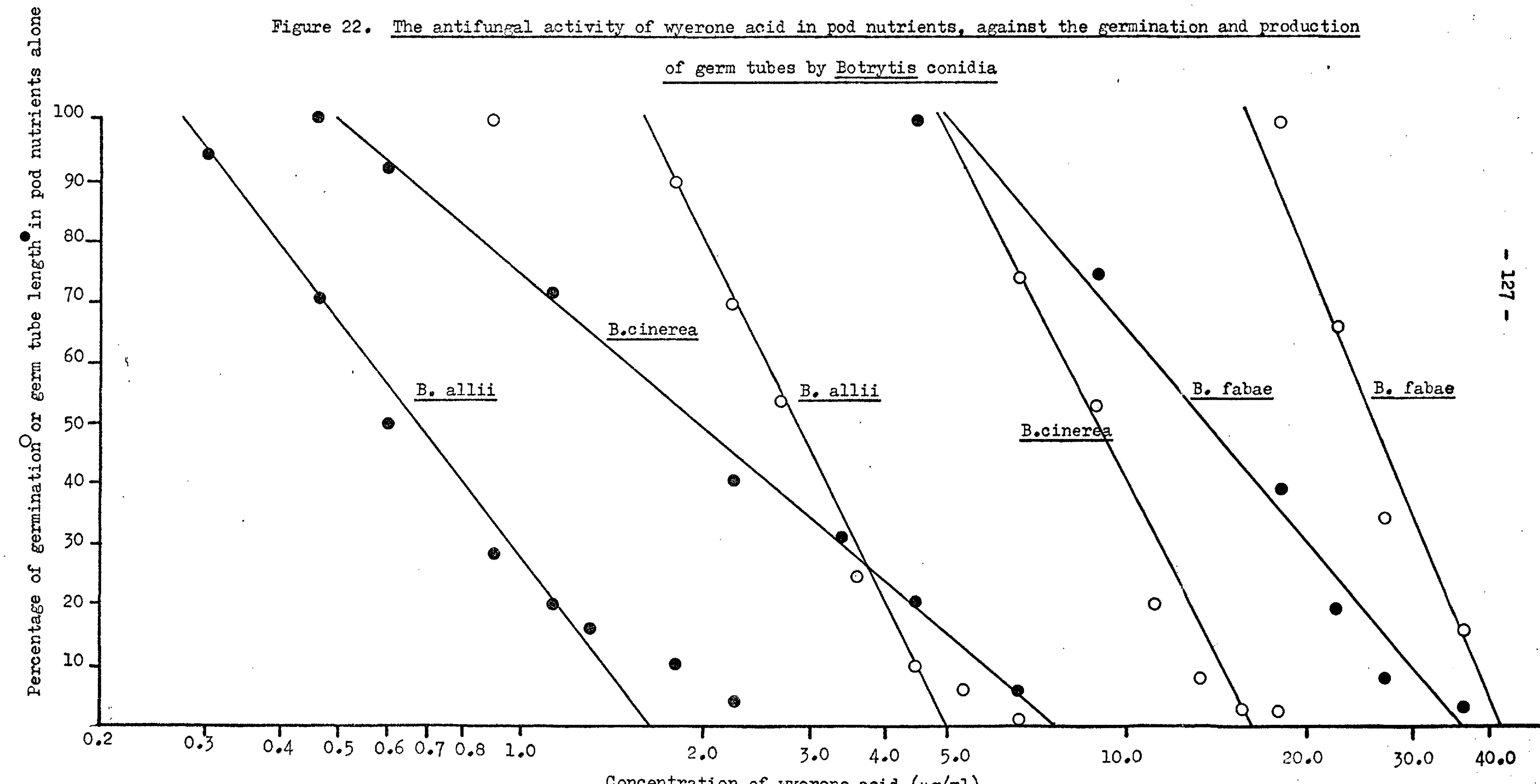


Figure 22. The antifungal activity of wyerone acid in pod nutrients, against the germination and production of germ tubes by *Botrytis* conidia

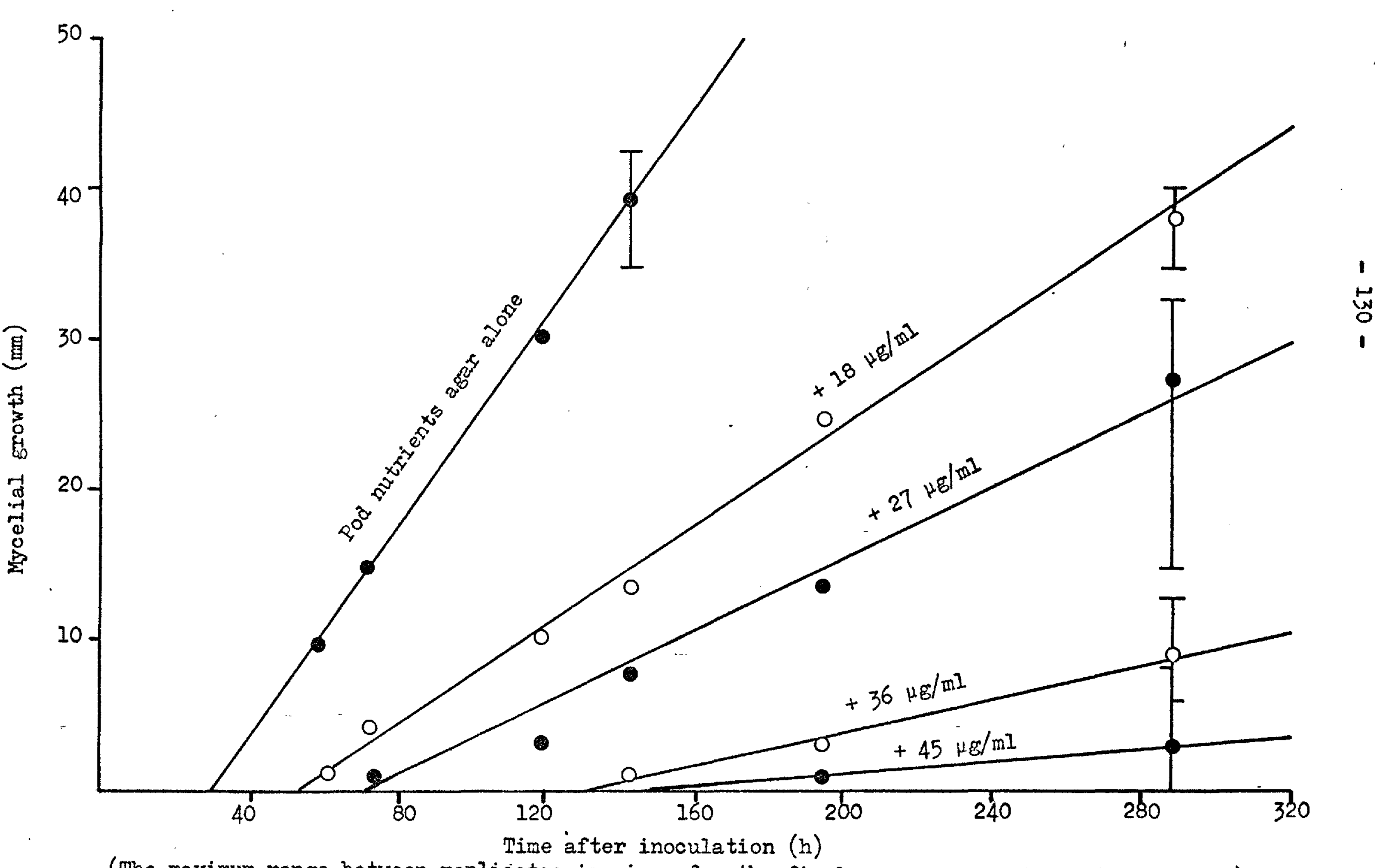
Table 21. Antifungal activity of wyerone acid against *Botrytis* conidia

Activity of wyerone acid(μg/ml)	*B. fabae*	*B. cinerea*	*B. allii*
E.D. 50 against germination	26.0	9.0	2.9
E.D. 50 against germ tube growth	13.5	2.0	0.7
Lowest concentration preventing all germination	45.0	18.0	9.0

allowing a growth rate of 0.1785 mm/h. In this way the E.D.50 was calculated to be 16.5 μg/ml.

In the dark B. fabae developed only sparse aerial mycelium even in the absence of wyerone acid. Quantitative measurement of mycelial growth was not attempted. However the development of a tuft of aerial hyphae clearly visible with the naked eye was recorded. In this way it was possible to record the lowest concentration of wyerone acid which inhibited such mycelial growth. The lowest concentrations at which no growth of B. cinerea or B. fabae was recorded at different times after inoculation are shown in Table 22. Five days after inoculation growth of B. fabae was observed on all concentrations of wyerone acid tested. The level required to inhibit mycelial growth by B. fabae is therefore described as being >54 μg/ml. No growth by B. cinerea was recorded in any of the replicates of pod nutrients agar + 54 μg wyerone acid/ml on the final day of measurement, 12 days after inoculation.

Figure 23. The growth of B. cinerea mycelium in blocks of pod nutrients agar + wyerone acid



(The maximum range between replicates is given for the final measurement of mycelial growth)

Figure 24. Effect of wyerone acid on the growth rate of B. cinerea mycelium in pod nutrients agar

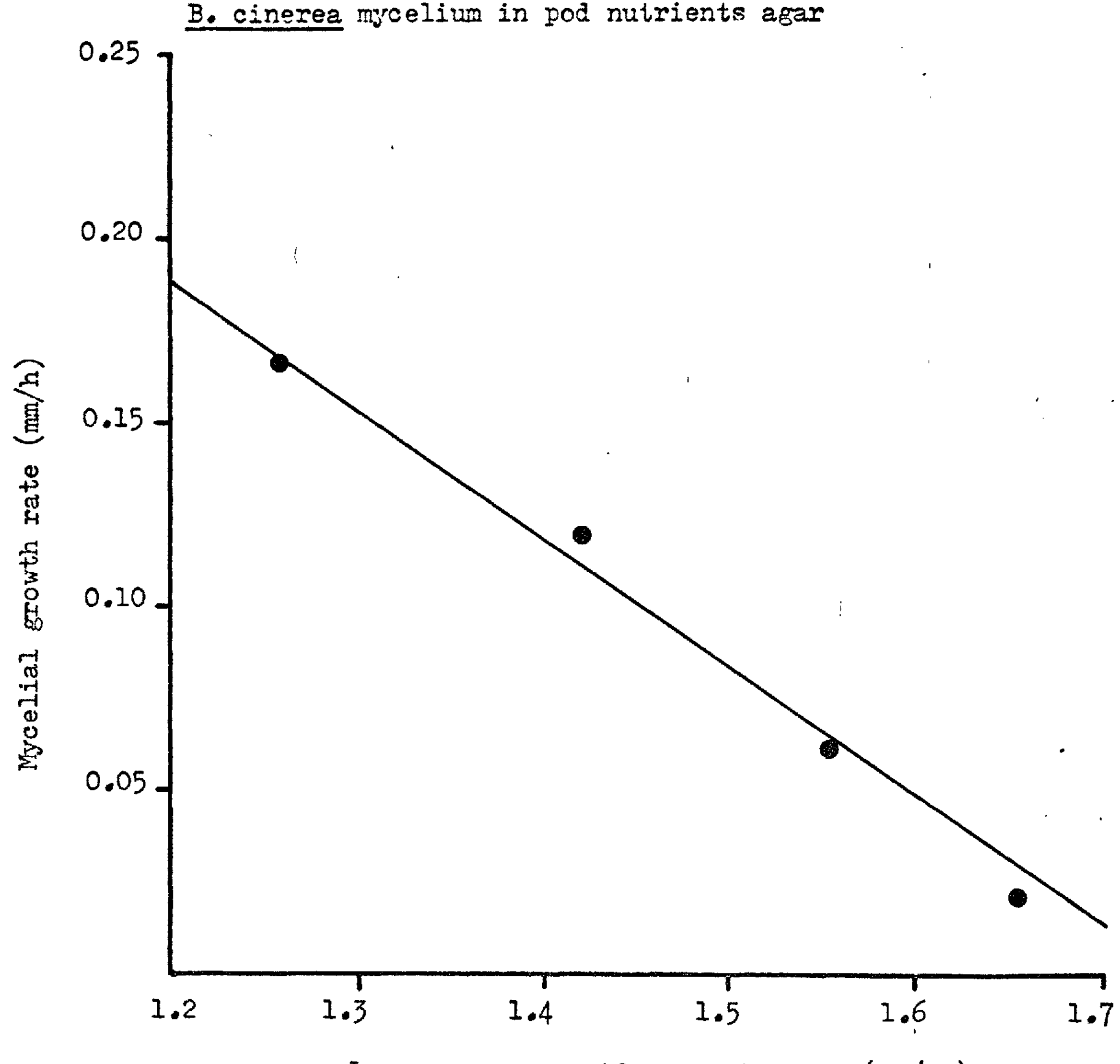


Table 22. Antifungal activity of wyerone acid against mycelial growth by *Botrytis*

Activity of wyerone acid (μg/ml)	Time after inoculation (Days)	*B. fabae*	*B. cinerea*
Lowest concentration which inhibited all mycelial growth	1	36	27
	3	45	36
	5	>54	36
	8	>54	45
	12	>54	54

Chapter 5. The extraction of wyerone acid from leaves before and after fungal infection

a) The extraction of wyerone acid following inoculation with *Botrytis cinerea* in water

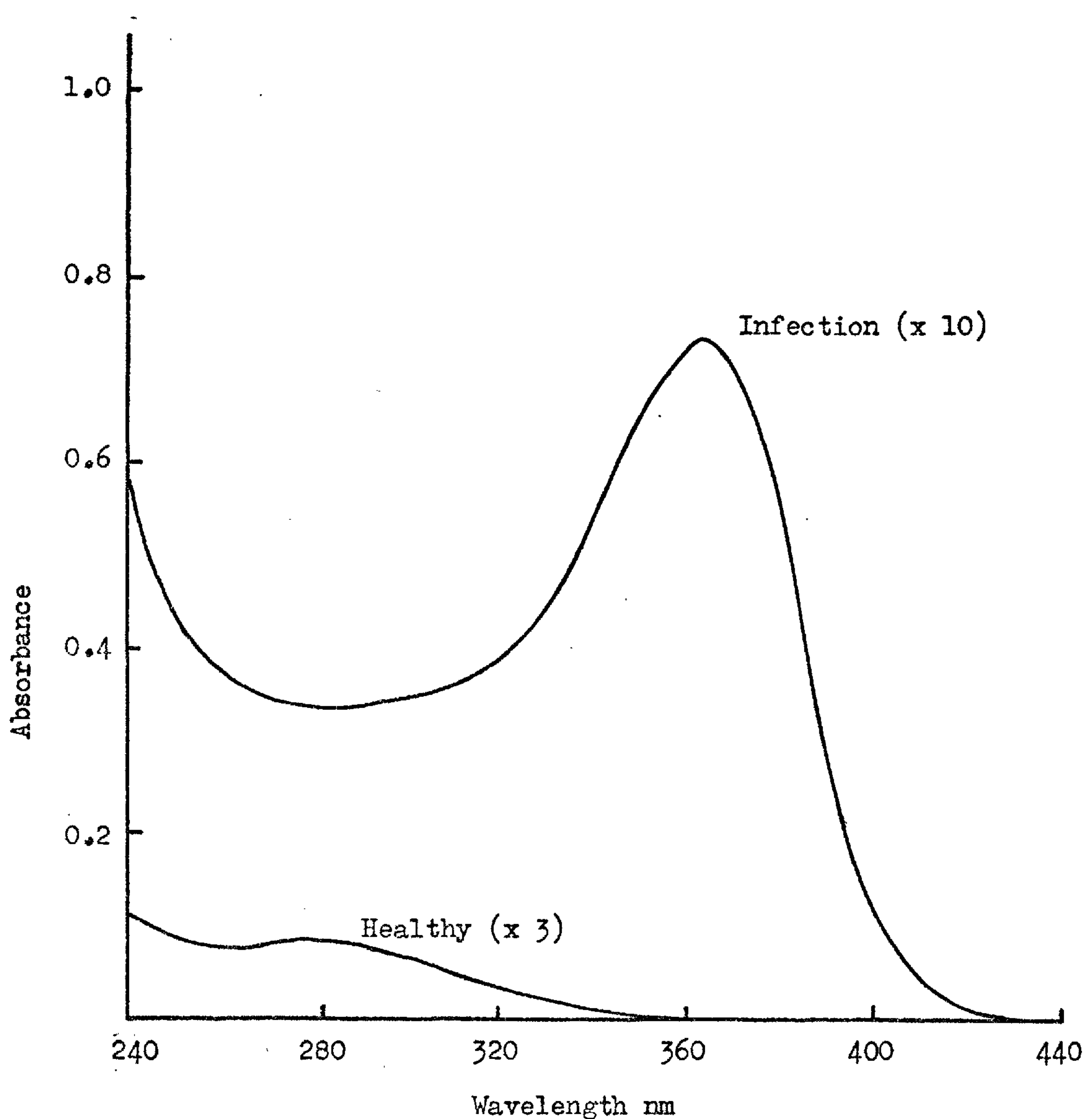
i) After incubation of healthy and infected leaves for 5 days

One leaflet of each of numerous detached leaves was inoculated with *B. cinerea*, the other with sterile distilled water. Five days later the droplets were removed, and the tissues beneath 216 water droplets, and infection droplets which had produced lesions, were cut out for extraction of ether soluble acids as described in "Materials and Methods".

A blue fluorescent band was detected under U.V. light at R_F 0.9 in the paper chromatogram (n-propanol/water) of the extract from infected tissue. The corresponding band extracted from healthy leaves did not fluoresce under U.V. These two bands were eluted in 50% ethanol and examined by U.V. spectrophotometry giving the absorption spectra shown in Figure 25. The peak of absorbance at 360 nm in the 50% ethanol eluate from infected leaves shifted to 350 nm when the eluate was dried and resuspended in ethanol; a characteristic of wyerone acid.

The two eluted fractions were bioassayed in pod nutrients against *B. cinerea*. The fraction from healthy tissue was reconstituted at fresh weight concentration, and that from infected tissue at a concentration (based on the O.D. at 360 nm, assuming that all absorbance

Figure 25. Chromatographic eluates in 50% ethanol, from healthy leaves, and B. cinerea infections.



(The volume of aqueous ethanol in relation to the fresh weight of extracted tissue is indicated in parentheses)

was due to wyerone acid) of 18 μg wyerone acid/ml. In pod nutrients alone, and in the solution containing the extract of healthy tissue, germ tubes grew to 450 μm in length. No conidia germinated in pod nutrients containing the eluate from infected leaves, which was consistent with the presence of wyerone acid at the estimated concentration. The concentration of wyerone acid in the 1.08 g fresh wt. infection sites was calculated from the O.D. to be 60 μg/g. It was concluded that wyerone acid was present 5 days after infection by B. cinerea, at a concentration greater than that which prevented mycelial growth by the fungus in pod nutrients agar for 12 days, and that wyerone acid could not be detected in healthy tissue.

ii) Changes in wyerone acid concentration with time, and symptom development

In Chapter 3 it was shown that the time of cessation of fungal growth varied between infection sites. In order to examine the role of wyerone acid in controlling B. cinerea infection it was considered essential to relate changes in wyerone acid concentration to the development of different grades of infection. Infections developing to grades 19 and 100 after 6 days were described in detail (Chapters 2 and 3), and it was therefore proposed to study the changes in wyerone acid as these infections developed for 6 days. This work necessitated the selection of certain grades, and groups of different grades of infections for extraction. In general these selections represented the minority of inoculations, and therefore, wherever possible examples of the remaining grades of infection were also extracted. The results of

these additional measurements are included in the following sections.

Infections developing to grade 100 after 6 days

This experiment was primarily designed to measure the concentration of wyerone acid in infection droplets and underlying tissues as grade 100 infections developed.

Those infection sites known to be most likely to develop to grade 100 after 6 days (as described in Chapter 2) were excised for extraction 1, 2, 3, and 4 days after inoculation. In addition, when the development of symptoms had virtually ceased after 6 days the concentration of wyerone acid was measured in the various grades of infection which then existed.

Leaves (135) of varying age but without signs of senescence were inoculated with B. cinerea in water. Two batches of 30 leaves were separated for examination 1 and 6 days after inoculation. The other leaves were divided into three groups for examination after 2, 3, and 4 days. Each group contained the same number of infections in each grade after 21 - 23 h as shown in Table 23. In this way homogeneous samples of infections were examined 2, 3, and 4 days after inoculation.

Infections in grade 100 after 6 days developed from lesions in grade 6.5 or higher one day after inoculation (Chapter 2). Diffusates and underlying tissues were separately collected from sites showing infection grades 0, 6.5, and higher on day 1, and from combined sites showing infection grades higher than 19, 38, and 63 on days 2, 3, and 4 respectively. The mean infection grades calculated for the later

Table 23. Number of infection sites showing different infection grades 1 day after inoculation with *B. cinerea*

	Infection grade				
	0	6.5	19	38	63
No. of infections	114	177	72	43	6

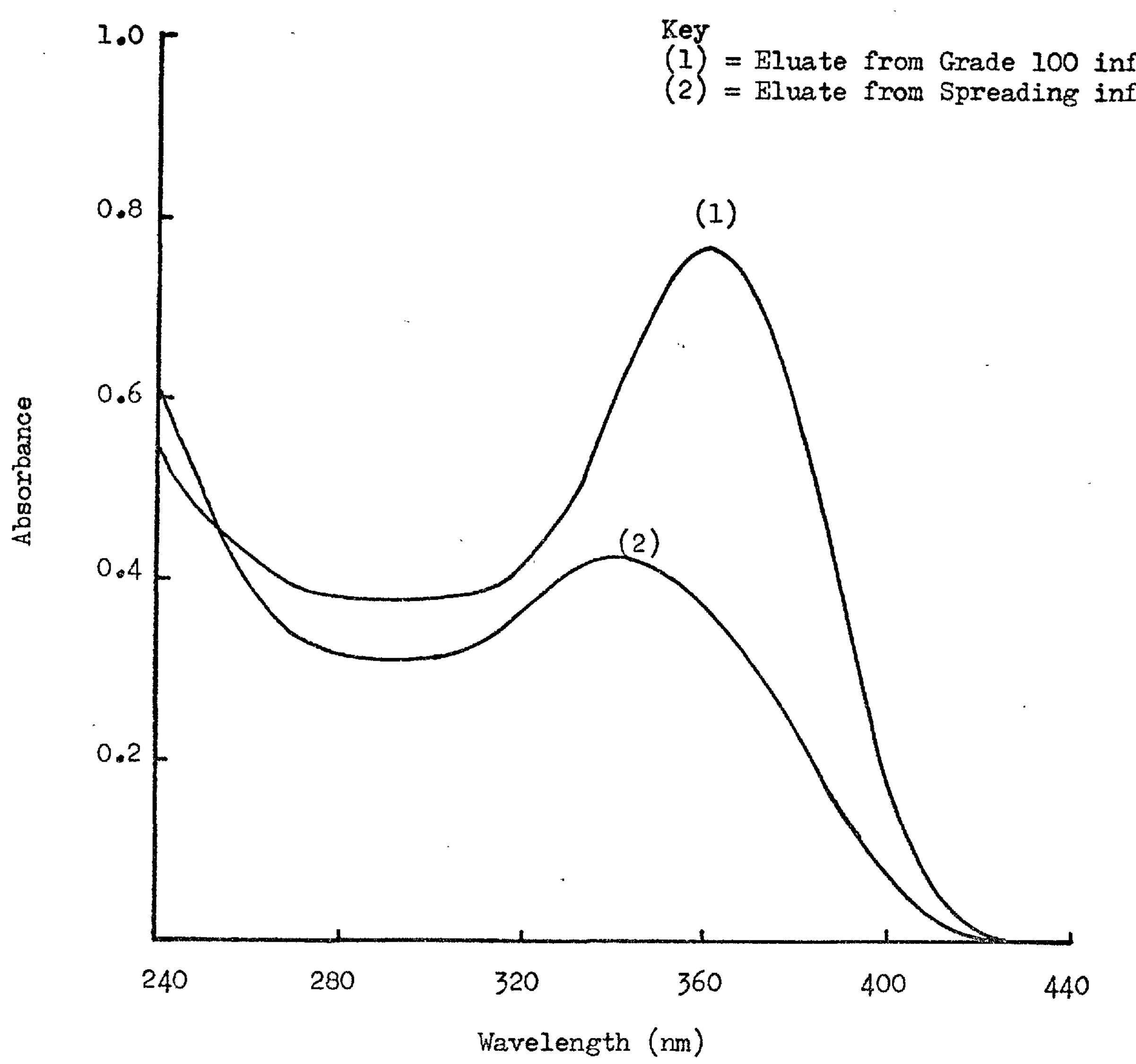
days were similar to the daily mean infection grades calculated for developing grade 100 infections in Chapter 2.

Several sites examined 6 days after inoculation were completely black and from some of these sites the blackening had spread into the surrounding tissues. Microscopic examinations revealed that B. cinerea had colonised this necrotic tissue. After infection droplets had been collected all blackened tissues were excised for extraction as samples of 'spreading infections'.

Wyerone acid was identified by U.V. spectrophotometry in chromatographic eluates prepared from diffusates and most tissue extracts. The absorption spectra of the appropriate eluates from chromatograms of extracts from grade 100 and 'spreading infections', 6 days after inoculation (Figure 26) show the characteristic presence of wyerone acid in the former but not the latter. The eluate from 'spreading infections' had λmax at 335 nm, and it was obviously impossible to know if wyerone acid was present in this extract.

The antifungal activities of solutions of the eluted substances at concentrations of one absorbance units at respective λmax 335 nm or 350 nm per ml of pod nutrients were tested. In pod nutrients alone all conidia germinated, and produced germ tubes about 500 μm long. The eluate from grade 100 infections allowed only 49% germination of conidia, which was characteristic of the calculated amount of wyerone acid. All conidia germinated in the solution of the 335 nm absorbing substance but germ tube growth of 180 μm was markedly less than in the control. This eluate was therefore somewhat inhibitory which could

Figure 26. U.V. absorption spectra in 50% ethanol of chromatographic eluates from extracts fo <u>B. cinerea</u> infected tissues.



have been caused by the 335 nm absorbing compound or by wyerone acid.

Assuming that wyerone acid was the only antifungal compound in the 335 nm absorbing eluate, the concentration of wyerone acid/g. fresh leaf was calculated as follows; using Figure 22 Chapter 4, to estimate the amount of wyerone acid which would have permitted the observed growth.

Yield of λmax 335 nm material	(a)	= 3.2A/g.fresh leaf
Wyerone acid equivalent of antifungal activity of a solution of 1A λmax 335 nm per ml pod nutrients	(b)	= 2.84 μg.(0.314A λ max 350 nm)
$\therefore$ Yield of wyerone acid	(ab)	= 9.1 μg/g.fresh leaf

Therefore this value of 9.1 μg per g.fresh material was considered to represent the maximum possible yield of wyerone acid from 'spreading infections', for comparison with the actual yield of wyerone acid from other infections. (Table 25)

Wyerone acid was present in diffusates (Table 24) collected from sites bearing visible lesions 1 day after inoculation. The highest concentration of acid was measured in diffusates from sites with an infection grade of 77, 3 days after inoculation. Very little, or no wyerone acid was detected in the small volumes of diffusates recovered after 6 days. The pH of diffusates declined as infection progressed, from 5.5 - 6.0 to 4.5 - 5.0 on the second day, but reverted to the original pH, 6 days after inoculation.

In an attempt to provide an estimate of the overall production

Table 24. Concentration of wyerone acid in leaf diffusates

Days after inoculation	Infection grade	pH +	Wyerone acid (µg/ml)
1	0	5.5 - 6.0	0
	6.5	4.5 - 5.0	1.3 x
	30.6*	4.5 - 5.0	9.8
2	35.7*	4.5 - 5.0	32.4
3	76.7*	5.5	58.8
4	86.1*	5.0 - 5.5	55.2
6	6.5	6.0	0
	0	6.0 - 6.5	< 0.1 ml collected
	19	5.5 - 6.0	< 0.1 ml collected
	42.2*	5.5 - 6.0	< 0.1 ml collected
	87.5	5.5 - 6.0	< 0.1 ml collected
	100	5.5 - 6.0	< 0.1 ml collected
	Spreading infections	5.5 - 6.0	< 0.1 ml collected

* Mean infection grade calculated for a mixed group
\+ pH of conidial suspension before inoculation = 5.5 - 6.0
x Estimated from O.D. at 350 nm of ether extraction ethanol

of wyerone acid after infection, a calculation was necessary to express the wyerone acid content of diffusates in terms of g.fresh leaf. This was done as follows: The volume of diffusate collected (a), was recorded. The concentration of wyerone acid per ml of diffusate was measured (b). The total yield of wyerone acid from the diffusate was then calculated (ab), and expressed per g.fresh leaf material. The yield of acid/g.fresh leaf extracted was calculated in the usual way and the total yield of wyerone acid from the infection was calculated as the sum of the yields from tissue and diffusate (Table 25). The yield of wyerone acid was always greater from the tissue than the associated diffusate.

The relationship between the concentration of wyerone acid in infected tissue, and the grade of infection 6 days after inoculation is illustrated in Figure 27. This shows that wyerone acid content increased with darkening infections grade 0 - 100, but the yield was not directly proportional to the infection grade. The completely blackened tissue of 'spreading infections' contained a very low level of wyerone acid.

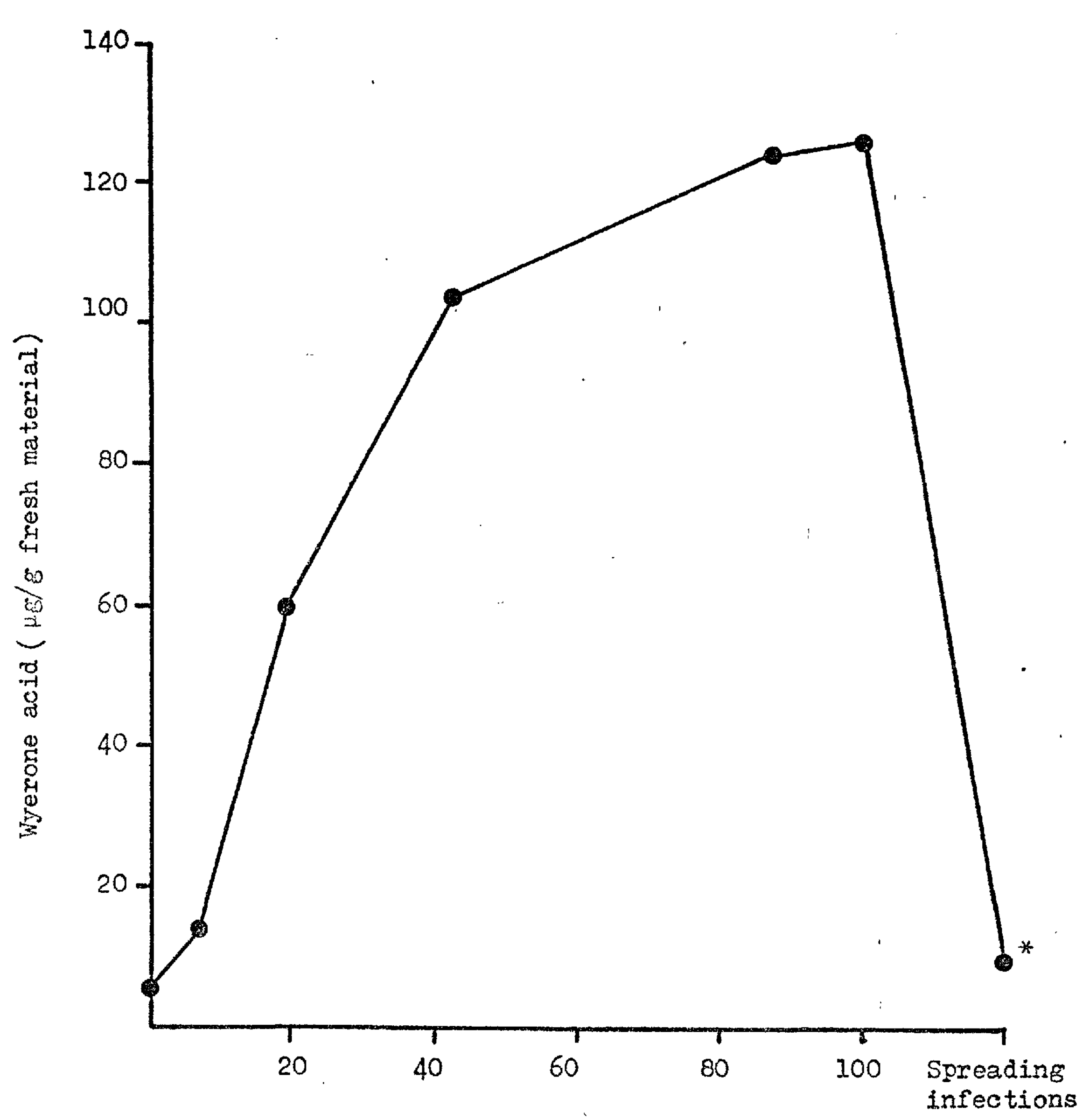
The change in concentration of wyerone acid during the 6 days as infections developed to grade 100 is illustrated in Figure 28. The total yield of acid increased rapidly between 1 and 2 days, reached a maximum after 3 days, and decreased thereafter. The decrease in total yield was caused by a decrease in yield from diffusate after 3 days. There was no net loss from the tissues but a slight increase between the second and sixth day and, in contrast, the percentage browning

Table 25. **Yield of wyerone acid from *B. cinerea* infection sites**

Days after inoculation	Infection grade	Yield of wyerone acid (μg/g. fresh material) Tissue	Diffusate	Total
1	0	1.8	0	1.8
	6.5	12.4	2.5[x]	14.9
	30.6*	31.6	19.1	50.7
2	35.7*	106	53.6	160
3	76.7*	106	62.3	168
4	86.1*	115	41.3	156
6	0	5.7	–[+]	5.7
	6.5	13.6	0	13.6
	19	59.6	–[+]	59.6
	42.2*	104	–[+]	104
	87.5	125	–[+]	125
	100	126	–[+]	126
	Spreading infections	9.1▲	–[+]	9.1

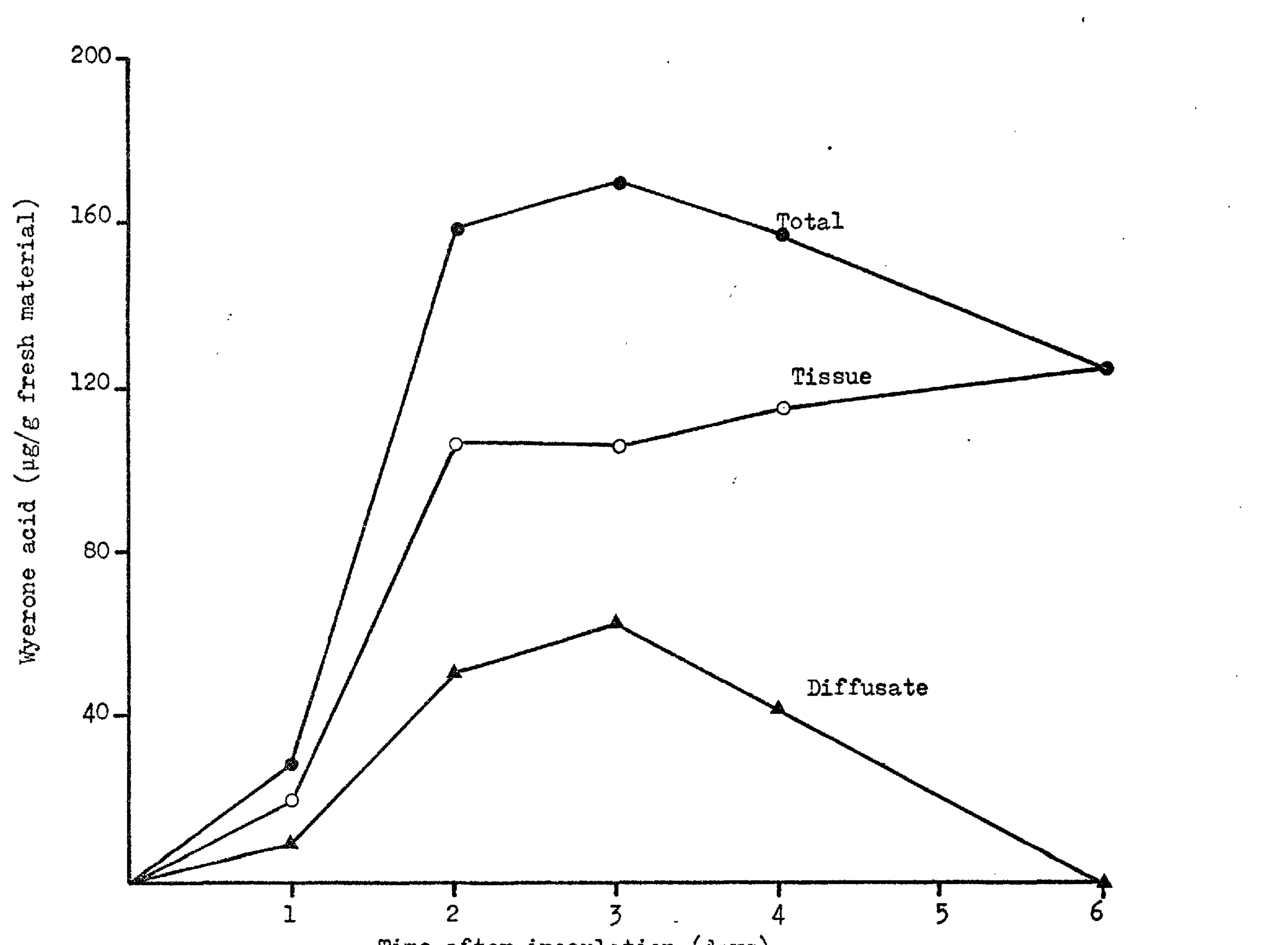
* Mean infection grade calculated for a mixed group
\+ $<$ 0.1 ml diffusate collected
x Estimated from O.D. at 350 nm of ether extract in ethanol
▲ Wyerone acid equivalent of antifungal activity

Figure 27. Yield of wyerone acid from infected tissues in relation to the symptoms produced 6 days after inoculation



(* Wyerone acid equivalent of antifungal activity)

Figure 28. Yield of wyerone acid from *B. cinerea* infections developing to grade 100



increased from 35.7% to 100% during this time. Assuming that no wyerone acid was lost from the tissue, this suggests that wyerone acid production was not confined to brown cells after 2 days. It should also be noted that the concentration of wyerone acid in tissue infected after the second day was more than twice that which inhibited all mycelial growth by B. cinerea in pod nutrients agar.

Infections developing to grade 19 after 6 days

The concentration of wyerone acid in grade 19 infections was measured 6 days after inoculation in the previous experiment. In the experiment (Chapter 2) in which continuous grading of B. cinerea infections was carried out, 21 of the lesions examined developed to grade 19 infections 6 days after inoculation. After 1 day 17 of these infections were grade 6.5, and 4 grade 0. The concentration of wyerone acid associated with 0 and 6.5 grade infections after 1 day are given in Table 25. A mean yield of wyerone acid from 1 day old developing grade 19 infections could therefore be calculated from these results. The calculation of the yield of wyerone acid from infected tissues was carried out as follows:

Infection grade	No. of lesions (a)	Yield of wyerone acid (b)	(ab)
0	4	1.8	7.2
6.5	17	12.4	210.8
	$\Sigma a = 21$		$\Sigma ab = 218$

$\therefore$ Mean yield of wyerone acid/g. fresh leaf tissue $= \frac{\Sigma ab}{\Sigma a} = 10.4\,\mu g$

The yield from diffusate/g. fresh leaf material was calculated in the same way. The concentrations of wyerone acid in developing grade 19 infections 1 and 6 days after inoculation are included in Figure 6.

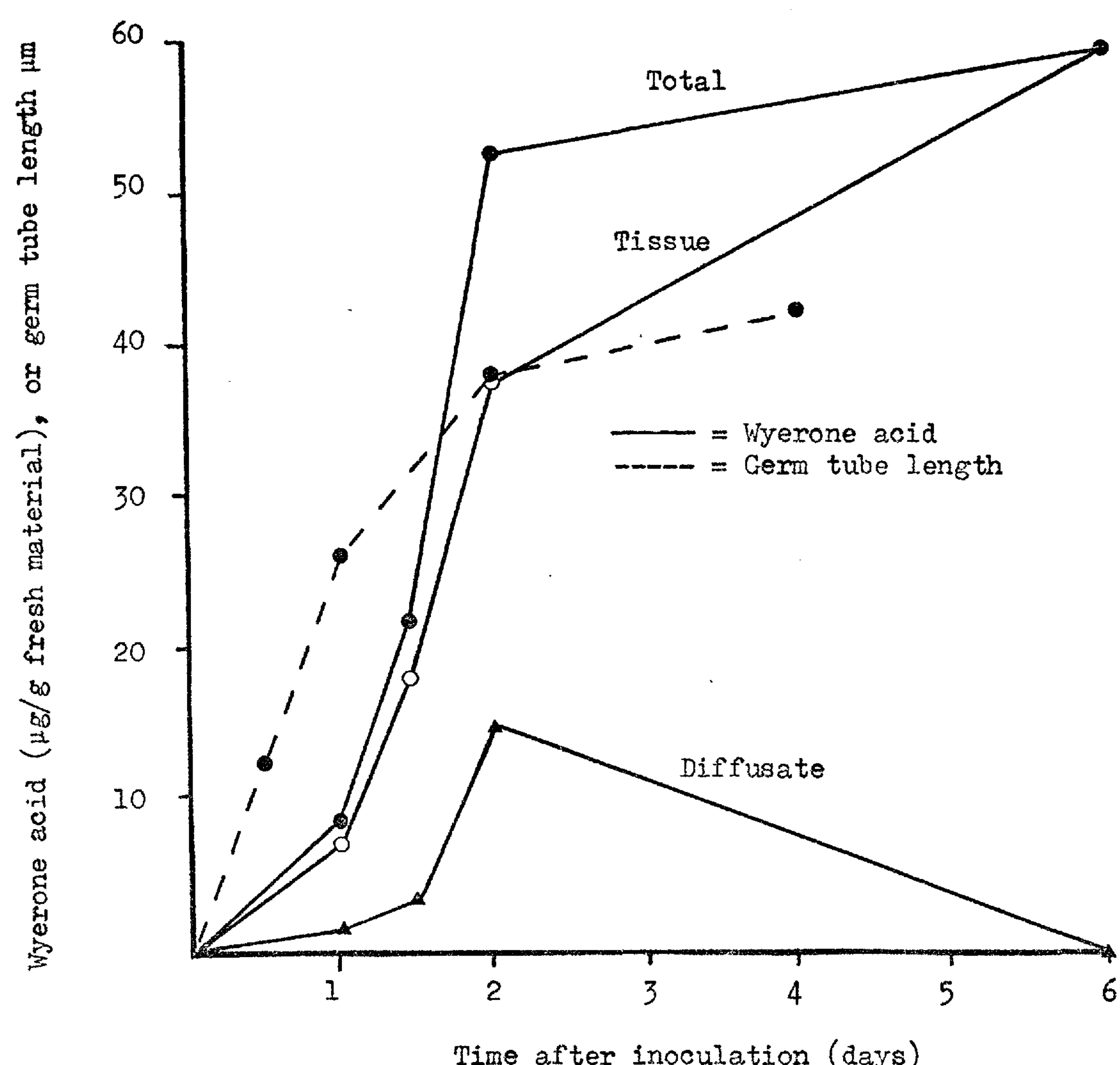
It was shown in Chapter 3 that the growth of germ tubes on the leaf surface over developing grade 19 infections ceased between 24 and 48 h after inoculation. An experiment was therefore designed to estimate the content of wyerone acid in developing grade 19 infections 36 h and 48 h after inoculation. These infections were shown in Chapter 2 to have a mean infection grade of 10.25 after 48 h; similarly, from the graph in Figure 9 it was estimated that 36 h after inoculation the mean infection grade was 8.5. For the purpose of this experiment it was assumed that grade 9.5 infections were representative of potential 6 day grade 19 infections, 36 h and 48 h after inoculation. One batch of leaves was examined after 36 h, and the other after 48 h. At each time grade 0, 3.5, and 9.5 infections were collected for extraction. The yields of wyerone acid recovered are recorded in Table 26. Wyerone acid was detectable in infections without microscopically visible symptoms, but highest concentrations were recovered at each time from infections in which most browning had developed (grade 9.5). There was a rapid increase in wyerone acid content in developing grade 19 infections between 36 h and 48 h after inoculation.

The yields of wyerone acid from developing grade 19 infections are expressed graphically (Figure 29) together with the increase in germ tube length associated with this type of lesion development (from Figure 18). The rapid increase in wyerone acid content between 24 and

Table 26. Yield of wyerone acid from *B. cinerea* infection sites

Time after inoculation h	Infection grade	Yield of wyerone acid (μg/g fresh leaf material)		
		Tissue	Diffusate	Total
36	0	1.0	0	1.0
	3.5	1.9	0	1.9
	9.5	18.5	3.6	22.1
48	0	2.1	0	2.1
	3.5	7.0	0	7.0
	9.5	38.0	14.9	52.9

Figure 29. Calculated yield of wyerone acid from, and germ tube growth on the leaf surface above, developing *B. cinerea* grade 19 infections.



48 h after inoculation coincides with the cessation of mean germ tube extension on the leaf surface; that is when the majority of hyphae probably penetrated the epidermis. This suggests that wyerone acid accumulated rapidly after penetration, and was therefore probably responsible for the inhibition of growth of invading hyphae.

The apparent loss of yield of wyerone acid from diffusates 2 - 6 days after inoculation was reflected by an almost identical increase in the concentration in the tissues. Thus, similar values were recorded for total yield 2 and 6 days after inoculation. This suggests that as infection droplets dried out from 2 - 6 days, wyerone acid was precipitated onto the leaf surface; and indicates that wyerone acid production had virtually ceased by 2 days after inoculation. The amount of browning at the infection site almost doubled between 2 and 6 days. This might suggest that wyerone acid was not confined to brown cells on the second day after inoculation.

iii) <u>The concentration of wyerone acid in and around grade 100 infections 6 days after inoculation</u>

In the experiment described in Section (ii) it was found that 6 days after inoculation the content of wyerone acid at the infection site was not directly proportional to the amount of cell browning. This suggested that high concentrations of wyerone acid were present in green apparently healthy cells, as well as in discoloured cells. In order to investigate the content of wyerone acid in healthy cells surrounding brown tissue it was decided to measure the concentration of acid in and around grade 100 infections 6 days after inoculation.

Table 27. Concentration of wyerone acid in and around limited *B. cinerea* lesions

Days after inoculation	Infection grade	Wyerone acid (µg/g fresh material)		
		Lesion	Periphery 1	Periphery 2
6	100	119	30.1	2.7

Twenty-five leaves were inoculated with droplets of conidial suspension in the usual way. After 6 days grade 100 infections were selected, and the lesion and two surrounding approx. 1 mm wide rings of tissue were cut out and extracted. Due to the inaccuracy of the cutting technique the first peripheral ring bore some brown tissue, the second ring was apparently healthy. The concentrations of wyerone acid extracted were measured from the characteristic absorption spectra of chromatographic eluates obtained, and are recorded in Table 27. Wyerone acid was present in the healthy tissue surrounding the lesions, but at a much lower concentration than within the infected tissue. This suggested that the highest concentrations of wyerone acid were probably further localized within the lesion, in brown tissue or adjacent healthy cells, and indicates that the concentration of wyerone acid to which B. cinerea hyphae were exposed was possibly higher than that extracted from the excised infection site.

b) The extraction of wyerone acid following inoculation with B. fabae in water

In order to measure the concentration of wyerone acid in, and around, B. fabae infection sites, I used the technique of cutting out the infection site and two 1 mm wide peripheral rings of tissue. In this way the effects of spread of infection to the lower leaf surface and of further lateral development on the concentration of wyerone acid within the leaf could be examined. Lesions at similar stages of development were selected 22, 64 and 88 h after inoculation.

Ether soluble acids were extracted and chromatographed. Eluates

from the band at R_F 0.9 which fluoresced blue under U.V. light had qualitatively different absorption spectra. The spectra of eluates derived from extracts of infection sites on the first day, immediately surrounding tissue on the third, and the outer periphery on the fourth day, were characteristic of wyerone acid (Figure 30-2). Absorption spectra of the other eluates, except those derived from extracts of completely blackened tissue after 88 h, had a pronounced peak at 360 nm which was thought to indicate the presence of wyerone acid, but there was also a second peak at 300 nm (Figure 30-1 and 3). It appeared that a compound absorbing light at λmax 300 nm was running to the same R_F as wyerone acid. In the extract from the completely blackened infection site the major peak of absorbance was at 300 nm (Figure 30-4). The low shoulder of absorbance at 360 nm might indicate the presence of a trace of wyerone acid.

The eluates derived from extracts of the infection site on the third day, and surrounding 1 mm ring on the fourth day (Figure 30-1 and 3), were bioassayed against B. cinerea at an estimated concentration (based on absorbance at 360 nm) of 18 μg wyerone acid/ml. In pod nutrients alone conidia developed germ tubes about 500 μm long. There was no growth in the presence of either eluate. The activity of the eluates was therefore consistent with the presence of the estimated concentration of wyerone acid. Therefore, despite the presence of the 300 nm absorbing substance the concentration of wyerone acid in these eluates was measured from the absorbance at 360 nm in 50% ethanol, assuming that wyerone acid was alone responsible for the absorbance

Figure 30. U.V. absorption spectra in 50% ethanol of chromatographic eluates of tissue extracts from in and around _B. fabae_ infection sites.

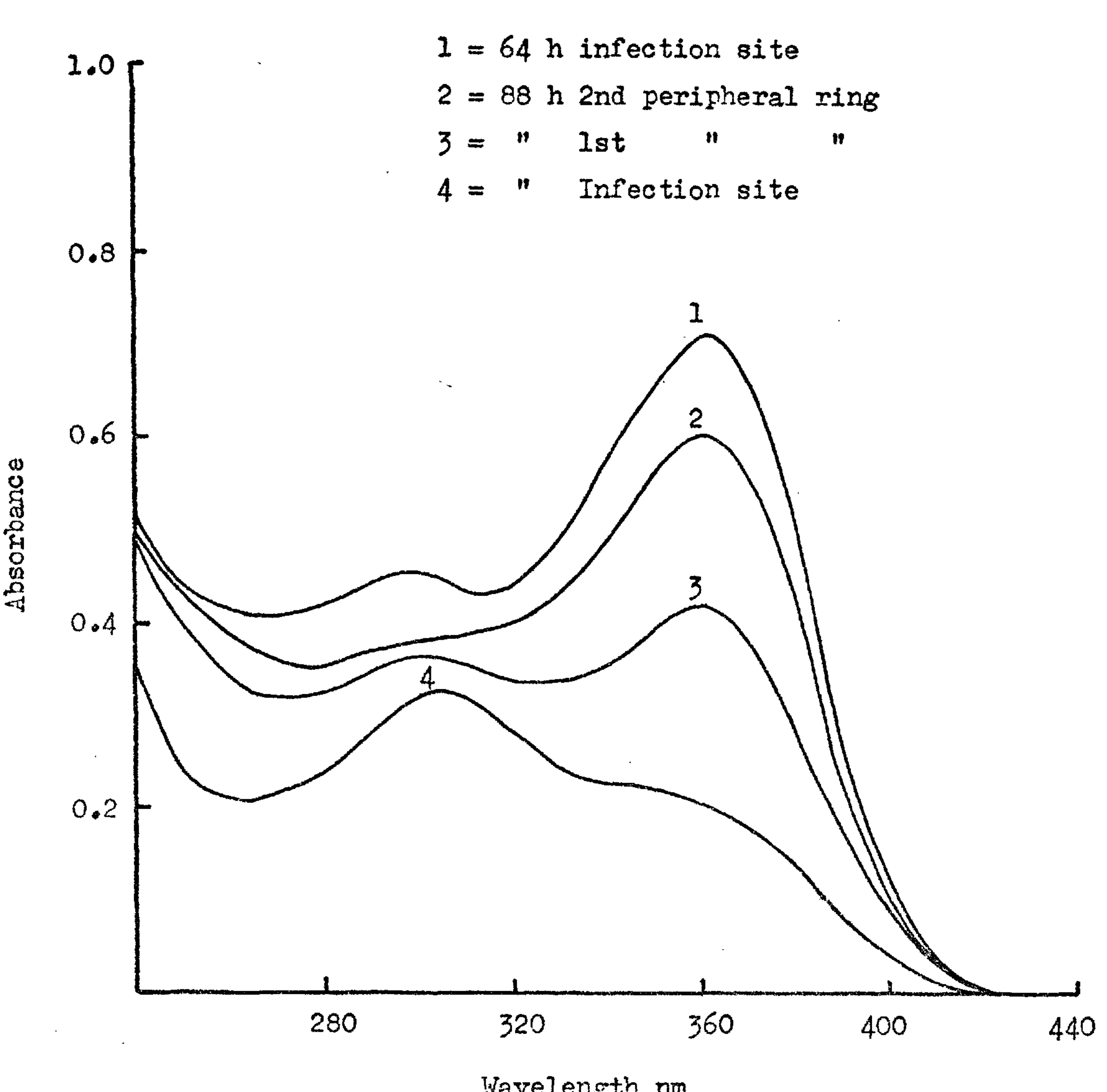


Table 28. Concentration of wyerone acid in and around B. fabae infection sites

Hours after inoculation	Appearance of leaf surface		Wyerone acid (μg/g fresh material)		
	Upper	Lower	Infection site	1st periphery	2nd periphery
22			8.8	3.3	-
64			30.7	47.5	16.5
88			< 7.6	18.0	21.0

of eluates at 360 nm.

The concentration of wyerone acid calculated in this way and the appearance of the lesions are recorded in Table 28. The yield of U.V. absorbing material in the eluate from the black centre of the lesion on the fourth day was too low to permit accurate measurement by spectrophotometry or bioassay. As there was a shoulder, not a peak of absorbance at 360 nm the concentration of wyerone acid in this tissue is shown as less than the estimated value in Table 28.

The following conclusions were drawn:-

Wyerone acid was present in healthy tissue surrounding the lesion. The partial blackening of the tissue at the infection site and in the surrounding peripheral tissue was accompanied by an increase in wyerone acid. There was a decrease in wyerone acid as these tissues became black and invaded by B. fabae. This suggested that B. fabae might metabolize wyerone acid in vivo.

A second experiment was designed to measure concentrations of wyerone acid in infection droplets, infection sites and two 1 mm wide peripheral rings of tissue, at four stages of lesion development. A third peripheral ring was excised at the final stage of infection examined. The infections were selected for extraction when: 1) The infection site was flecked black in the upper leaf surface, 18 h after inoculation 2) The upper leaf surface at the infection site was completely blackened, and the opposite leaf surface flecked black, after 46 h 3) The infection site and first peripheral ring were completely blackened, and the second periphery partly infected, after 85 h

4) The infection site and first and second peripheral rings were completely blackened, and the third ring partly infected, after 116 h. In order to investigate the possibility that aging of leaves after inoculation might reduce the ability of tissues to produce wyerone acid, control leaves were incubated in the growth cabinet and were inoculated when the original infections had reached the fourth stage of development. These control infections were extracted 36 h after inoculation when lesions were at the second stage as in the original infections.

Eluates derived from diffusates and tissues extracted 18, and 46 h after inoculation either possessed U.V. absorbance characteristic of wyerone acid or did not absorb light at 360 nm. Wyerone acid was recognised by U.V. spectrophotometry in eluates derived from diffusates and outer peripheries excised after 86 h and 116 h, when infection sites and inner peripheries yielded comparable eluates with λmax 300 nm. The U.V. absorption spectra of the latter (Figure 31-1) were similar to those previously recorded from completely blackened infection sites produced by B. fabae in 88 h. The second periphery extracted after 116 h yielded an eluate from R_F 0.9 with light absorbing properties different from any eluate previously examined (Figure 31-2). The eluates in which recognition of wyerone acid by spectrophotometry was impossible due to presence of other U.V. absorbing compounds were bioassayed against B. cinerea. The wyerone acid equivalents in terms of antifungal activity extracted per g. fresh material were calculated to enable quantitative comparison with the concentrations of wyerone

Figure 31. U.V. absorption spectra in 50% ethanol of eluates from R_f0.9 in chromatograms of ether soluble acids extracted from 1 mm peripheral rings of tissue surrounding the infection site 116 **h** after inoculation with B. fabae conidia in water.

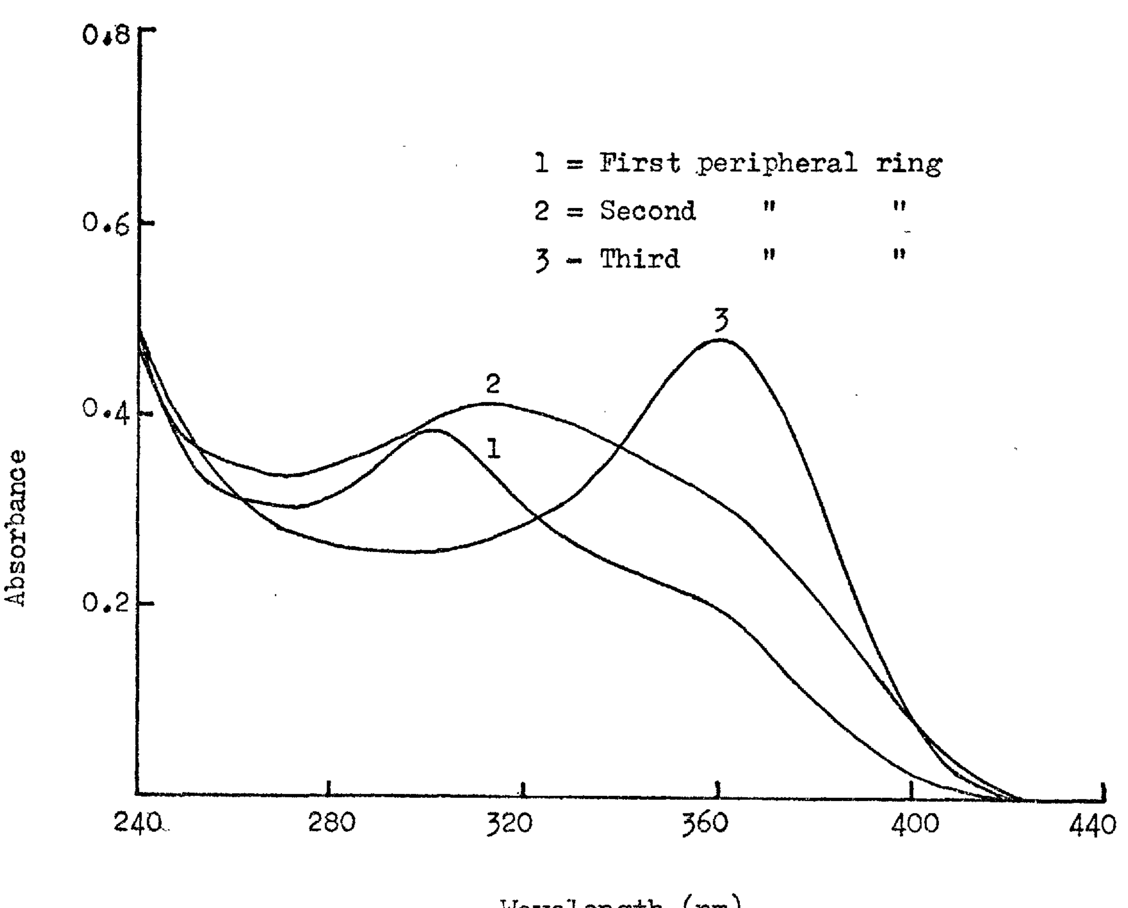


Table 29. Yield of wyerone acid from infection sites and surrounding peripheries after inoculation with *B. fabae*

Time after inoculation(h)	Appearance of upper leaf surface	Source	Yield of wyerone acid(μg per.g.fresh leaf tissue) Tissue	Diffusate	Total
18		Infection site	5.4	0	5.4
		1st periphery	0	-	-
		2nd "	0	-	0
46		Infection site	74.8	9.0(12.3)△	83.8
		1st periphery	30.0	-	30.0
		2nd "	5.7	-	5.7
48 (delayed inoculation)	As above	Infection site	72.3	8.0(10.0)△	80.3
		1st periphery	39.9	-	39.9
85		Infection site	6.0*	2.3(4.5)△	8.3
		1st periphery	10.1*	-	10.1
		2nd "	20.4	-	20.4
116		Infection site	5.8*	-+	5.8
		1st periphery	8.9*	-	8.9
		2nd "	7.3*	-	7.3
		3rd "	24.8	-	24.8

* Wyerone acid equivalent of antifungal activity

\+ Insufficient diffusate collected for extraction

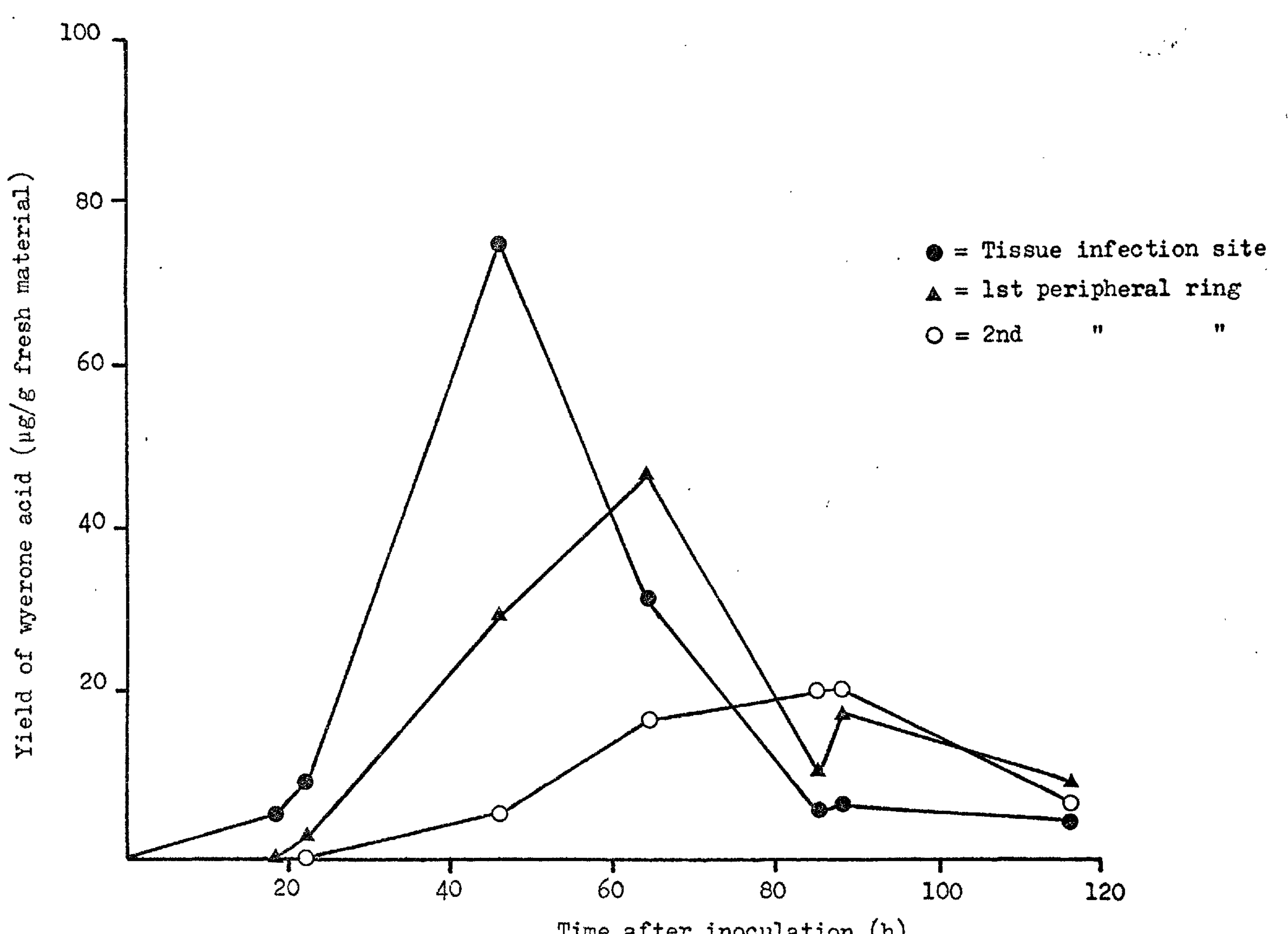
△ Yield of wyerone acid per ml. of diffusate in parentheses

acid extracted from other tissues. The calculated yields of wyerone acid from the infections and their appearance are given in Table 29.

i) Detection of antifungal ether soluble acids in *B. fabae* infections

Previous workers failed to detect antifungal compounds in infected leaves other than that which was subsequently identified as wyerone acid (Deverall, et al, 1968). It was decided to test their results to some extent by investigating the activity of bands of antifungal compounds in a chromatogram of ether soluble acids extracted from partially blackened infection sites collected 64 h after inoculation (see the first experiment described in this section). Extract from 0.64 g. fresh material was applied at a rate of 0.04 g/cm originto the paper which was developed until the solvent front was 18.6 cm above the origin. Before examination under U.V. a 0.5 cm wide vertical strip was cut from 0.6 cm below the centre of the origin to the solvent front. This strip was cut into 1 cm long pieces and bioassayed as described in the "Materials and Methods" section, so that substances on the segments were bioassayed as fresh weight concentration. In the segment from 15.6 - 16.6 cm above the origin only 4% of conidia germinated and these produced short germ tubes. In the control and all other segments germination was between 98 and 100% and germ tube length between 400 and 500 μm. Examination under U.V. revealed that the active segment coincided with a band of blue fluorescence, typical of wyerone acid, from 15.9 - 16.5 cm above the origin, R_F 0.92. It was concluded that wyerone acid was the only antifungal ether soluble acid which could be detected by bioassay of

Figure 32. Change in the concentration of wyerone acid* in tissue at and around *B. fabae* infection sites



(* All values $<$ 12 µg after 80 h are estimates from antifungal activity)

segments of the developed chromatogram.

The following conclusions were drawn:

Delayed inoculation had no effect on lesion formation or wyerone acid productivity. Wyerone acid was present in healthy tissue surrounding lesions, but the highest yield was obtained from the infection site on the second day. Much higher yields were obtained from tissues than from overlying diffusates particularly on the second day. The low concentration of wyerone acid in diffusates and in completely blackened tissue again suggested that _B. fabae_ metabolized the phytoalexin _in vivo_.

The changes in wyerone acid concentration in the tissues at infection sites, and in surrounding peripheries are compared in Figure 32. Yields of wyerone acid were lower from peripheral tissues than from infection sites. The implications of the lower productivity of successive peripheral rings will be considered more fully in the discussion.

c) _The extraction of wyerone acid from naturally infected field beans_

It was considered important to seek wyerone acid in naturally infected beans. Examples of the material collected are shown in Plates 28 - 31. These plates illustrate the difference between the grades of infection; limited lesions 1, and 2, and spreading lesions. Pieces of healthy leaves, and leaves bearing limited lesions, selected for even lesion distribution were cut out. Spreading lesions and two peripheral rings of tissue (approx. 2 mm wide) were also excised, for

Plate 28. Upper leaf surface of naturally infected field beans. The leaf on the left bears predominantly Limited lesion 1, and on the right Limited lesion 2, type infections.

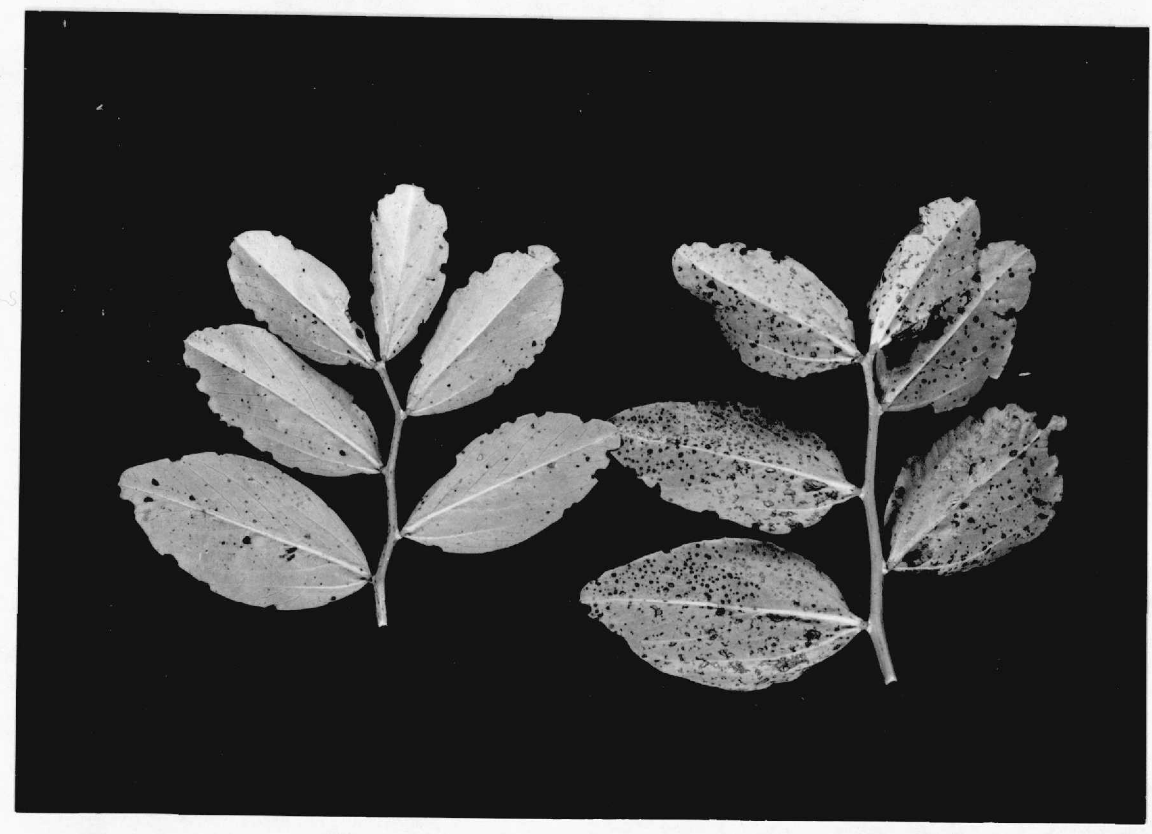


Plate 29. The lower leaf surface of leaves (from plate 28) bearing limited lesion 1 (leaf on the left), and limited lesion 2 (on the right) type infections.

Plate 30. Upper leaf surface of naturally infected field beans. The leaves bear both limited, and spreading lesions.

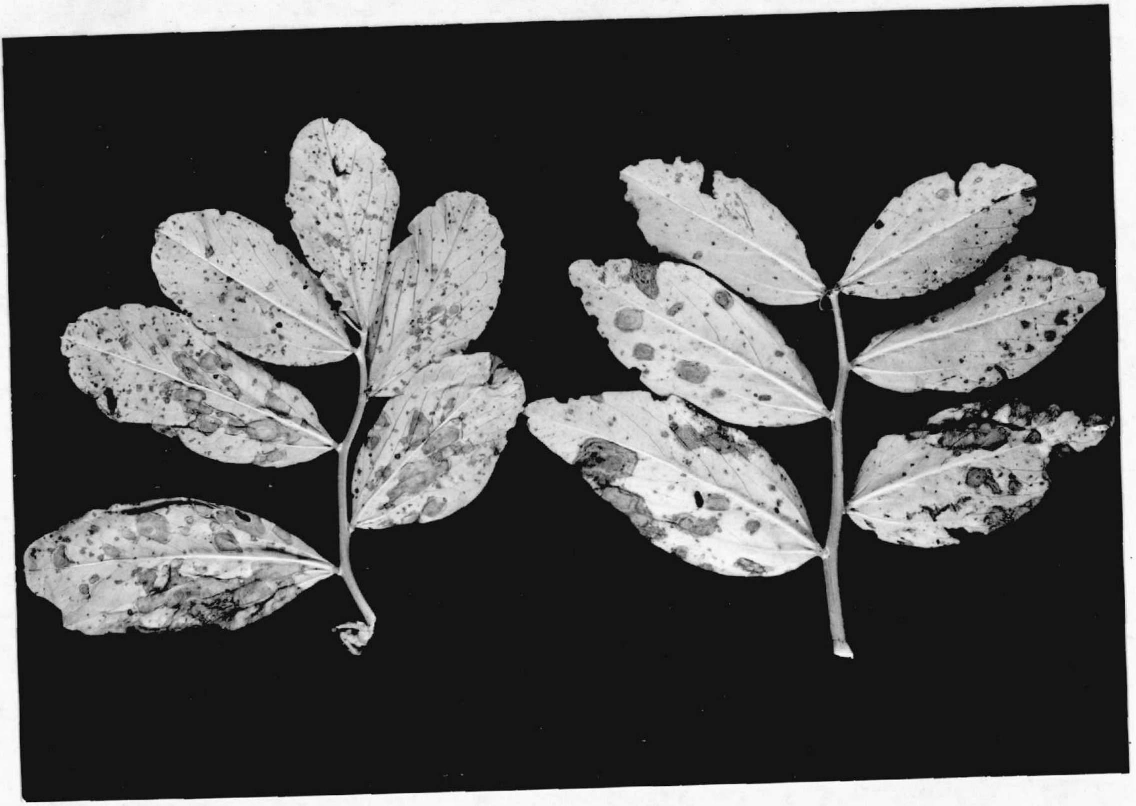


Plate 31. Lower leaf surface of leaves (from Plate 30), bearing both limited and spreading lesions.

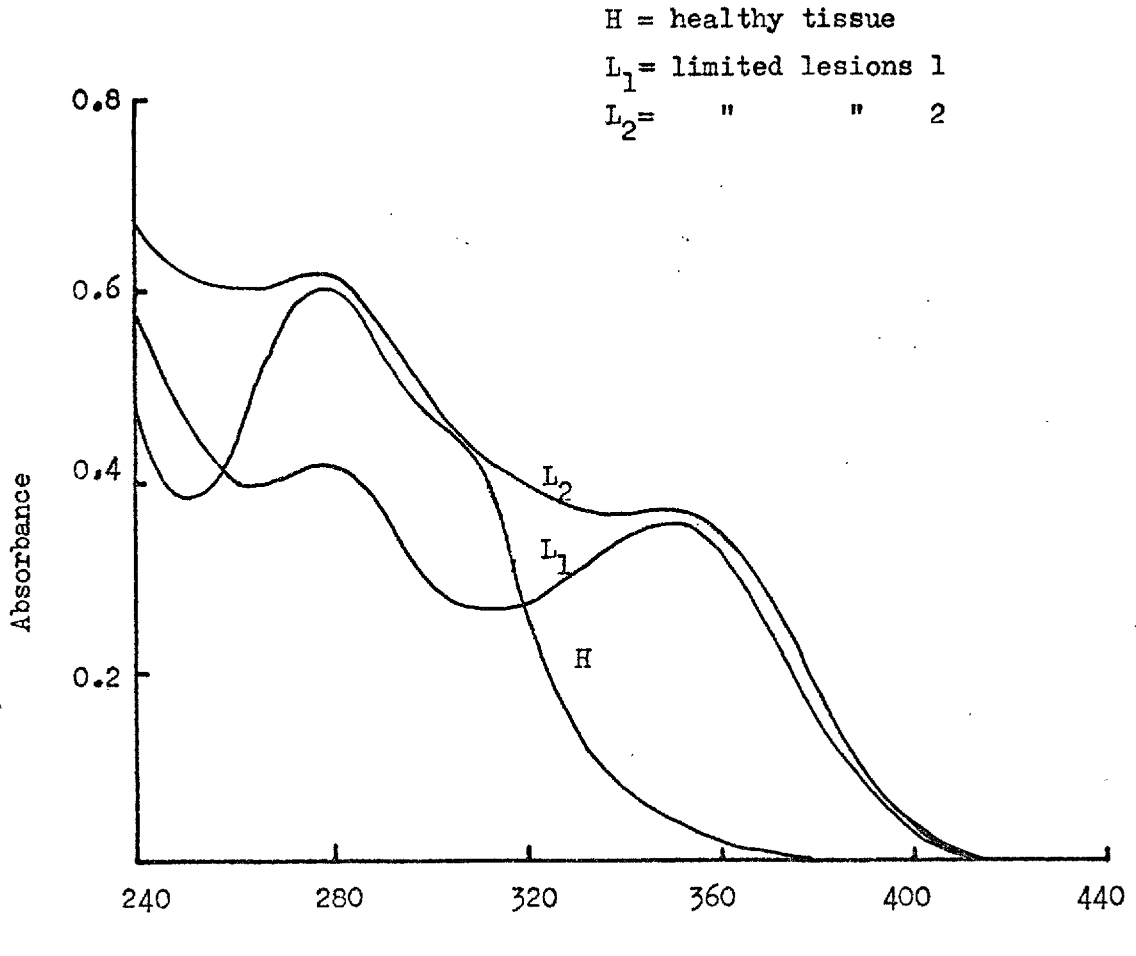
extraction. Large spreading lesions were selected so that the outer peripheral ring in fact represented the leaf tissue remaining after the other tissues had been collected. The peripheral rings bore several limited lesions. Leaves bearing examples of the different types of lesion were enclosed in moist transparent chambers and incubated in the growth cabinet used for experimental inoculations. Any development of the infections was recorded. Attempts were made to isolate fungi from the infected tissue.

The absorption spectra of chromatographic eluates redissolved in ethanol from the same R_F as wyerone acid are shown in Figures 33 and 34. The eluate from healthy tissue is included in both Figures.

In most eluates, the absorption spectrum of wyerone acid, if present, was masked by other U.V. absorbing sunstances, particularly with λmax 280 nm. Only in the spectra of eluates from the limited lesions were there peaks of absorbance at 350 nm. The spectra of these eluates also showed the peak shift characteristic of wyerone acid from 360 nm - 350 nm in 50% to absolute ethanol.

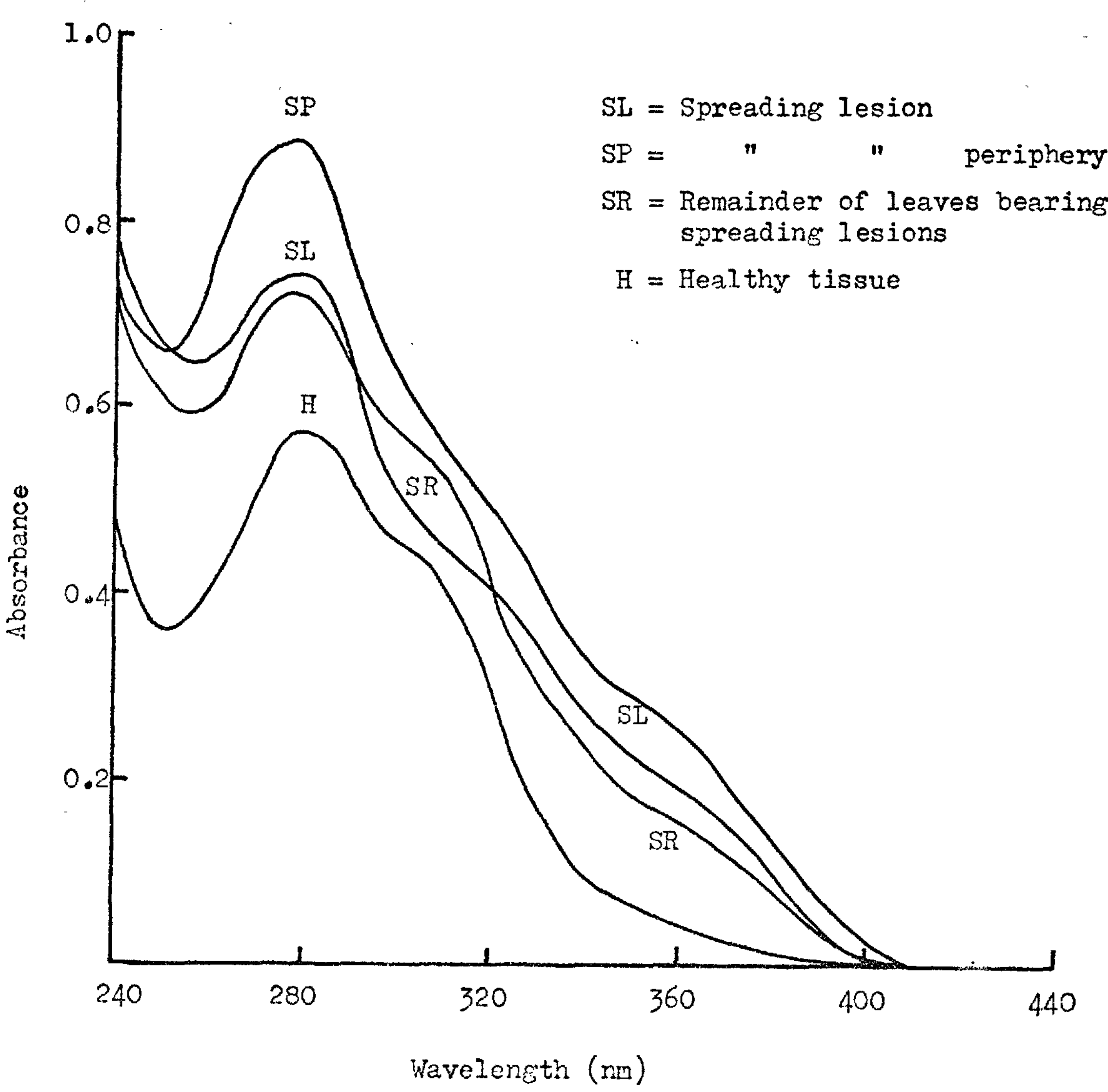
An estimate of the yield of wyerone acid was calculated from the O.D. of eluates at 350 nm, after subtraction of absorbance of the eluate from healthy tissue. The eluates were then bioassayed against <u>B. cinerea</u> at fresh weight concentration, and wyerone acid equivalents estimated from antifungal activity. Estimates from bioassay and spectrophotometry of wyerone acid/g. fresh material are shown in Table 30. Despite the presence of other U.V. absorbing substances in the eluates from limited lesions, similar values for wyerone acid

Figure 33. U.V. absorption spectra in ethanol of chromatographic eluates from extracts of healthy and naturally infected field beans.



(The eluates were in a volume of ethanol 3 x fresh wt. of tissue extracted)

Figure 34. U.V. absorption spectra in ethanol of chromatographic eluates from extracts of healthy and naturally infected field beans.



(The extracts were from similar areas of leaf)

Table 30. Concentration of wyerone acid in naturally infected field beans

Tissue	Wyerone acid (µg/g fresh material	
	Estimated from antifungal activity	Estimated from absorbance at 350 nm.
Limited lesions 1.	8.2	8.1
Limited lesions 2.	7.8	8.4
Spreading lesion	3.2	13.5
Spreading lesion periphery	4.1	5.4
Remainder of leaves bearing spreading lesions	< 1	2.7
Healthy	0	0

concentration were calculated by both methods. It was concluded from spectrophorometric evidence that wyerone acid was present in the tissues bearing limited lesions.

Healthy tissue comprised the major part of the fresh weight of tissue bearing limited lesions. It was estimated that only about $\frac{1}{20}$ of the tissue was infected. Assuming that wyerone acid production was principally restricted to the infected brown tissues, the concentration of wyerone acid in limited lesions was probably c. 20 x the recorded value, that is greater than 150 µg/g. fresh material. This contrasts with the low concentration that may have been present in and around spreading lesions.

After incubation for six days the most development of limited lesions recorded was the occasional transition from limited lesions 1 to limited lesions 2. In contrast, after two days, aerial hyphae developed from spreading lesions and fresh areas of necrotic tissue could be recognised at the edge of the lesions. After six days leaves which had borne spreading lesions were completely rotten and covered in sporulating mycelium.

Fungi were isolated from tissue bearing limited and spreading lesions, and from sporulating aerial hyphae. The most common isolate from limited lesions was a species which developed a mass of green/black conidia on either acid V8 agar or medium X. Plate 32 is a photomicrograph of conidia of the isolate. Their structure is indicative of a species of _Alternaria_, possibly _A. tenuis_, a known pathogen of _Vicia faba_. Celloidin peels of the surface of leaves

Plate 32. Conidia of fungal isolate from limited lesions.

bearing limited lesions revealed the presence of similar conidia which had germinated. Several isolates of B. fabae were obtained from spreading lesions, and from aerial hyphae.

The results obtained from extractions of wyerone acid from naturally infected tissue were consistent with the main features of laboratory inoculations. Wyerone acid was extracted from limited lesions in which the tissue was partially necrotic but was present at very low levels, if at all, where B. fabae had advanced through leaf tissue.

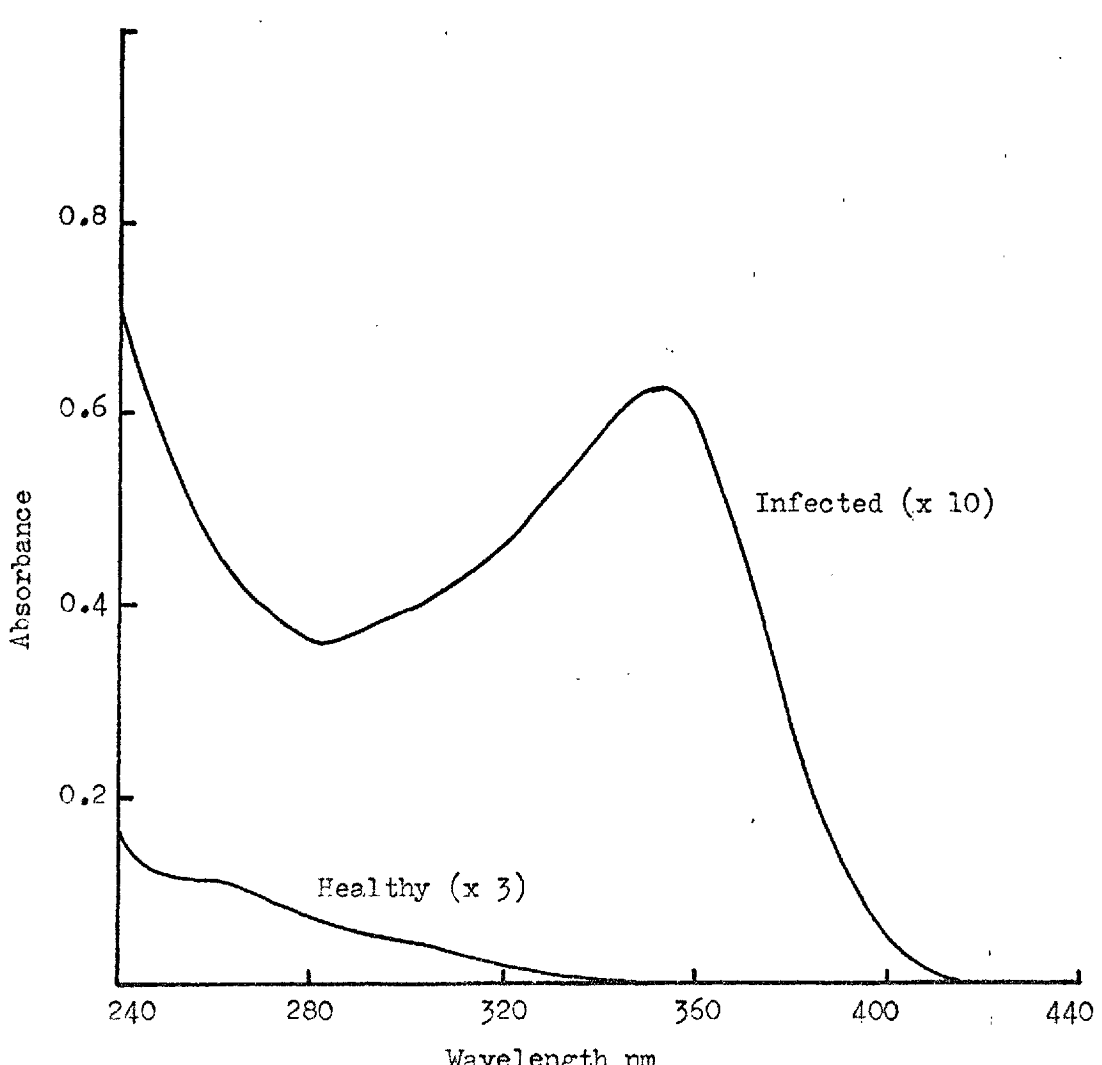
Chapter 6. The biological activity of diffusates from different bean tissues

In Chapter 4 it was shown that the concentrations of wyerone acid which accumulated in droplets over many infection sites caused by *Botrytis cinerea* in leaves were sufficient to prevent germination of conidia in the usual medium used for bioassays, pod nutrients; namely the stimulatory aqueous solution remaining after combined *B. cinerea* infection droplets collected from pod cavities 24 h after inoculation had been extracted with ether. This Chapter reports the surprising discovery that many of the infection droplets from leaves were not in fact inhibitory to test conidia despite their content of wyerone acid, and describes some analyses of the influence of water-soluble components of test solutions, particularly those derived from leaf surfaces, on the activity of wyerone acid.

a) The biological activity of diffusates from leaves.

Leaves were inoculated with *B. cinerea* conidia in sterile distilled water, or with water alone. One hundred hours after inoculation, diffusates were collected from healthy leaves and from above infections with more than 26% browning of the area beneath the infection droplet. Samples of the diffusates were extracted with ether. The U.V. absorption spectra of the ether solubles are illustrated in Figure 35, and these indicate the presence of wyerone acid in infection droplets, but not in water from healthy leaves. The concentration of acid, based on the O.D. at 350 nm in ethanol, was calculated to be

Figure 35. U.V. absorption spectra in ethanol of the ether solubles extracted from healthy <u>B. cinerea</u> infected leaves 100 h after inoculation.



(Figures in parentheses indicate the volume of ethanol relative to the volume of diffusate extracted)

55.8 μg/ml. of diffusate, confirming the results obtained in Chapter 5. This concentration greatly exceeds that shown in Chapter 4, Table 21, to cause complete inhibition of germination and germ tube growth in normal bioassays.

Table 31 shows the effect of the unextracted diffusates on germination of B. cinerea, and it can be seen that relative to water controls diffusates from healthy leaves were inhibitory and, very surprisingly, those from infected leaves were stimulatory. Table 31 also shows that the aqueous phase remaining after ether extraction of diffusates from infected leaves was highly stimulatory even in the presence of added wyerone acid. Although the comparable aqueous phase derived from healthy leaves had no effect on conidia, it also rendered added wyerone acid ineffective.

Table 32 shows that the ether phase from diffusates on infected leaves was inhibitory to conidia when incorporated in pod nutrients, at a concentration of 9 μg wyerone acid/ml based upon the absorbance shown in Figure 31. This result confirmed the presence of wyerone acid as indicated by U.V. absorbance. Furthermore the ether phase from diffusates on healthy leaves had no effect on conidia in this system.

Thus this experiment confirmed the accumulation of wyerone acid in infection droplets on leaves, but showed that wyerone acid was inactive in these infection droplets. Furthermore the acid was inactive when added to the water phase from droplets from healthy leaves.

This experiment was based upon droplets collected after

Table 31. The biological activity of diffusates and their extracts collected 100 h after inoculation, from healthy and *B. cinerea* infected leaves

Extract	Growth of *B. cinerea* conidia in bioassays*			
	B. cinerea infected leaves		Healthy leaves	
	% germination	Germ tube length(μm)	% germination	Germ tube length(μm)
Sterile distilled water control	97(1.2)	43(3.7)	-	-
Crude diffusate	100(0.3)	127(0.4)	33(7.7)	7(0.4)
Water phase+	100(0.1)	416(23)	92(0.4)	44(3.2)
Water phase + 9μg wyerone acid/ml	100(-)	370(15)	99(1.0)	110(0.2)

* Measurements of fungal growth are expressed as the mean of values recorded in two replicate bioassay drops. The difference between replicates and the mean is given in parentheses.

\+ Water phase remaining after an ether-extraction of the crude diffusate.

Table 32. Antifungal activity of the ether solubles, extracted from diffusates from healthy and *B. cinerea* infected in leaves, suspended in pod nutrients solution

Source of extract	Growth of *B. cinerea* conidia in bioassays*	
	% Germination	Germ tube length (μm)
Pod nutrients	100 (-)	500 †
'Healthy diffusate' Δ	100 (0.5)	500 †
'Infected diffusate' +	57 (3.0)	9 (0.1)

* Measurements of fungal growth are expressed as the mean of values recorded in two replicate bioassay drops. The difference between replicates and the mean is given in parentheses.

† Germ tube length estimated to the nearest 50 μm.

Δ Ether solubles at their concentration in diffusates per ml pod nutrients.

\+ Ether solubles at a concentration of 9 μg wyerone acid per ml. pod nutrients.

incubation for 4 days on leaves. It was decided to study the way in which the activity of diffusates changed during earlier stages of incubation to provide information of possibly greater relevance to the infection process.

Thirty-two boxes of leaves were inoculated with a suspension of B. cinerea conidia in water. The leaves from 8 boxes after 24 h and 10 after 71 h were examined, and diffusates were collected in batches depending on grading of infection sites. Five boxes of leaves were inoculated with water and diffusates were collected at the prescribed times to act as healthy controls. The diffusates were bioassayed against conidia of B. cinerea. The water phase remaining after ether extraction of the diffusates was bioassayed against B. cinerea conidia. The concentration of wyerone acid was estimated from the O.D. at 350 nm of ether extracts in ethanol. Unless stated it was estimated that no wyerone acid was present. The bioassay results and the pH of diffusates are given in Tables 33 and 34.

The diffusates collected after different periods of incubation, and from above different grades of infection had dissimilar physical and biological properties. Results obtained 24 h after inoculation are given in Table 33. The diffusate from healthy leaves supported similar fungal growth as water. The diffusate from infections without visible symptoms (grade 0) was inhibitory, and the inhibitory principle was removed in ether as shown by the activity of the remaining water phase. The diffusate from grade 6.5 infections stimulated growth; however the greater stimulation of growth by the water phase indicated

Table 33. The growth of *B. cinerea* conidia in diffusates, and their extracts collected from infected leaves 24h after inoculation

Infection grade	Colour	Crude diffusate pH+	Crude diffusate % Germination	Crude diffusate Germ tube length(µm)	Water phase after an ether extraction % Germination	Water phase after an ether extraction Germ tube length (µm)
Water Control	-	5.5	97(0.5)	32(0.3)	95(0.8)	32(4.0)
Healthy leaves	-	5.5-6	96(1.4)	44(4.0)	98(0.8)	40(3.7)
0	-	5.5-6	77(1.4)	17(0.8)	93(2.8)	24(3.5)
6.5	pale yellow/ brown	5.0-5.5	100(0.5)	113(4.7)	98(0.7)	143(10.4)

* Measurements of fungal growth mean of values recorded in two replicate bioassay drops. Difference between replicates and the mean is given in parentheses.

\+ pH was measured using "Whatman , BDH" narrow range indicator papers.

Table 34. The growth of *B. cinerea* conidia* in diffusates and their extracts collected from healthy and *B. cinerea* infected leaves 71h after inoculation

Infection grade	Crude diffusate				Water phase after an ether extraction	
	Colour	pH+	% Germination	Germ tube length(μm)	% Germination	Germ tube length(μm)
Healthy leaves	-	5.5-6.0	62(2.8)	19(0.22)	82(2.3)	13(1.2)
0	very pale yellow/brown	5.5-6.0	55(4.0)	14(0.5)	86(2.0)	16(2.1)
6.5	pale yellow/ brown	5.5-6.0	90(1.7)	35(0.3)	98(0.3)	54(9.3)
38	pale brown	5.0	99(1.5)	124(17.6)	98(1.0)	330(18.2)

* Measurements of fungal growth mean of values recorded in two replicate bioassay drops. Difference between replicates and the mean is given in parentheses.

\+ pH was measured using "Whatman , BDH" narrow range indicator papers.

Table 35. Antifungal activity of the ether soluble fraction of leaf diffusates suspended in sterile distilled water at their concentration in the diffusates, against *B. cinerea* conidia

Source of ether soluble extract of diffusate	% Germination*	Germ tube length* (μm)
Sterile distilled water	96 (1.4)	38 (4.1)
71 h grade 0 infections	62 (5.7)	8 (1.4)
71 h healthy leaves	69 (2.3)	11 (0.9)

* Measurements of fungal growth are expressed as the mean of values recorded in two replicate bioassay drops. The difference between replicates and the mean is given in parentheses.

that a growth retardant had been removed by ether. U.V. spectrophotometry indicated that 2 μg wyerone acid had been present per ml of diffusate.

The results of the examination of diffusates collected 71 h after inoculation are given in Table 34. The diffusate from healthy leaves was now somewhat inhibitory to B. cinerea and the diffusate from infection sites without visible symptoms was even more so, but no wyerone acid was detectable. Diffusates from grade 6.5 and $\gg$38 infections contained 0.5 and 41 μg wyerone acid per ml, and had a slightly inhibitory, and a stimulatory action respectively on germination and growth. Thus as shown in Table 31, high levels of wyerone acid failed to prevent growth of B. cinerea in leaf diffusates although wyerone acid may have been the retardant removed by ether to leave the highly stimulatory water phase from infections $\gg$grade 38. As shown in Table 34 diffusates from over more heavily damaged sites had slightly lower pH values and more intense brown colouration.

Ether solubles extracted from the most inhibitory diffusates, (from healthy leaves and from grade 0 infections 71 h after inoculation) were bioassayed at diffusate concentration in sterile distilled water against conidia of B. cinerea. The results of the bioassays are given in Table 35. The ether extracts were both antifungal despite the absence of wyerone acid. This experiment confirmed the presence of an ether soluble inhibitor in these diffusates, which was not wyerone acid.

A third experiment was carried out to confirm the activity of diffusates from healthy and infected tissues. One quarter of each

leaf was inoculated with water, and the rest of the leaf with B. cinerea. In this way diffusates of each type were obtained from the same leaves. The results of bioassays of diffusates collected 36, 48 and 68 h after inoculation and their extracts, against B. cinerea are given in Tables 36 - 38.

The results confirmed the main points revealed in the previous experiment. Diffusates from healthy leaves or low grade infections became highly inhibitory to B. cinerea only after prolonged incubation on the leaf surface. In general the diffusates from low grade infections were more inhibitory than those from healthy leaves. The development of antifungal activity of diffusates was associated with an increase in pH. The water phases of some diffusates were shown to be inhibitory to B. cinerea conidia, although inhibitory activity was usually removed by ether extraction. There were no pronounced maxima in the U.V. absorption spectra of the ether solubles from inhibitory diffusates, suggesting that wyerone acid was not responsible for the inhibition. Diffusates collected from higher grades of infection stimulated fungal growth. Diffusates collected after 36 and 48 h from grade 9.5 infections were highly stimulatory despite the presence of 10.8 μg and 32.4 μg wyerone acid/ml respectively. These infections were considered (Chapter 5) to represent developing 6 day old grade 19 infections. Germ tube growth on the leaf surface was shown to have ceased in these infections within 48 h after inoculation and thus it was concluded that, despite the presence of relatively high concentrations of wyerone acid, the development of B. cinerea in

Table 36. The biological activity of diffusates from healthy and *B. cinerea* infected leaves in relation to the symptoms produced

The growth of *B. cinerea* conidia in 36h leaf diffusates and their extracts*

Infection grade	pH^Δ	Crude Diffusate		Water phase+	
		% Germ-ination	Germ tube length µm	% Germ-ination	Germ tube length µm
Water control	5.5	97(0.6)	33(2.2)	97(0.7)	38(1.6)
Healthy	5.5-6	78(0.2)	12(1.4)	90(2.4)	20(0.6)
0	5.5-6	90(4.5)	29(0.6)	97(0.6)	46(0.2)
3.5	5.5	92(1.1)	31(2.0)	97(0.3)	44(4.4)
9.5	5.5	100(0.3)	155(1.7)	100(0)	172(6.3)

\+ Water phase after an ether extraction of crude diffusate.

* Measurements of fungal growth are expressed as the mean of values recorded in two replicate bioassay drops. The difference between the replicates, and the mean is given in parentheses.

Δ pH was measured using "Whatman BDH" narrow range indicator papers.

Table 37. <u>The biological activity of diffusates from healthy and *B. cinerea* infected leaves in relation to the symptoms produced</u>

<u>The growth of *B. cinerea* conidia in 48h leaf diffusates and their extracts</u>[+]

Infection grade	pH[△]	Crude diffusate		Water phase[*]	
		% Germ-ination	Germ tube length µm	% Germ-ination	Germ tube length µm
Healthy	6.0	84(0.6)	17(1.0)	95(1.7)	49(0.2)
0	6.0	64(3.8)	14(1.9)	80(5.5)	32(1.4)
3.5	5.5-6.0	77(0.7)	15(0.3)	91(1.6)	41(3.3)
9.5	5.0	100(0.3)	140(7.3)	100(0)	263(19.4)

* Water phase after an ether extraction of crude diffusate

\+ Measurements of fungal growth are expressed as the mean of values recorded in two replicate bioassay drops. The difference between the replicates and the mean is given in parentheses.

△ pH was measured using "Whatman BDH" narrow range indicator papers.

Table 38. The biological activity of diffusates from healthy and B. cinerea infected leaves in relation to the symptoms produced

The growth of B. cinerea conidia in 68h leaf diffusates and their extracts+

Infection grade	pH $^{\Delta}$	Crude diffusate		Water phase*	
		% Germ-ination	Germ tube length µm	% Germ-ination	Germ tube length µm
Healthy	6.5	27(3.0)	12(1.5)	68(5.0)	13(2.0)
0	6.5	6(1.3)	5(0.1)	60(3.4)	10(1.2)
3.5	6-6.5	42(0.4)	12(1.7)	81(6.2)	17(0.9)

* Water phase after an ether extraction of crude diffusate

\+ Measurements of fungal growth are expressed as the mean of values recorded in two replicate bioassay drops. The difference between the replicate and the mean is given in parentheses

Δ pH was measured using "Whatman BDH" narrow range indicator papers

these infections was not arrested by the content of the infection droplets.

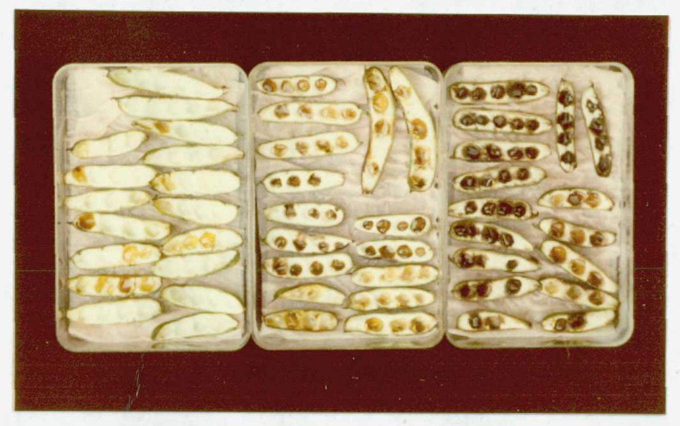
b) The biological activity of diffusates from pods

A major difficulty in studying diffusates from leaves was the variation in symptoms produced and partially in consequence, the low volumes of diffusates collected from some types of infection. In contrast the development of infections in pod seed cavities was less variable, and larger volumes of diffusates could be readily collected. It was therefore decided to examine the biological activity of diffusates, and their extracts, from pods inoculated with sterile distilled water and suspensions of B. cinerea, or B. fabae conidia.

Pods of "Minor" field beans grown at Silwood Park were used in these experiments. Diffusates were collected 12, 24, 43, 62, and 86 hours after inoculation, and their pH recorded. The crude diffusates and their constituent water phase alone and supplemented with 9 μg wyerone acid/ml, and the constituent ether solubles dissolved at diffusate concentration in 'pod nutrients', were bioassayed against B. cinerea in the usual way.

Though described as diffusates from healthy pods browning developed beneath many droplets of sterile distilled water within 43 h of inoculation. This is illustrated in Plate 32. The number of water droplets associated with browning and its intensity increased with time. This was reflected by the colour of diffusates. This diffusate collected after 24 h, when there was no browning of pod endocarp, was pale yellow/brown; and after 86 h, when many seed cavities were

Plate 33. Field bean pods 62 h after inoculation with sterile distilled water, or suspension of B. cinerea or B. fabae conidia in water.



Water B.cinerea B.fabae

Note the browning beneath several water droplets, the limitation of B. cinerea infection to the infection site, and in contrast, the spread of lesions caused by B. fabae into the surrounding tissues.

discoloured, was light brown in colour. Diffusate collected 86 h after inoculation was examined microscopically before centrifugation, and found to contain numerous bacteria, but no fungal contaminants.

The bioassay results, and pH of diffusates from healthy pods are given in Table 39. The pH of diffusates from healthy pods was slightly less than that of water. The diffusates were all stimulatory to development of B. cinerea. Extraction with ether enhanced the stimulatory activity of the diffusates collected after 43 h. The degree of stimulation by water phase increased with time of incubation. Though of similar length, the germ tubes produced in 86 h water phase were noticeably thicker than those in extracts of other "healthy diffusates". This suggested that the water phase collected 86 h after inoculation was the most stimulatory to fungal growth. The greater stimulation of fungal growth observed after ether extraction of crude diffusates was reflected by the antifungal activity of the extracted ether solubles when they were returned to pod nutrients. The antifungal activity of wyerone acid added to water phases increased with the period of incubation of the original infection droplets but only became marked in the solution obtained after 86 h incubation.

The results of the examination of diffusates from B. cinerea infected pods are recorded in Table 40. The diffusate collected 12 h after inoculation of a suspension of B. cinerea conidia was pale brown in colour; the later diffusates were darker. Diffusate pH was always less than that of water. The 12 h diffusate stimulated fungal growth, but twenty-four hours after inoculation the diffusate had become highly

Table 39. <u>The biological activity of water droplets incubated in pod seed cavities</u>

Time after inoculation	pH of crude diffusate	Germ tube length µm of *B. cinerea* in bioassays[*]			
		Crude diffusate	Water phase[Δ]	Water phase[+] wyerone acid (9µg/ml)	Ether solubles[x] in pod nutrients
0h (water control)	5.0	50	60	80	500
12h	4.5-5.0	350	350	300	500
24h	4.5	350	350	200	500
43h	5.0	250	500	200	200
62h	4.5	200	550	150	200
86h	4.5	300	550	10(49%)[+]	200

* Expressed as the mean of 3 replicate bioassay drops; germ tube length was estimated to the nearest 10µm $<$ 200µm and the nearest 50µm $>$ 200µm.

Δ Water phase remaining after an ether extraction of crude diffusate

x Ether solubles at their concentration in the original diffusate

\+ Percentage germination unless 98 - 100%

Table 40. **The biological activity of *B. cinerea* infection droplets incubated in pod seed cavities**

Time after inoculation	pH of crude diffusate	Germ tube length µm of *B. cinerea* in bioassays*			
		Crude diffusate	Water phase△	Water phase+ wyerone acid (9µg/ml)	Ether solubles x in pod nutrients
12	4.5	500	550	60(71%)+	500
24	4-4.5	10(30%)+	550	20(62%)+	10(26%)
43	4.0	Zero	550	10(23%)+	Zero
62	4.0	Zero	450	10(27%)+	Zero
86	4.0	Zero	500	20(60%)+	Zero

* Expressed as the mean of 3 replicate bioassay drops; germ tube length was estimated to the nearest 10 µm $<$ 200 µm and the nearest 50 µm $>$ 200 µm.

△ Water phase remaining after an ether extraction of crude diffusate.

x Ether solubles at their concentration in the original diffusate.

+ Percentage germination unless 98 - 100%.

Table 41. The biological activity of *B. fabae* infection droplets incubated in pod seed cavities

Time after inoculation	pH of crude diffusate	Germ tube length µm of *B. cinerea* in bioassays*			
		Crude diffusate	Water phase △	Water phase+ wyerone acid (9µg/ml)	Ether solubles x in pod nutrients
12	4.5	450	550	60(87%)+	500
24	4-4.5	400	500	30(55%)+	300
43	4.0	250	500	20(60%)+	140
62	4.0	250	500	20(66%)+	250
86	4.0	300	300	30(71%)+	400

* Expressed as the mean of 3 replicate bioassay drops; germ tube length was estimated to the nearest 10µm $<$ 200µm and the nearest 50µm $>$ 200µm.

△ Water phase remaining after an ether extraction of crude diffusate

x Ether solubles at their concentration in the original diffusate

\+ Percentage germination unless 98 - 100%

inhibitory to the development of B. cinerea. The water phases of diffusates were all highly stimulatory, and added wyerone acid restricted germ tube growth to less than 60 µm in each water phase. The diffusate ether solubles permitted similar growth in pod nutrients to that observed in the diffusates of their origin (i.e. to a very slight extent except after 12 h).

The results of studies on the B. fabae diffusates are given in Table 41. The colour of diffusates progressed with time of collection from pale brown after 12 h to dark brown after 86 h. The diffusates had pH values less than water, and to varying degrees stimulated the development of B. cinerea conidia. This stimulatory activity was enhanced by extraction with ether except in the 86 h diffusate. The ether solubles of most diffusates were therefore considered to have been inhibitory within the crude diffusate. This antifungal activity was also shown in the bioassays of the ether solubles returned to pod nutrients. The activity of wyerone acid was high in each water phase.

Therefore the activity of pod diffusates depended on the initial inoculum. Thus diffusates from B. cinerea infection became highly inhibitory 24 h after inoculation, but diffusates collected after inoculation with water, or B. fabae, stimulated fungal growth. In most cases the removal of ether solubles enhanced the stimulatory activity. This implied the presence of ether soluble inhibitors; a conclusion which was supported by the antifungal activity of the extracted ether solubles in pod nutrients. It was considered, in view of results reported earlier, that this antifungal activity was caused

by wyerone acid within the diffusates. Although residual water phases of all types stimulated growth to similar extents, the addition of wyerone acid caused variable inhibition in the different water phases. There was no obvious explanation for this interesting phenomenon.

Comparison of diffusates from leaves and pods revealed:

1) The slow accumulation of fungal inhibitors in droplets on the surfaces of healthy leaves, but the rapid accumulation of fungal stimulants in droplets inside healthy pods, although there was an indication of an ether soluble growth retardant released along with the stimulants from pods.

2) A similar inactivity of endogenous or added wyerone acid in most diffusates from healthy pods, and from healthy or diseased leaves.

3) The high activity of endogenous or added wyerone acid in diffusates from infected pods.

c) The antifungal activity of wyerone acid

The results of previous experiments prompted further investigation of the antifungal activity of wyerone acid to assist in the interpretation of some of the observations on the biological activity of diffusates.

The antifungal activity of wyerone acid (9 μg/ml) in sterile distilled water against B. cinerea was further examined. In distilled water alone 97% of conidia germinated, and produced germ tubes with a mean length of 39 μm. In water + wyerone acid 99% germinated and these produced germ tubes 93 μm in length. As previously observed in sterile distilled water, and the water phase of the diffusate from

healthy leaves, wyerone acid appeared to slightly stimulate germ tube growth.

It was considered that wyerone acid may be insoluble in water, and that the observed stimulation was due to the presence of stimulatory substances eluted with wyerone acid from chromatography papers. Both these possibilities were investigated.

First, a solution of wyerone acid (9 μg/ml) in distilled water was prepared and examined in the U.V. spectrophotometer. The absorption spectrum was identical to that of wyerone acid in 50% ethanol, with λmax at 360 nm. It was concluded, from measurements of O.D. at 360 nm, that all the wyerone acid had been dissolved.

Secondly, "Whatman 3 MM" papers without extract were developed in n-propanol/water and a band from the same R_F as wyerone acid eluted. This eluate was bioassayed in sterile distilled water at a concentration equivalent to that in the stock solution of wyerone acid used for bioassays. The development of conidia in the solution of the eluate was similar to that in water alone.

Therefore, it was concluded that wyerone acid was soluble in water at a concentration of 9 μg/ml, and that the slight stimulation of germ tube growth observed was not due to the presence of stimulatory substances from chromatography papers.

Chapter 7. The effect of additives to the infection droplet on the resistance of bean leaves to infection by *Botrytis cinerea*

Pollen grains were shown by Chou and Preece (1968) to greatly stimulate the pathogenicity of *B. cinerea* towards the broad bean. The following studies on the effect of additives to the infection droplet on the resistance of bean leaves to infection by *B. cinerea* were primarily designed as an investigation of the pollen phenomenon.

a) The effect of extracts of pollen grains on pathogenicity

Chou and Preece (1968) showed that the active fraction of pollen grains could be extracted as a diffusate into water, and was dialysable. A diffusate was prepared at the same concentration as that used by Chou and Preece. The pathogenicities of conidia of *B. fabae* and *B. cinerea* suspended in water, and *B. cinerea* suspended in pollen diffusate were compared after inoculation onto detached leaves. Nine leaves were inoculated with four 10 µl droplets of a different treatment on each half leaflet. There were therefore 48 droplets of each treatment. Using grading system 1, the infections which developed were recorded 2, 4 and 6 days after inoculation. The results are given in Table 42. *B. cinerea* conidia in pollen diffusate produced lesions as rapidly as *B. fabae* in water.

The effect of dialysis on the ability of pollen diffusate to promote infection was then examined. The pathogenicities of *B. cinerea* conidia suspended in dialysed pollen diffusate, or sterile distilled water were compared. The results are recorded in Table 43. No

Table 42. The effect of pollen diffusate on the development of lesions by *B. cinerea*

Inoculum	Time after inoculation					
	2 days		4 days		6 days	
	% Lesions*	% Spreading*	% Lesions	% Spreading	% Lesions	% Spreading
B. cinerea	71	0	88	2	94	2
B.cinerea + pollen	100	5	100	100	100	100
B. fabae	100	0	100	90	100	100

* Expressed as percentage of total number of droplets (48)

Table 43. The effect of dialysed pollen diffusate on the development of lesions by *B. cinerea*

Inoculum	Time after inoculation			
	2 days		4 days	
	% Lesions*	% Spreading*	% Lesions	% Spreading
B. cinerea	48	0	78	0
B. cinerea + pollen	100	0	100	100

* Expressed as a percentage of total number of droplets (40)

stimulatory activity was lost from pollen diffusate by dialysis.

These experiments confirmed the findings of Chou and Preece (1968).

b) Changes in the concentration of wyerone acid in leaves inoculated with B. cinerea conidia suspended in dialysed pollen diffusate.

Numerous leaves were inoculated with B. cinerea conidia suspended in dialysed pollen diffusate. The concentration of wyerone acid extracted from lesions and peripheral rings of tissue, at different times after inoculation are shown in Table 44.

Wyerone acid was found in the peripheral tissues surrounding the lesions in amounts similar to those found around lesions caused by B. fabae. However, unlike the results obtained from infections produced by B. fabae conidia in water, there was more wyerone acid in the completely blackened lesions than in the surrounding mainly green peripheries.

In a second experiment the concentration of wyerone acid in tissue at the infection site was measured during the first 4 days after inoculation. The first extraction was performed 21 h after inoculation, when the development of B. cinerea on the leaf surface was examined by the chlorine bleaching technique and a mesh of hyphae was seen above the infection sites. The last extraction was made from blackened 5 mm diameter leaf discs cut 82 h after inoculation from the centre of lesions which had spread beyond this area. At this stage of infection tufts of fungal hyphae were emerging from the lower surface of the leaf opposite infection droplets. The absorption spectrum of

Table 44. Concentration of wyerone acid in and around lesions produced by *B. cinerea* conidia suspended in dialysed pollen diffusate

Hours after inoculation	Appearance of Leaf Surface		Wyerone acid (µg/g fresh material)	
	Upper	Lower	Lesion	Periphery
21			16.9	3.2
45			94.2	36.9
75			71.6	31.9

Figure 36. U.V. absorption in 50% ethanol spectrum of wyerone acid extracted from infection sites 82h after inoculation with *B. cinerea* conidia suspended in dialysed pollen diffusate

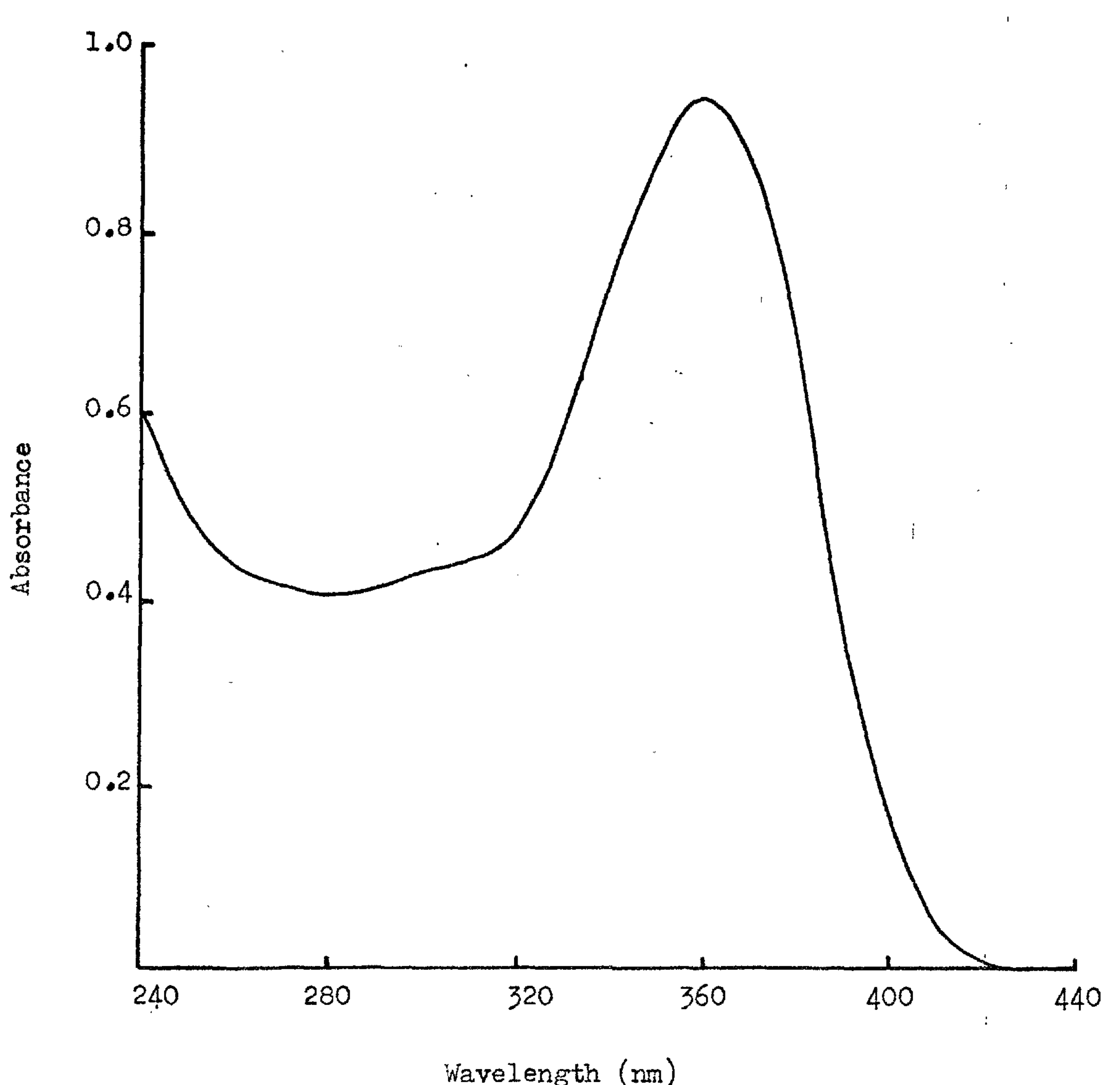


Table 45. Concentration of wyerone acid at infection sites during the first 4 days after infection with *B. cinerea* conidia suspended in dialysed pollen diffusate

Hours after inoculation	Appearance of leaf surface Upper	Lower	Wyerone acid (μg/g fresh material)
20			11.0
65			103.5
82			97.2

the chromatographic eluate containing wyerone acid extracted from the blackened centre of the _B. cinerea_ lesion 82 h after inoculation is illustrated in Figure 36. Eluates with similar absorption spectra were obtained from the younger infections examined. A solution of wyerone acid (18 μg/ml pod nutrients) extracted from the spreading infection allowed only 2% germination of conidia after incubation for 21 h at 20°C. This activity was similar to that recorded in Chapter 4 for wyerone acid extracted from pod diffusates. The concentrations of wyerone acid at infection sites during the first four days after inoculation are recorded in Table 45.

The accumulation of wyerone acid was not followed by a marked decrease in wyerone acid concentration, when the infection site became completely colonised by the fungus and blackened, as was observed in similar lesions caused by _B. fabae_.

An experiment was designed to determine whether wyerone acid remained at a high level in spreading lesions produced 5 days after inoculation with _B. cinerea_ in dialysed pollen diffusate. Thirty-two leaves were inoculated and half of these were sampled after incubation for 41 h, when the developing lesions and surrounding 2 mm wide peripheries of apparently healthy tissue were excised. After 128 h from inoculation lesions greater than 10 mm in diameter, and surrounding 2 mm wide peripheries were excised from the remaining leaves.

The absorption spectra of appropriate chromatographic eluates from extracts of tissues collected 41 h after inoculation were characteristic of wyerone acid. Those from tissues extracted after

128 h, differed in having λmax at 330 nm in both 50% and absolute ethanol. The absorption spectra of the two types of eluates are compared in Figure 37, and the eluates from the respective peripheries were similar.

The eluates obtained from the spreading infection (128 h) were bioassayed at fresh weight concentration per ml in pod nutrients, against B. cinerea. Control germ tubes of B. cinerea grew to 500 μm in length. In presence of the extracts from the lesion and periphery germ tube length was reduced to 120 μm, and 80 μm respectively. This low activity correlates with the low absorbance at 360 nm (Figure 37 - 128 h) and the apparent absence of wyerone acid recorded by spectrophotometry. The wyerone acid equivalents, of the unknown inhibitor were calculated from the anitfungal activity of the eluates in order to allow direct comparison with the measurements of wyerone acid extracted from the 41 h infections, in Table 46. It was concluded that the continuing development of spreading lesions caused by B. cinerea + pollen was associated with a loss of wyerone acid. Failure to extract a compound with the spectrophotometric characteristics of wyerone acid from the peripheral healthy tissues surrounding the large spreading infections repeated the experience with similar tissues surrounding natural B. fabae infections (Chapter 5, Section c.).

It was concluded that pollen extracts did not prevent the accumulation of wyerone acid in infected leaf tissues. In the presence of dialysed pollen diffusate B. cinerea was able to completely colonize the infection site and adjacent tissues, despite the presence of high

Figure 37. U.V. absorption spectra in 50% ethanol of eluates from Rf 0.9 in chromatograms of ether soluble acids extracted from lesions produced by B. cinerea conidia suspended in dialysed pollen diffusate, 41h and 128h after inoculation

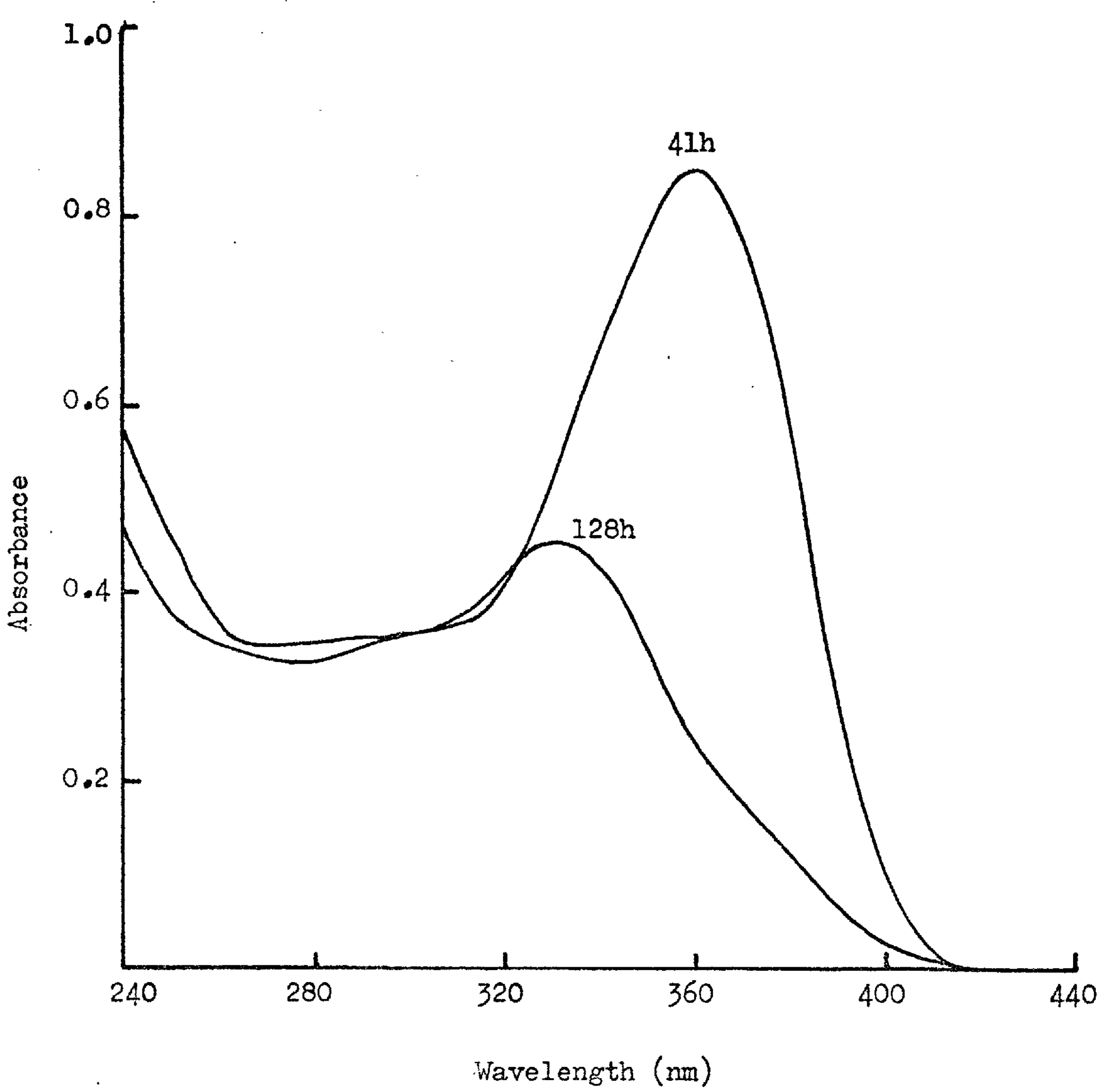


Table 46. Concentrations of wyerone acid in and around lesions, 2 and 5 days after inoculation with *B. cinerea* conidia suspended in dialysed pollen diffusate

Hours after inoculation	Appearance of leaf surface Upper	Lower	Wyerone acid (µg/g fresh material) Lesion	Periphery
41			120	41.3
128			4.0*	5.0*

* Wyerone acid equivalent of antifungal activity

concentrations of wyerone acid. The loss of wyerone acid from spreading lesions between the third and fifth day after inoculation suggested that the subsequent spread of the infection through the leaf was associated with the degradation of the phytoalexin by the fungus.

c) The antifungal activity of wyerone acid dissolved in dialysed pollen diffusate

The concentrations of wyerone acid extracted from lesions caused by B. cinerea in dialysed pollen diffusate were frequently higher than those which prevented mycelial growth in vitro (Table 22). The sensitivity of Botrytis to wyerone acid in pollen diffusate was therefore examined in vitro.

The antifungal activity of wyerone acid in dialysed pollen diffusate (pH 5.3) was compared with its activity in pod nutrients (pH 4.5) at a concentration of 20 μg/ml. The mean results of triplicate bioassays are shown in Table 47. In the absence of wyerone acid pollen diffusate stimulated the growth of Botrytis in a manner similar to pod nutrients, and germ tubes produced in the two solutions were indistinguishable. Pollen diffusate, unlike pod nutrients, to a large extent overcame the inhibitory effect of wyerone acid.

In order to test whether pollen diffusate was having a direct effect on wyerone acid, 20 μg of wyerone acid/ml were incubated in dialysed pollen diffusate or pod nutrients for 24 h at 22°C in test tubes on a wrist action shaker. Wyerone acid was extracted by solvent partition with diethyl ether and measured spectrophotometrically and by its antifungal activity. The results given in Table 48 indicate

Table 47. Antifungal activity of wyerone acid (20 µg/ml) dissolved in dialysed pollen diffusate or pod nutrients, against germ tube growth from conidia

Solution	B. fabae	B. cinerea
Pod nutrients	450µm[*]	500µm
Pod nutrient + wyerone acid	56%[+]	Zero
Pollen diffusate	500µm	600µm
Pollen diffusate + wyerone acid	400µm	400µm

* Germ tube length estimated to nearest 50µ unless < 20µm

\+ Germination of conidia unless 98 - 100%

Table 48. Recovery of wyerone acid after incubation for 24h in pod nutrients or dialysed pollen diffusate at a concentration of 20µg/ml.

a) Estimated from absorbance at 350 nm.

Incubation solvent	Wyerone acid (µg/ml) extracted
Pod nutrients	17.8
Dialysed pollen diffusate	18.0

b) Indicated by the antifungal activity of ether extracts against germ tube growth from *B. cinerea* conidia

x Initial concentration	Incubation solvent: Pod nutrients	Incubation solvent: Dialysed pollen diffusate
1	Zero	Zero
$\frac{1}{2}$	51%*	40%*
$\frac{1}{4}$	40µm+	30µm+

* Germination unless 98 - 100%

\+ Estimated germ tube length unless < 20µm

that there was no substantial loss of wyerone acid after incubation in either solvent, and no change in antifungal activity. It was concluded that pollen diffusate did not have a direct effect on wyerone acid.

Further bioassays were therefore carried out to examine the degree of insensitivity of the fungi to wyerone acid dissolved in dialysed pollen diffusate. A solution of 1000 μg wyerone acid/ml dialysed pollen diffusate was prepared. Solutions of 500, 250 and 125 μg/ml were prepared from this by serial dilution with dialysed diffusate. The solubility of wyerone acid at these concentrations was confirmed by extraction of the acid into ether from the 500 μg/ml solution. The results of standard bioassays are shown in Table 49. Wyerone acid at 1000 μg/ml prevented the germination of both Botrytis species. Germination in all other treatments was between 48 - 100%. The lowest concentration which prevented all germination would have been between 500 and 1000 μg/ml. This contrasts with the concentrations of 18 μg/ml and 45 μg/ml which had a similar effect in pod nutrients, on B. cinerea and B. fabae respectively. (Table 21). Wyerone acid at 125 μg/ml, a concentration similar to that found in the spreading lesions caused by B. cinerea + pollen (Tables 44 and 45) caused only a 42% reduction in the length of germ tubes produced by B. cinerea. Even in dialysed pollen diffusate B. fabae appeared to be less sensitive to wyerone acid than B. cinerea.

Table 49. Antifungal activity of wyerone acid dissolved in dialysed pollen diffusate pH 5.3 against length of germ tubes (μm)* produced by *Botrytis* conidia

Wyerone acid (μg/ml)	*B. fabae*	*B. cinerea*
0	600	600
125	500	350
250	350	200
500	200	50
1000	Zero+	Zero

* Estimated to nearest 50μm

\+ No germination of conidia

d) The metabolism of wyerone acid dissolved in dialysed pollen diffusate or pod nutrients, by *Botrytis*

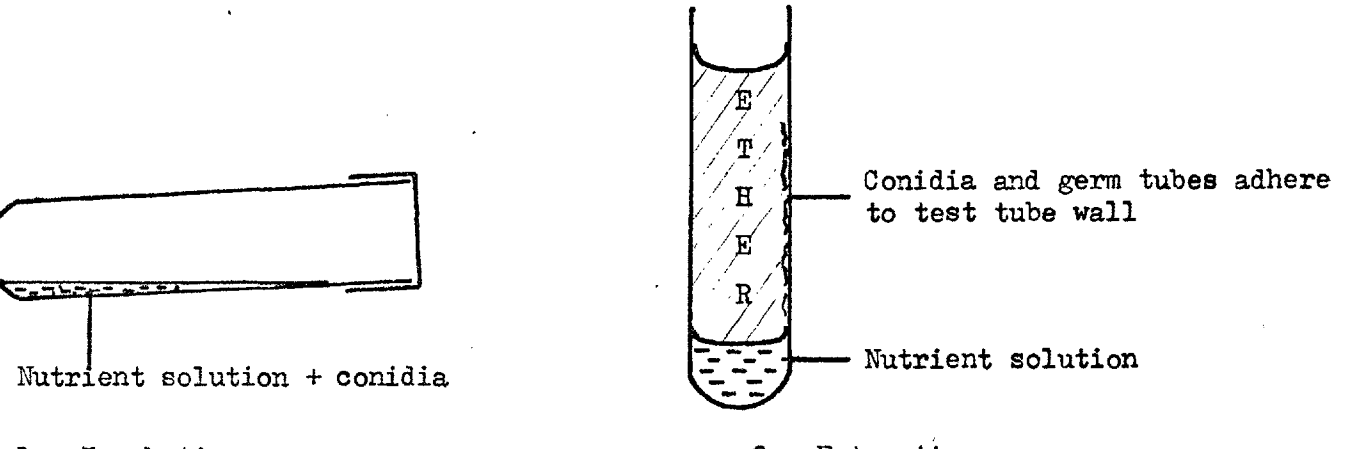
The high concentration of wyerone acid found in spreading lesions caused by *B. cinerea* + pollen indicated that the fungus developed without the loss of wyerone acid which was apparent after *B. fabae* caused similar symptoms. This suggested that the observed effect of pollen on the sensitivity of *B. cinerea* to wyerone acid *in vitro* was not mediated by increased metabolism of the acid by the fungus. To test this hypothesis, the relationship between wyerone acid matabolism and fungal growth in pod nutrients and dialysed pollen diffusate was examined.

Several small scale systems for incubation of developing conidia were examined. Conidial suspension was added to 1 ml aliquots of pod nutrients in test tubes held vertically in a rack. After incubation for 24 h at 22°C the nutrient solution was decanted and fungus development examined through the test tube wall. Growth was much less than that observed in comparable bioassays. Conidia which had adhered to the wall near the meniscus developed much more than the tube which had settled to the bottom of the tube. This suggested that oxygen tension might have been a growth limiting factor. Similar tubes of solution were therefore incubated in the same way on a wrist action shaker. Conidia grew on the surface of the solution, and on the wall of the tube above the actual liquid surface in the "splash zone", produced by agitation. This was clearly not a satisfactory method of incubation. The possibility of using an extended bioassay

droplet system was examined. One ml of pod nutrients was pipetted into an almost horizontal test tube. A drop (0.05 ml) of conidial suspension was added to the elliptical 1 ml droplet using a 0.1 ml graduated pipette. The development of conidia in the droplet was similar to that in comparable bioassays on slides.

An experiment was designed to test the use of the system for the measurement of wyerone acid metabolism. Three test tubes were incubated nearly horizontally with 1 ml droplets of a solution of 15 μg wyerone acid/ml pod nutrients. One tube was incubated without conidia. B. cinerea and B. fabae conidia respectively, were added to the other tubes. After 21 h incubation at 20°C the tubes were returned to the vertical. The solutions settled to the bottom of the tube, but conidia and germ tubes adhered to the wall. Ether was added and wyerone acid extracted from the solution in the manner described for leaf diffusates. The recovery of wyerone acid, measured from the absorption spectra of the ether extracts, was calculated as a percentage of the original concentration. Ninety-nine per cent was recovered in the absence of conidia; 100% from the B. cinerea and 76% from the B. fabae treatment. The residual solutions were decanted, and conidial development observed through the tube wall. Only 5% of B. cinerea conidia, but 84% of B. fabae conidia had germinated. The latter had produced germ tubes estimated to be 100 μm in length. It was concluded that this system allowed the easy extraction of wyerone acid, and the measurement of growth of the fungi. The system is illustrated in Figure 38.

Figure 38. System designed to examine the metabolism of wyerone acid by *Botrytis*



It was considered that washing developing conidia with ether might not be adequate to extract wyerone acid which was absorbed by or absorbed onto the fungi. In this way a false representation would be made of wyerone acid metabolism, which had not in fact occurred. An experiment was therefore designed to examine the efficiency of the proposed system, and also the production of ether soluble substances by Botrytis in the absence of wyerone acid.

One ml aliquots of test solutions were dispensed into hand homogenizing tubes, or test tubes held at c 1° from the horizontal. Suspensions of conidia (0.05 ml) were added to the appropriate solutions. The following treatments were prepared for incubation.

Pod nutrients + B. cinerea or B. fabae conidia
Pod nutrients + 20 μg wyerone acid/ml
Pod nutrients + 20 μg wyerone acid/ml + B. cinerea or B. fabae conidia

Where the solutions were incubated in the absence of conidia they were prepared in test tubes. There were four replicates of this, and two of other treatments. After 40 h incubation at 21°C, the treatments without wyerone acid were examined. The supernatant was decanted into a fresh tube and extracted with ether. Enough ether was then added to the original tube to immerse the mesh of hyphae which had adhered to the tube wall. The ether was collected with a Pasteur pipette after 30 min. This mycelial soaking was repeated. The pooled ether washes were taken to dryness, resuspended in 1.5 ml ethanol and examined in the spectrophotometer. Hyphae were then scraped from the walls, and pushed to the bottom of the homogeniser. The mycelium was macerated twice in 5 ml aliquots of 80% ethanol. The macerate was decanted,

fungal debris was removed by centrifugation and the supernatant dried and resuspended in 1.5 ml ethanol for spectrophotometry.

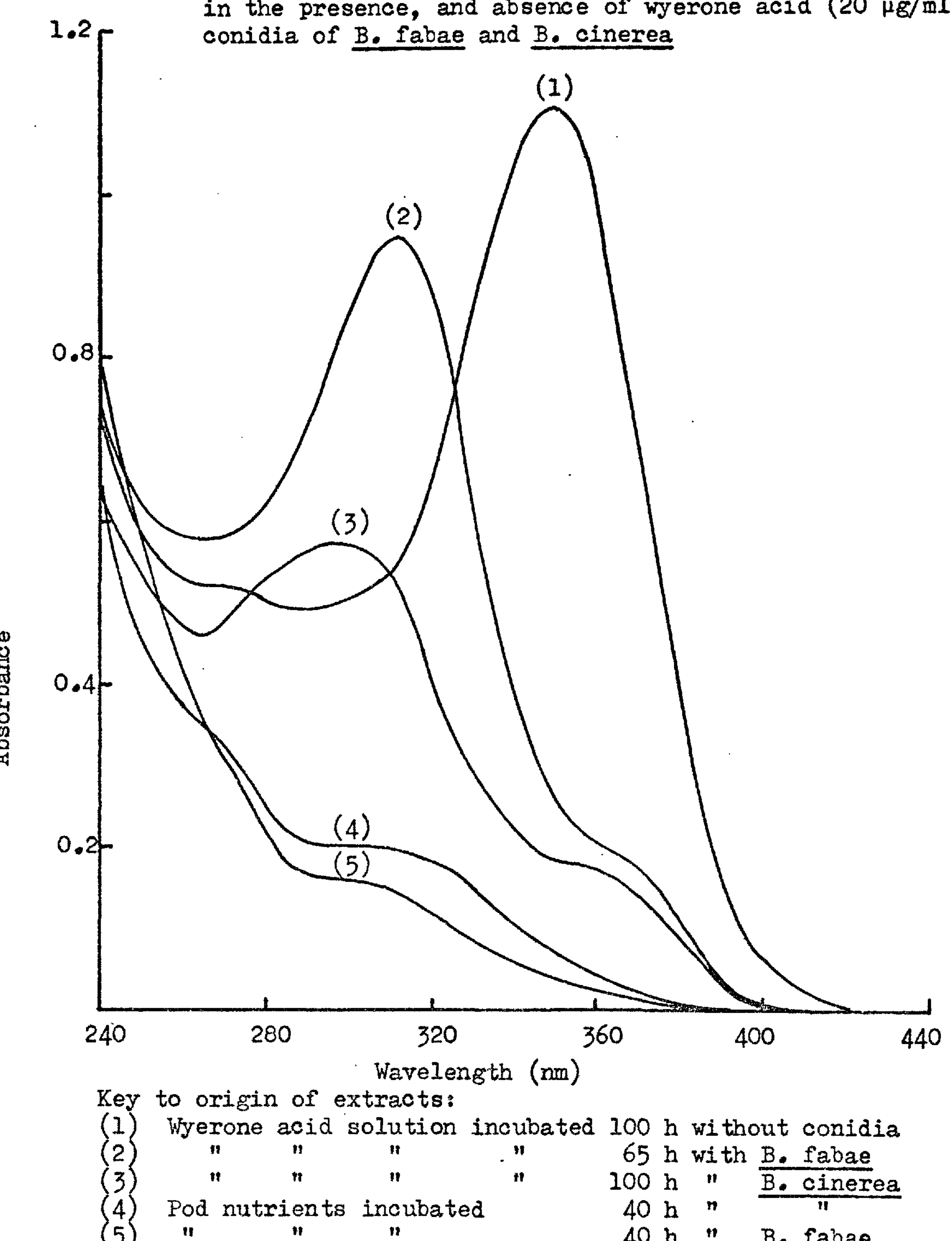
A mesh of hyphae had developed from B. fabae conidia in wyerone acid solution 65 h after inoculation. At this time the remaining tubes inoculated with B. fabae conidia were processed as described above. Two replicates of wyerone acid solution without conidia were extracted in the same way, except that the test tube walls were rinsed with ethanol in place of the maceration of the fungal mycelium.

A mesh of hyphae had developed from B. cinerea conidia 100 h after inoculation when this and the remaining treatments were processed.

Examples of the absorption spectra of ether extracts of the decanted bathing solutions from each treatment are shown in Figure 39. Clearly most of the wyerone acid had disappeared from the bathing solutions around conidia and hyphae of B. fabae and B. cinerea. The amounts of wyerone acid recovered from bathing solutions, residues and macerates were estimated from the absorbance of extracts at 350 nm in ethanol. The recorded values are given in Table 50. The wyerone acid lost from the bathing solutions could not be recovered from the mycelial extracts. It was concluded that the metabolism of wyerone acid by Botrytis could be examined by the system described in Figure 38.

This experiment demonstrated that both B. cinerea and B. fabae were capable of metabolizing wyerone acid. Neither fungus produced substantial amounts of ether soluble substances in pod nutrients alone. However the loss of wyerone acid from incubation mixtures was associated with the appearance of other U.V. absorbing substances. The production of novel substances by B. fabae was most marked. The

Figure 39. U.V. absorption spectra of ether solubles in 1.5 ml ethanol extracted from bathing solutions of pod nutrients incubated in the presence, and absence of wyerone acid (20 µg/ml) and conidia of <u>B. fabae</u> and <u>B. cinerea</u>



Key to origin of extracts:

(1)	Wyerone acid solution incubated	100 h	without conidia
(2)	" " " "	65 h	with <u>B. fabae</u>
(3)	" " " "	100 h	" <u>B. cinerea</u>
(4)	Pod nutrients incubated	40 h	" "
(5)	" " "	40 h	" <u>B. fabae</u>

Table 50. Metabolism of 20μg wyerone acid by *Botrytis*

Recovery of wyerone acid μg.	*B. fabae*	*B. cinerea*	No conidia control
Supernatant	2.44	1.89	16.72
Walls of test tube	0.50	0.43	1.54
Mycelial macerate	0	0	0
Total	2.94	2.32	18.26
Recovery as % no conidia control	16.1	12.7	100

distinct peak at 310 nm illustrated in Figure 39 was recorded from extracts of both replicates. The peak of absorbance at 297 nm in the spectrum of the B. cinerea extract shown in the figure was not seen in the second replicate. In this replicate spectrum there was not a pronounced maximum of absorbance but a broad peak from 260 - 290 nm, with an O.D. greater than that of extracts of mycelium growth in the absence of wyerone acid. It was considered that the substances responsible for this absorbance, produced by the fungi in the presence of wyerone acid, may have been fungal metabolites of the acid.

The metabolism by Botrytis of wyerone acid dissolved at a concentration of 6 μg/ml was then examined in dialysed pollen diffusate with pod nutrients as a comparison. Separate controls were prepared for each time of extraction. Absorption spectra of appropriate eluates were measured after chromatography of the ether extracts. The results of estimated germ tube length and percentage loss of wyerone acid in different treatments and after different times of incubation are recorded in Table 51. The loss of acid was calculated from the percentage loss of absorbance at 350 nm relative to recovery from control solutions without conidia.

Dialysed pollen diffusate was again shown to overcome the inhibitory action of wyerone acid. The growth of Botrytis conidia in pollen diffusate was not associated with a loss of wyerone acid (Table 51a) comparable to that of pod nutrients. This was further illustrated by the loss of acid in pod nutrients (Table 51b) after the germ tubes had been allowed to grow as far as those in pollen

Table 51. Metabolism of wyerone acid (6μg/ml) dissolved in pod nutrients or dialysed pollen diffusate, by *Botrytis*[+]

a) After incubation for 20h at 20°C

Fungus	Pod nutrients		Dialysed pollen diffusate	
	Germ tube length	% Loss of acid	Germ tube length	% Loss of acid
B. fabae	200μm	50	800μm	29
B. cinerea	50μm	11	600μm	16

b) After incubation in pod nutrients to allow growth similar to that in dialysed pollen diffusate after 20h

Fungus	Incubation (h)	Germ tube length	% Loss of acid
B. fabae	32	600μm	65
B. cinerea	45	700μm	61

\+ All measurements expressed as the mean of two replicates

Table 52. Metabolism of wyerone acid (9μg/ml) dissolved in pod nutrients or dialysed pollen diffusate, by Botrytis +

a) After incubation for 22h at 20°C

Fungus	Pod nutrients		Dialysed pollen diffusate	
	Germ tube length	% Loss of acid	Germ tube length	% Loss of acid
B. fabae	170μm	44	700μm	9
B. cinerea	56%*	1	800μm	2

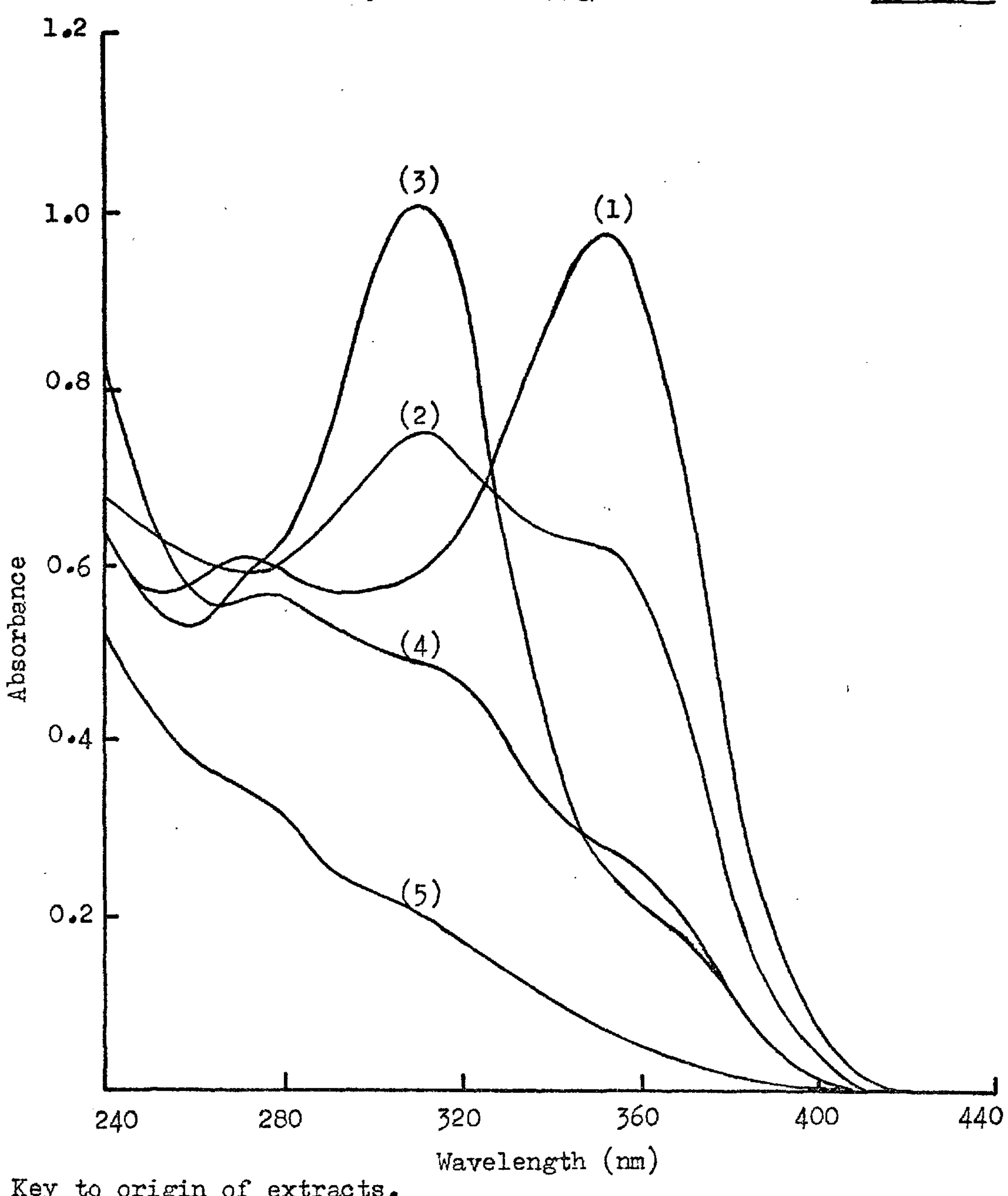
b) After incubation in pod nutrients to allow growth similar to that in dialysed pollen diffusate after 22h

Fungus	Incubation (h)	Germ tube length	% Loss of acid
B. fabae	44	700μm	76
B. cinerea	80	800μm	78

* Percentage germination, germ tube length $<$ 20μm

\+ All measurements expressed as the mean of two replicates

Figure 40. U.V. absorption spectra of ether solubles in 1 ml ethanol extracted from pod nutrients incubated in the presence or absence of wyerone acid 9µg/ml and conidia of Botrytis



Key to origin of extracts.

1) Wyerone acid solution incubated 80h without conidia
2) " " " " 22h with B. fabae
3) " " " " 44h " "
4) " " " " 80h " B. cinerea
5) Pod nutrients incubated for 80h without wyerone acid or conidia

diffusate in 20 h.

A second experiment designed to examine the metabolism of wyerone acid was prepared with solutions of 9 μg wyerone acid/ml of pod nutrients, or freshly prepared dialysed pollen diffusate. Wyerone acid levels were recorded after chromatography as described in the previous experiment, except that the ether extracts from the replicates of prolonged incubation of conidia in pod nutrients were pooled before chromatography. The results expressed as replicate means are shown in Table 52. In contrast to their development in pod nutrients, the growth of both species of Botrytis in pollen diffusate was not associated with substantial loss of wyerone acid.

The U.V. absorption spectra of the ether extracts of solutions in which Botrytis had grown with wyerone acid and pod nutrients are illustrated in Figure 40. The loss of wyerone acid (λ max 350 nm) from the B. fabae treatment was associated with the appearance of a peak of absorbance at 310 nm. Loss of absorbance at 350 nm from the B. cinerea treatment was not associated with any such defined peak of novel absorbance. Further investigations of the nature of the 310 nm absorbing substance produced in B. fabae treatments, are described in Chapter 8.

e) The ability of various nutrient sources to enable B. cinerea conidia to develop in the presence of wyerone acid, and to enhance the pathogenicity of B. cinerea when added to the infection droplet

Chou and Preece (1968) reported that orange juice, from an

Table 53. The effect of different nutrient solutions on the growth of *B. cinerea* conidia in the presence, and absence of wyerone acid (8µg/ml), and on pathogenicity when added to the infection droplet

Nutrient source	Conidial growth[4]		Production of spreading lesions 6 days after inoculation *
	Alone	+ Wyerone acid	
Orange juice			
1. Outspan 10/68	508µm	247µm	++++
2. " 9/69 i)	435µm	51%	++
3. " ii)	450µm	85%	+
4. " 6/69	435µm	61%	++
5. Maroc navel "	451µm	9%	+
6. Jaffa "	363µm	Zero	+
7. Tangerine "	292µm	Zero	0
8. Brazil "	406µm	50%	+
9. Canned jaffa"	435µm	Zero	0
Other nutrients			
Pod nutrients	450µm	83%	++++
Pollen diffusate	465µm	262µm	+++++

µm = germ tube length unless < 20µm

% = germination unless 98 - 100%

*+ = some spreading infections 0 = no spreading infections

4 = mean of 2 replicate bioassays

unspecified source, had the same effect as pollen grains and their extracts in promoting the pathogenicity of B. cinerea. An experiment was therefore designed to compare the antifungal activity of wyerone acid dissolved in various orange juices, pod nutrients and undialysed pollen diffusate, and the ability of these nutrients to stimulate the development of lesions by B. cinerea. The juice was squeezed from oranges and filtered before use or storage at -20°C. The experiment was carried out with fresh orange juice unless the source shown in Table 53 is before 6/69. The results of bioassays and the development of spreading lesions assessed from 40 infections six days after inoculation are given in Table 53.

Attention is drawn to the following features of interest in Table 53. Outspan orange juice No.1 and pollen diffusate stimulated the development of spreading infections, and rendered spores insensitive to wyerone acid. Wyerone acid was much more antifungal in the other orange juices, which were much less stimulatory to the development of infections. By contrast, pod nutrients promoted lesion development but did not overcome the sensitivity of conidia to wyerone acid *in vitro*.

f) Comparative studies of the pathogenicity of *B. fabae* and *B. cinerea* conidia in water, and *B. cinerea* in dialysed pollen diffusate, or pod nutrients

The discovery reported in the previous section that B. cinerea in pod nutrients developed spreading infections was further examined. thirteen detached leaves were inoculated, depending on their size, with up to 5 droplets of conidia of B. cinerea in water, dialysed

pollen diffusate, or pod nutrients, and of B. fabae in water. Each treatment was applied to one half leaflet of each leaf. The development of infections was measured daily up to 6 days after inoculation, and after grading by system 2, the results shown in Table 54 were obtained. Although pod nutrients did stimulate the development of spreading B. cinerea infections, they were not as active as dialysed pollen diffusate.

The effect of dilution of both dialysed pollen diffusate, and pod nutrients, on ability to induce development of spreading infections by B. cinerea was examined in two experiments. In each experiment three leaves were inoculated with each dilution on three of the half leaflets. The fourth quarter of each leaf was inoculated with B. cinerea conidia in water. Droplet numbers varied on each leaf, but there was a total of about 40 droplets of each dilution, and 100 of B. cinerea in water. The results obtained six days after inoculation are given in Tables 55 and 56. The leaves were observed to be similarly susceptible to infection by Botrytis cinerea in water.

The dilution of both stimulants resulted in a progressive loss in their ability to promote pathogenicity, but the loss occurred after fewer dilutions of pod nutrients than of pollen diffusate. The difference between the rate of loss of activity by dilution of pollen diffusate and pod nutrients is further illustrated in Figure 41.

Table 54. Comparison of lesion development caused by *B. cinerea* and *B. fabae* conidia suspended in water, and *B. cinerea* conidia suspended in dialysed pollen diffusate or pod nutrients, six days after inoculation

Inoculum		Percentage of infections in grades				
		1	2	3	4	5
B. cinerea	in water	14	70	14	2	-
"	+ pod nutrient	-	2	16	27	55
"	+ pollen diffusate	-	-	2	5	92
B. fabae	in water	-	-	2	3	95

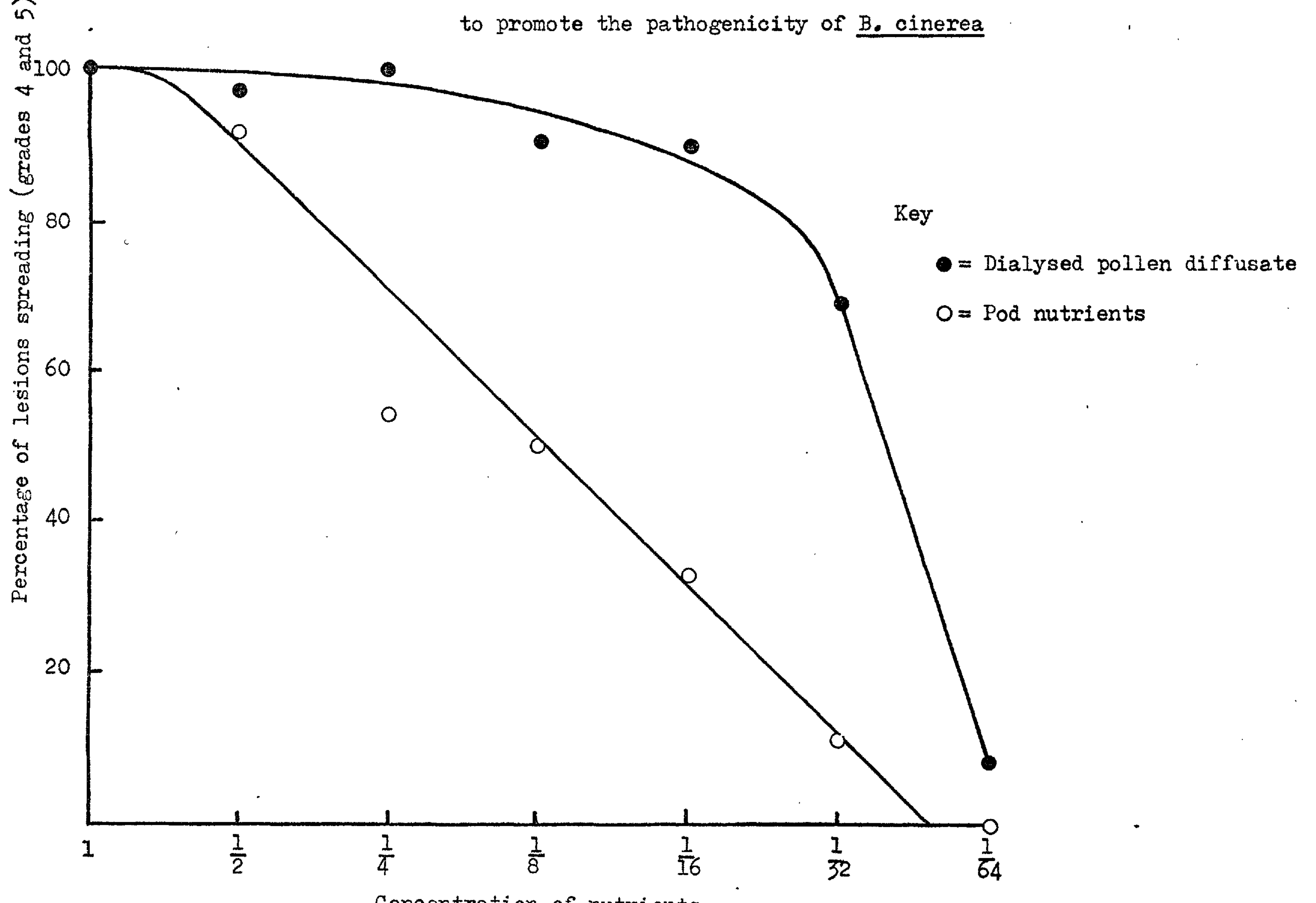
Table 55. The effect of dilution of dialysed pollen diffusate on its capacity to promote the pathogenicity of *B. cinerea* conidia

Concentration of dialysed pollen diffusate	Percentage of infections in grades 1	2	3	4	5
x 1					100
x $\frac{1}{2}$			3	6	91
x $\frac{1}{4}$				13	87
x $\frac{1}{8}$			10	29	61
x $\frac{1}{16}$			10	78	12
x $\frac{1}{32}$			31	69	
x $\frac{1}{64}$	4	84	4	8	
Water control	19	63	15	4	

Table 56. The effect of dilution of pod nutrients on its capacity to promote the pathogenicity of *B. cinerea* conidia

Concentration of pod nutrients	Percentage of infections in grades				
	1	2	3	4	5
x 1	-	-	-	8	92
x $\frac{1}{2}$	-	-	8	56	36
x $\frac{1}{4}$	-	2	44	52	2
x $\frac{1}{8}$	-	26	24	46	4
x $\frac{1}{16}$	-	54	13	33	-
x $\frac{1}{32}$	7	67	15	11	-
x $\frac{1}{64}$	25	69	6	-	-
Water control	3	78	15	4	-

Figure 41. Effect of dilution of dialysed pollen diffusate and of pod nutrients on their capacity to promote the pathogenicity of <u>B. cinerea</u>



g) Changes in the concentration of wyerone acid in leaves inoculated with *B. cinerea* in pod nutrients, or *B.fabae* in water

Though less effective than pollen diffusate, pod nutrients were clearly shown (Section f.) to break resistance to *B. cinerea*. This phenomenon provided another situation in which to examine the role of wyerone acid in controlling lesion development. It was decided to measure the changes in concentration of wyerone acid at infection sites during the first 4 days after inoculation. Lesions were selected for extraction when their appearance was similar to that of lesions produced by *B. cinerea* conidia in pollen diffusate which had already been examined (Table 45). Microscopic examination of the present infections revealed that a mesh of hyphae developed on the leaf surface within 24 h after inoculation. The last extraction was carried out on completely blackened infection sites, 88 h after inoculation, by which time tufts of hyphae had emerged from the underside of the leaf in some of the lesions.

The absorption spectra of chromatographic eluates from extracts of partially blackened infection sites excised 23 h and 44 h after inoculation, were characteristic of wyerone acid. Wyerone acid could not be identified from the U.V. absorption spectrum of the appropriate eluate derived from extracts of the completely blackened infection site excised 88 h after inoculation, despite the characteristic fluorescence of wyerone acid on the developed chromatogram under U.V. The absorption spectra of eluates from infection sites 44 h and 88 h after inoculation

are illustrated in Figure 42. The peak of absorbance of the eluate from spreading infections was at 335 nm in both 50% and absolute ethanol. The failure to identify wyerone acid by U.V. spectrophotometry and the presence of unknown U.V. absorbing compounds with similar chromatographic properties with λmax 330 - 340 nm had been previously experienced when attempting to extract wyerone acid from certain spreading infections caused by <u>B. cinerea</u> conidia suspended in water, or dialysed pollen diffusate.

The yield of substance with λmax at 335 nm was 3.8 Absorbance units at 335 nm/g fresh material. This eluate was bioassayed at a concentration of $2A_{335\ nm}$ /ml pod nutrients, against <u>B. cinerea</u> conidia in the standard manner. In pod nutrients alone conidia produced germ tubes about 450 μm in length . The addition of the eluted compounds reduced germ tube length to about 200 μm. In contrast, wyerone acid extracted from 44 h infections bioassayed in the same way at a concentration of $2A_{350\ nm}$ (18 μg) /ml pod nutrients prevented all germination of conidia.

The appearance of the lesions,and concentration of wyerone acid extracted are shown in Table 57. There was an initial increase in wyerone acid in tissues after infection. The acid was subsequently lost from the infection site as it became blackened, and colonized by the invading fungus.

Similar results were obtained after infection by <u>B. fabae</u> (Chapter 5, Section b.). The levels of wyerone acid at infection sites in leaves infected in one half leaflet with <u>B. fabae</u> in water, and in

Figure 42. U.V. absorption spectra in 50% ethanol of eluates from Rf 0.9 in chromatograms of ether soluble acids extracted from infection sites 44h and 88h after inoculation with B. cinerea conidia suspended in pod nutrients.

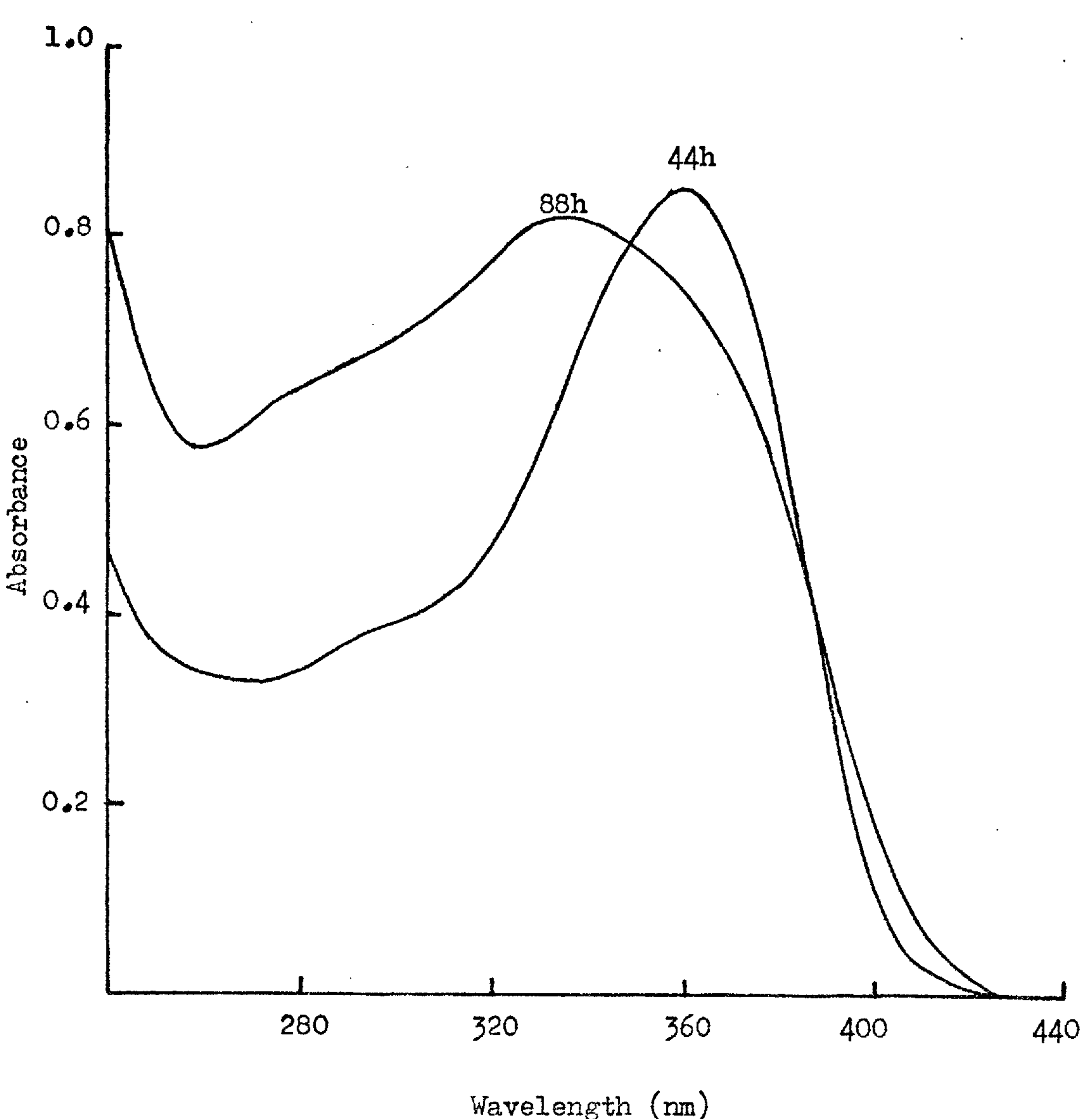


Table 57. Concentration of wyerone acid at infection sites after inoculation with *B. cinerea* conidia suspended in pod nutrients

Hours after inoculation	Appearance of leaf surface		wyerone acid (µg/g fresh material)
	Upper	Lower	
23			35.3
48			78
88			4.9*

* Wyerone acid equivalent based on antifungal activity

the other with B. cinerea in pod nutrients were therefore measured. There was more variability in the type of lesions produced by B. cinerea + pod nutrients, than by B. fabae in water. However, only lesions with similar appearance were selected for extraction 41 h and 100 h after inoculation.

Wyerone acid could be identified from the characteristic absorption spectra obtained from chromatographic eluates of infection sites extracted after 41 h. Wyerone acid, if present in the spreading infections extracted 100 h after inoculation, could not be identified by U.V. spectrophotometry because of the presence of other U.V. absorbing substances. The absorption spectrum of the eluate from spreading infections caused by B. cinerea was similar to that recorded in the previous experiment with λmax at 333 nm. The eluate from B. fabae infections extracted 100 h after inoculation had a similar U.V. absorption spectrum in 50% ethanol (λmax 300 nm infl.360 nm) to that recorded in Chapter 5, Figure 30, for an eluate similarly extracted from the infection site at the centre of a spreading lesion caused by B. fabae.

The antifungal activity of the eluates from spreading infections at fresh weight concentration/ml pod nutrients was measured against B. cinerea conidia by the standard bioassay. The presence of the eluate from B. fabae infection reduced the length of germ tubes produced in pod nutrients from 500 μm to 120 μm. The eluate from B. cinerea + pod nutrients infection allowed germ tube growth of 180 μm. The wyerone acid equivalents of inhibitor extracted/g fresh

Table 58. Levels of wyerone acid at infection sites after inoculation with *B. cinerea* conidia suspended in pod nutrients or *B. fabae* conidia suspended in water

Hours after inoculation	Appearance of leaf surface		Wyerone acid (µg/g fresh material)	
	Upper	Lower	*B. fabae*	*B. cinerea* + pod nutrient
44			84.1	83.4
100			3.7*	2.9*

* Wyerone acid equivalent based on antifungal activity

material were calculated from the results of bioassays on these eluates in order to enable comparison with the concentrations of wyerone acid extracted from infections at the earlier stage of development, as shown in Table 58.

The experiment on the ability of dilutions of pod nutrients to enhance the pathogenicity of B. cinerea, described in Section 6, was examined eight days after inoculation; although several of the leaves were yellowing, there remained a green ring of tissue around numerous Grade 3 and 4 lesions which had developed after various treatments. It was considered that the delayed senescence of the cells within the green ring might have been associated with the production of wyerone acid, as the leaf's last attempt to resist infection.

Despite the spread of some of the lesions beyond the infection site in the upper part of the leaf, there was very little browning on the underside of the leaf, beneath the droplet area. These limited lesions, and their peripheral green islands were cut out and extracted. Absorption spectra characteristic of wyerone acid were obtained for chromatographic eluates from both extracts. The concentrations of wyerone acid in these tissues are reported in Table 59. Very high concentrations of wyerone acid were obtained from the limited lesions. Wyerone acid was present in the green islands, but at a much lower level than within the lesions. It is conceivable that if large amounts of wyerone acid were produced in the green islands they were translocated into the tissue bearing the lesion.

Table 59. Concentration of wyerone acid in limited lesions and surrounding "green islands" produced by *B. cinerea* conidia suspended in pod nutrients

Tissue	Wyerone acid (µg/g fresh material)
Lesion	156
Green island	36

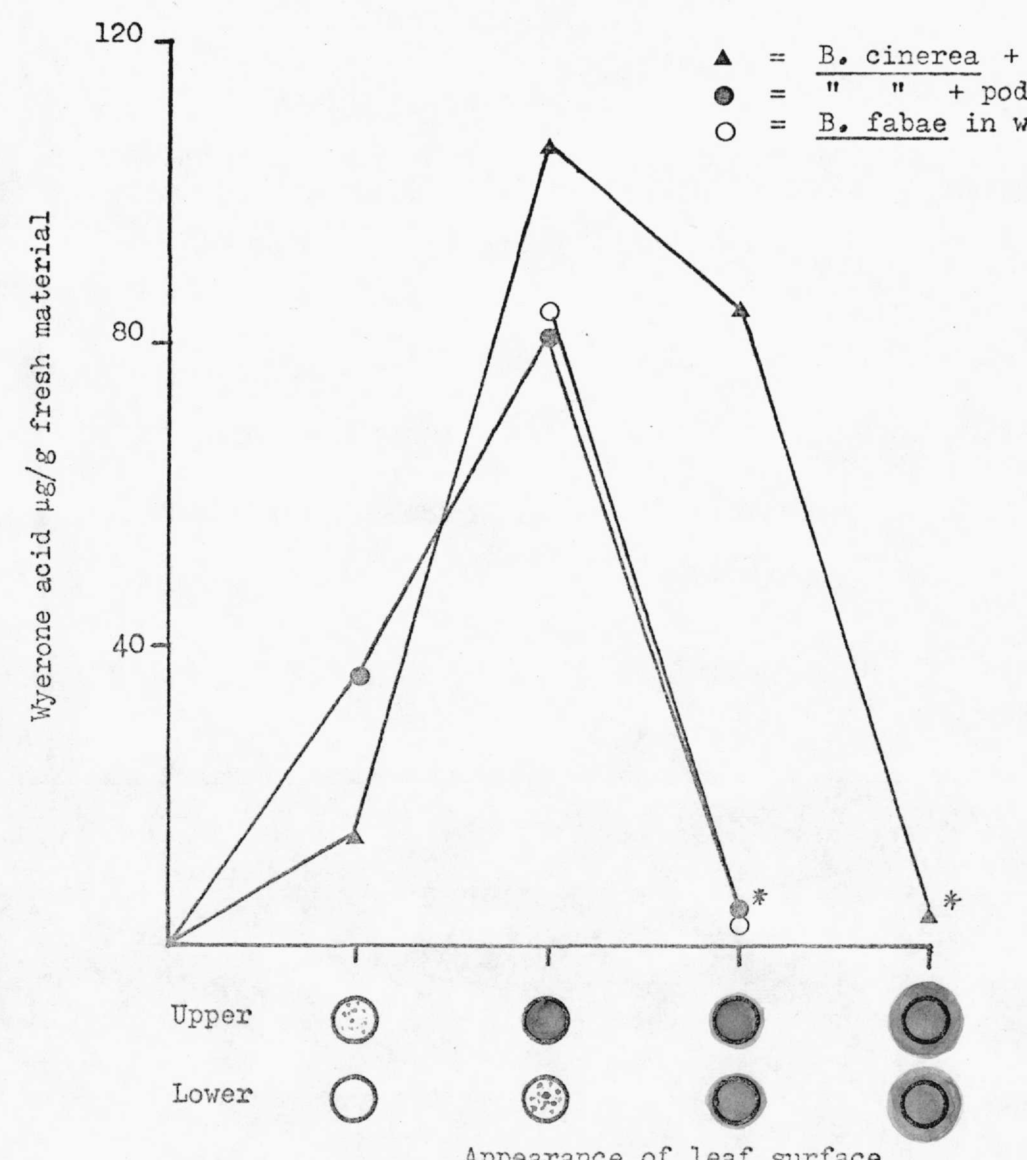
h) Summary

B. cinerea conidia suspended in pollen extracts, pod nutrients, and one source of orange juice produced a high percentage of spreading lesions in leaves. The effects of dialysed pollen diffusate, and pod nutrients were examined in detail. The pattern of loss, by dilution, of the ability to promote infection was different for the two stimulants. This suggested that different factors were active in pollen and pod nutrients.

The changes in concentration of wyerone acid extracted as infections progressed are summarized in Figure 43. Wyerone acid accumulated in lesions produced after inoculation with B. cinerea in either dialysed pollen diffusate, or pod nutrients; but the highest concentrations were detected in tissues infected with B. cinerea in pollen. The changes in concentration of wyerone acid after inoculation with B. cinerea in pod nutrients were similar to those found after inoculation with B. fabae in water. Thus the development of spreading lesions after inoculation with B. cinerea in pod nutrients was associated with a loss of wyerone acid from the infection site. In contrast, high yields of wyerone acid were extracted from spreading infections after inoculation with B. cinerea in dialysed pollen diffusate. Eventually, however, wyerone acid disappeared from large spreading lesions caused by B. cinerea in pollen diffusate.

The activity against B. cinerea of wyerone acid in dialysed pollen diffusate (pH 5.3) was examined. Pollen diffusate overcame the antifungal activity of wyerone acid observed when it was dissolved in

Figure 43. The average concentration of wyerone acid in lesions extracted at similar stages of development after inoculation with conidia of B. fabae suspended in water, or B. cinerea in pod nutrients, or dialysed pollen diffusate.



(* At these stages of lesion development wyerone acid equivalents of antifungal activity were measured.)

pod nutrients (pH 4.5). Dialysed pollen diffusate did not have a direct effect on wyerone acid. It was therefore concluded that pollen extracts changed the sensitivity of Botrytis to wyerone acid.

The development of B. cinerea and B. fabae from conidia in pod nutrients was associated with the metabolism of wyerone acid. B. fabae appeared to metabolize the acid at a faster rate than B. cinerea. Growth of either species in dialysed pollen diffusate was not associated with a similar loss of acid. It was concluded that the decreased sensitivity of Botrytis to wyerone acid in pollen extract in vitro was not mediated by an increased rate of metabolism of the acid.

Chapter 8. Investigation of a metabolite produced by the interaction of *B. fabae* with wyerone acid

It was shown in Chapter 7 that an ether soluble fraction with λmax in ethanol at 310 nm was produced by *B. fabae* as wyerone acid disappeared from solution in pod nutrients. It was suggested that the novel U.V. substance was a conversion product of wyerone acid. Deverall (1967) recorded an increase in absorbance at 310 nm in ethanol of the ether solubles extracted from *B. fabae* infection droplets during 28 h incubation in pod seed cavities. These observations suggested that the same metabolite was produced by *B. fabae* *in vivo* and *in vitro*. It was decided to investigate the appearance of the 310 nm absorbing fraction and its relationship, if any, to wyerone acid.

a) Chromatographic separation of the metabolite derived from *B. fabae* and wyerone acid, *in vitro*

It was decided to attempt to separate the material with λmax 310 nm in ethanol produced by *B. fabae*, which replaced wyerone acid (λ max 350 nm) as the principal U.V. absorbing substance in ether extracts of incubation mixtures. The ether solubles with λ max in ethanol 310 nm, extracted from incubation mixtures after prolonged incubation of *B. fabae* in pod nutrients + 9 μg wyerone acid/ml. (Chapter 7 Section d) were applied to 5 cm origins on "Whatman 3 MM" paper. The paper was developed in n-propanol/water (12.5/87.5) until

the front was 17 cm above the origin. The dried chromatograms were examined under U.V., and bands of fluorescence marked, cut out, and eluted with 50% ethanol. The remainders of the chromatograms were cut into bands of known R_F, not more than 1 cm in width. The eluates were examined by U.V. spectrophotometry.

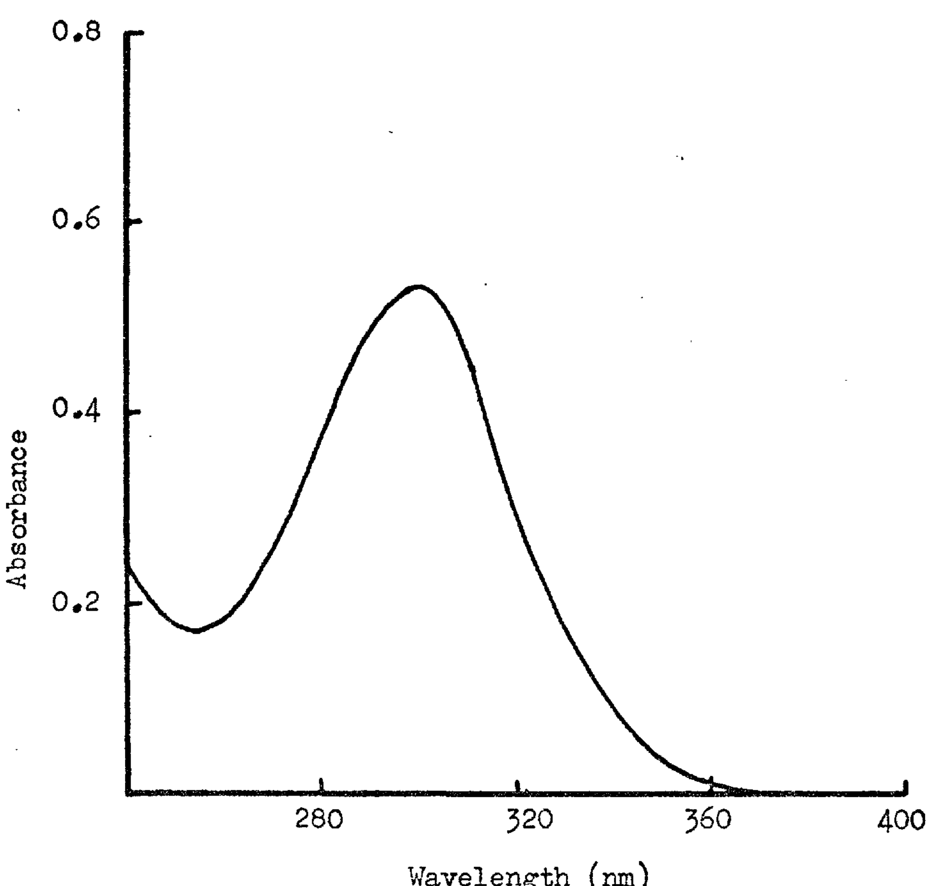
Wyerone acid was recognised by its characteristic absorption spectrum in the eluates from a band with blue fluorescence under U.V., which ran to R_F 0.9 on chromatograms. Apart from this, only the eluate from R_F 0.95 absorbed light at wavelengths greater than 280 nm, and this had been derived from a dark U.V. absorbing band on the paper. The U.V. absorption spectrum of the eluate from R_F 0.95 in 50% ethanol is illustrated in Figure 44, and it can be seen that the λmax was 300 nm.

This eluate, from R_F 0.95, λmax 300 nm, was taken to dryness, resuspended in ethanol and re-examined by U.V. spectrophotometry. The λmax was now 310 nm. It was concluded that the fraction had different λmax of 300 nm and 310 nm in 50% ethanol and ethanol respectively. A shift of 10 nm in λmax in aqueous and absolute ethanol is a characteristic of wyerone acid (Chapter 4).

It was calculated that the loss of 10 μg of wyerone acid in the presence of <u>B. fabae</u> was associated with the appearance of 0.49 Absorbance units at λmax 300 nm (50% ethanol). The compound responsible for this absorbance will henceforth be termed 'substance I'.

(The production of 'substance I' by <u>B. fabae</u> <u>in vitro</u> was confirmed when this experiment was repeated. In the second experiment

Figure 44. U.V. absorption spectra in 50% ethanol of the eluate Rf 0.95 in the chromatogram of ether solubles of bathing solutions of wyerone acid incubated in the presence of B. fabae.



the loss of 10 μg of wyerone acid was associated with the appearance of 0.55 Absorbance units of 'substance I').

b) Investigation of the partition of the metabolite between aqueous solutions and ether

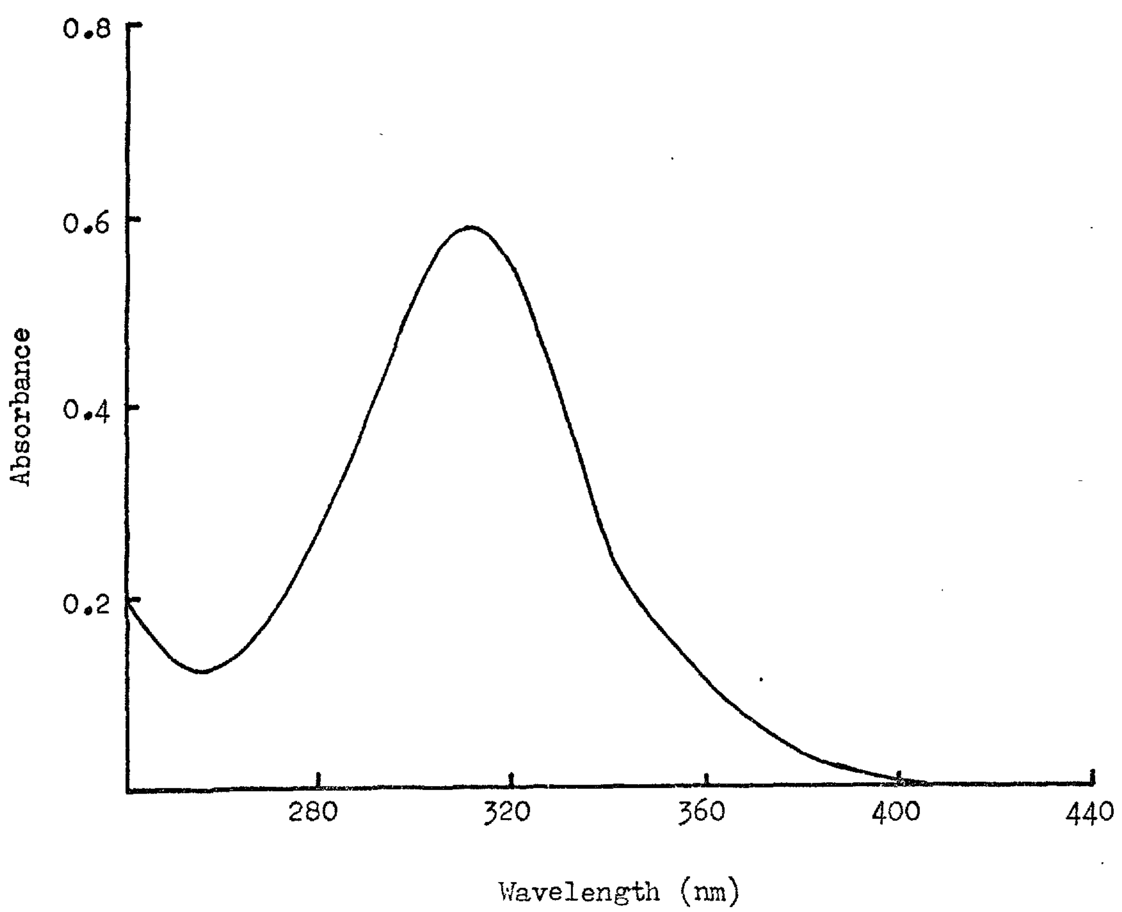
The ethanolic solution of 'substance I' prepared in the previous experiment was taken to dryness and the basic plus neutral, and acidic ether solubles were extracted from aqueous solution of the residue as described for leaf diffusates in Materials and Methods. All absorbance at 310 nm in ethanol was recovered in the fraction of ether soluble acids. It was concluded that 'substance I' behaved as an ether soluble acid.

c) Extraction of the metabolite and wyerone acid from *B. fabae* infection droplets incubated in pod seed cavities.

Deverall (1967) reported that ether soluble material absorbing light at 310 nm in ethanol accumulated in *B. fabae* infection droplets in pod seed cavities, and possibly marked the spectrophotometric detection of the 360 nm absorbing phytoalexin. An experiment was therefore designed to detect and measure the concentration of 'substance I' and wyerone acid in *B. fabae* infection droplets incubated in pod seed cavities.

Pods of the field bean 'Minor' were inoculated with a suspension of *B. fabae* conidia in water, and incubated at 17°C in the dark. Diffusates were collected 12, 24, 43, 62 and 86 h after inoculation. The ether solubles were extracted from the diffusates and examined in ethanolic solution by U.V. spectrophotometry.

Figure 45. U.V. absorption spectrum in ethanol of total ether solubles extracted from B. fabae infection droplets incubated in pod seed cavities for 43h.



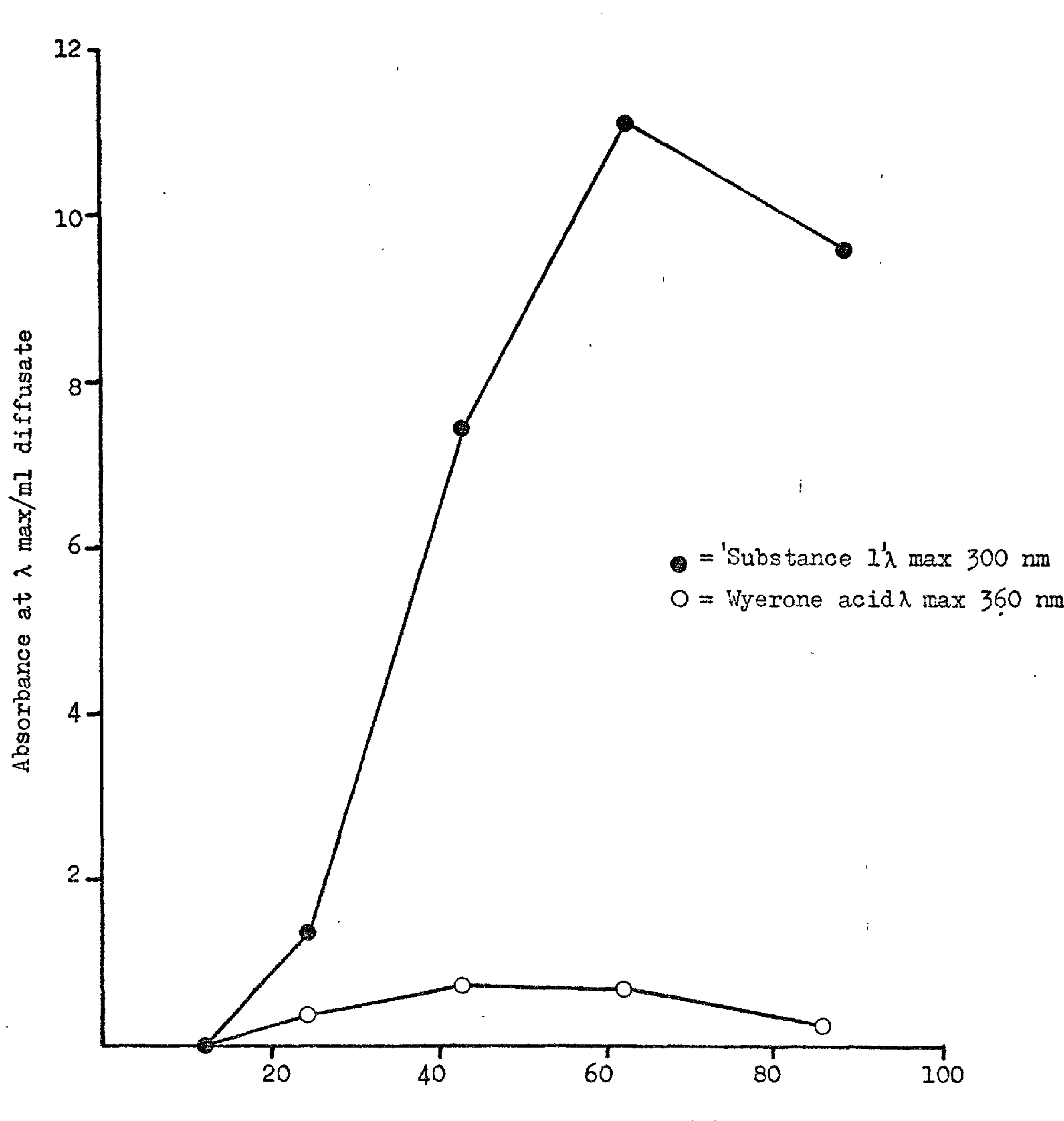
(The extract was dissolved in a volume of ethanol 15 x the volume of the diffusate from which it was derived)

The extracts all had λmax at 310 nm but the absorbance at 310 nm per ml of diffusate varied from 0.16 after 12 h to about 14.0 after 62 h. The U.V. absorption spectrum of the ether extract of diffusates collected 43 h after inoculation is illustrated in Figure 45. A sample of this extract was taken to dryness and resuspended in 50% ethanol. The λmax shifted to 300 nm indicating the presence of a large amount of 'substance I'.

The acidic fraction was separated from the ether solubles. These ether soluble acids were chromatographed on "Whatman 3 MM" in n-propanol/water, before examination under U.V. light. A blue fluorescent band had run to R_F 0.9, and a dark U.V. absorbing band to R_F 0.95 on all chromatograms except that of the extract of diffusates collected 12 h after inoculation. Bands at R_F 0.9 and 0.95 were eluted and examined by U.V. spectrophotometry. Where wyerone acid and 'substance I' could be recognised by their characteristic U.V. absorption spectra, the O.D. at λmax in 50% ethanol, 360 nm and 300 nm respectively, were calculated per ml of diffusate and these are expressed graphically in Figure 46. Wyerone acid and 'substance I' could not be identified by U.V. spectrophotometry in the diffusate collected 12 h after inoculation. At all other times 'substance I' was the principal U.V. absorbing ether soluble substance in these diffusates from B. fabae infected pods.

It was estimated from the change in concentration of 'substance I' that the maximum rate of increase per ml of diffusate was achieved 38 h after inoculation, when the tissue underlying the

Figure 46. Concentrations of 'Substance I' and wyerone acid in diffusates from pods infected with B. fabae



infection droplet was only partially blackened. The maximum concentration of 'substance I' extracted 60 h after inoculation when the underlying pod tissues were completely blackened and the infection had started to spread into the surrounding endocarp. (see plate 32)

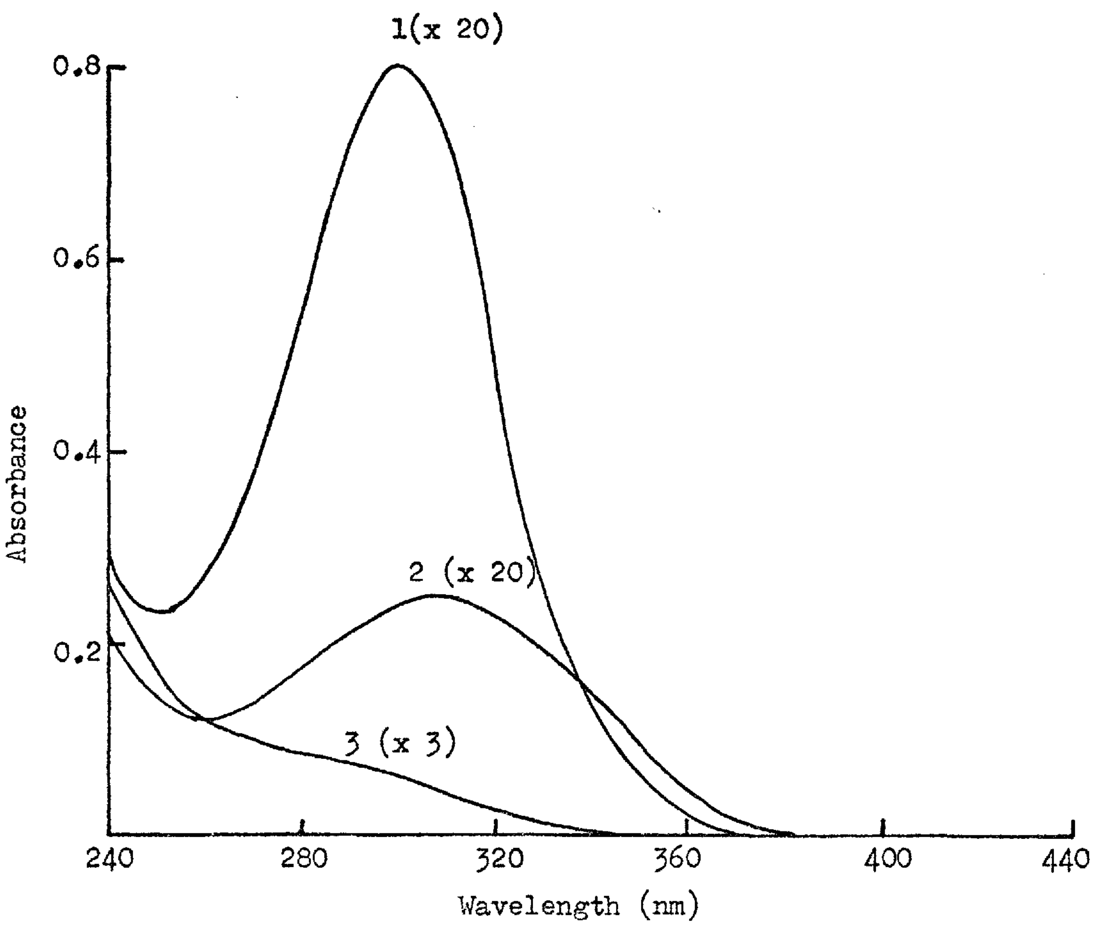
d) The extraction of the metabolite from healthy leaves, and spreading Botrytis infections

This section reports attempts to detect 'substance I' in healthy leaves, and in leaves which had succumbed to major infections caused by B. fabae or B. cinerea supplemented with stimulants.

Leaves were inoculated with 10 μl droplets of water alone or suspensions of B. fabae conidia in sterile distilled water, or B. cinerea conidia in pod nutrients. The infection droplets above spreading infections that developed were removed 100 h after inoculation, and the lesions cut out for extraction of the ether soluble acids. Similarly water droplets were removed, and underlying discs of healthy tissue cut out for extraction at the same time.

The U.V. absorption spectra of aqueous ethanolic eluates from R_F 0.95 in chromatograms of the ether soluble acids extracted from infected and healthy tissue are illustrated in Figure 47. The spectrum of the eluate from B. fabae lesions was characteristic of 'substance I', λmax 300 nm. There was no 'substance I' in healthy tissue. If present 'substance I' could not be identified by U.V. spectrophotometry in the eluate from the B. cinerea lesions, due to the presence of other U.V. absorbing compounds with λmax 310 nm in

Figure 47. U.V. absorption spectra in 50% ethanol of eluates from Rf 0.95 in chromatograms of ether soluble acids extracted from healthy leaves and spreading *Botrytis* infections.



Key to origin of chromatographic eluates
(1) Spreading lesion produced by *B. fabae* conidia in water
(2) Spreading lesion produced by *B. cinerea* conidia in pod nutrients
(3) Healthy leaves

(Figures in parentheses indicate the volume of aqueous ethanol relative to fresh weight of tissue extracted)

50% ethanol.

It was calculated that 'substance I' was extracted from the spreading lesion caused by B. fabae at a concentration of 17.3 absorbance units at 300 nm (50% ethanol) per g. fresh material.

e) Changes in concentrations of the metabolite at B. fabae infection sites in leaves

It was decided to measure the concentration of 'substance I' at B. fabae infection sites in leaves at different times after inoculation. The diffusates and tissues cut from the infection site were collected 18, 46, 85, and 116 h after inoculation. The concentrations of wyerone acid recovered from infection sites at these times have already been described in Chapter 5.

The concentrations of 'substance I' extracted from tissue and diffusates and calculated total yield are recorded in Table 60. 'Substance I' could not be identified by U.V. spectrophotometry at the infection site 18 h after inoculation. 'Substance I'concentration increased until the tissue became completely blackened and infected by B. fabae. This contrasts with the decrease in wyerone acid concentration (Table 29) between 46 and 85 h, and is consistent with the concept of 'substance I' as a product of the metabolism of wyerone acid by B. fabae.

f) Antifungal activity of the metabolites against B. cinerea

The antifungal activity of 'substance I' was tested against B. cinerea conidia. Samples of 'substance I' extracted from both leaf diffusates and tissues infected with B. fabae were bioassayed at

Table 60. Yield of 'Substance I' from infection sites inoculated with *B. fabae* conidia

Hours after inoculation	Appearance of leaf surface Upper	Appearance of leaf surface Lower	Yield* of 'Substance I'(per g.fresh leaf tissue) Tissue	Diffusate	Total
18h			0	0	0
46h			6.3	1.9	8.2
85h			11.0	4.2	15.2
116h			16.1	-[+]	16.1

* Absorbance units at 300 nm in 50% ethanol

\+ Insufficient diffusate collected for extraction

Table 61. Antifungal activity of Substance I against *B. cinerea* conidia

Source	Concentration (Absorbance/ml)	Germ tube length (μm*)
Leaf diffusate	2	500
	20	200
Leaf tissue	2	500
	20	200
Pod nutrients alone		500

* Mean of 3 replicate bioassay drops germ tube length estimated to nearest 50 μm

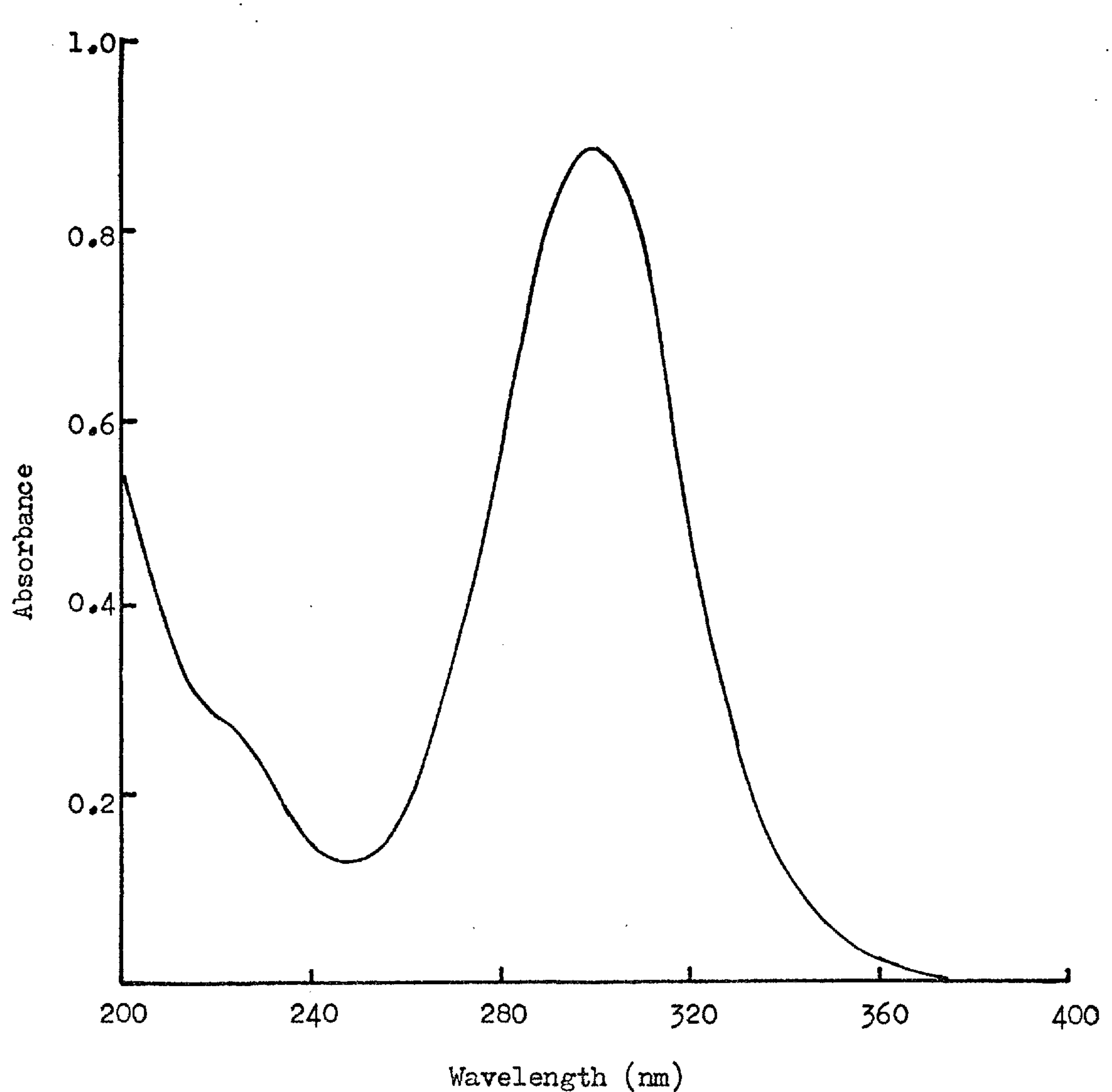
concentrations of 2 and 20 Absorbance units at λmax 300 nm (50% ethanol) per ml of pod nutrients. The results of the bioassays are given in Table 61. 'Substance I' possessed the same activity whether extracted from diffusates or tissues. It was concluded that on the basis of O.D. at λmax 'substance I' was much less antifungal than wyerone acid, because 2 such Absorbance units of wyerone acid (18 μg/ml) were completely inhibitory.

g) Preparation and identification of the metabolite from *B. fabae* pod diffusate

Some of the previous experiments provided evidence in favour of the hypothesis that 'substance I' was a metabolite of wyerone acid produced by *B. fabae* both *in vitro* and *in vivo*. In order further to test this hypothesis it was decided to identify 'substance I' prepared from *B. fabae* pod diffusates by solvent partition and thin layer chromatography.

Fifty-one ml of *B. fabae* pod diffusate were collected 70 h after inoculation. The ether soluble acids were extracted by solvent partition, and chromatographed on a Merck 2.5 mm thick T.L.C. plate developed in ether : methanol (2:1). 'Substance I' was eluted with 50% ethanol from a band at R_F 0.8, which quenched the fluorescence of the silica gel under U.V. (254 nm). None of the eluates from the remainder of the chromatogram possessed λmax at 300 nm in aqueous ethanol. The eluate containing 'substance I' was taken to dryness, and the residue applied in ethanol to a second T.L.C. plate which was developed in chloroform : methanol (1:1). The principal band detected

Figure 48. U.V. absorption spectrum in 50% ethanol of 'Substance I' prepared from B. fabae pod diffusates by solvent partition and TLC.



under U.V. was eluted. The U.V. absorption spectrum of the eluate from T.L.C. is illustrated in Figure 48.

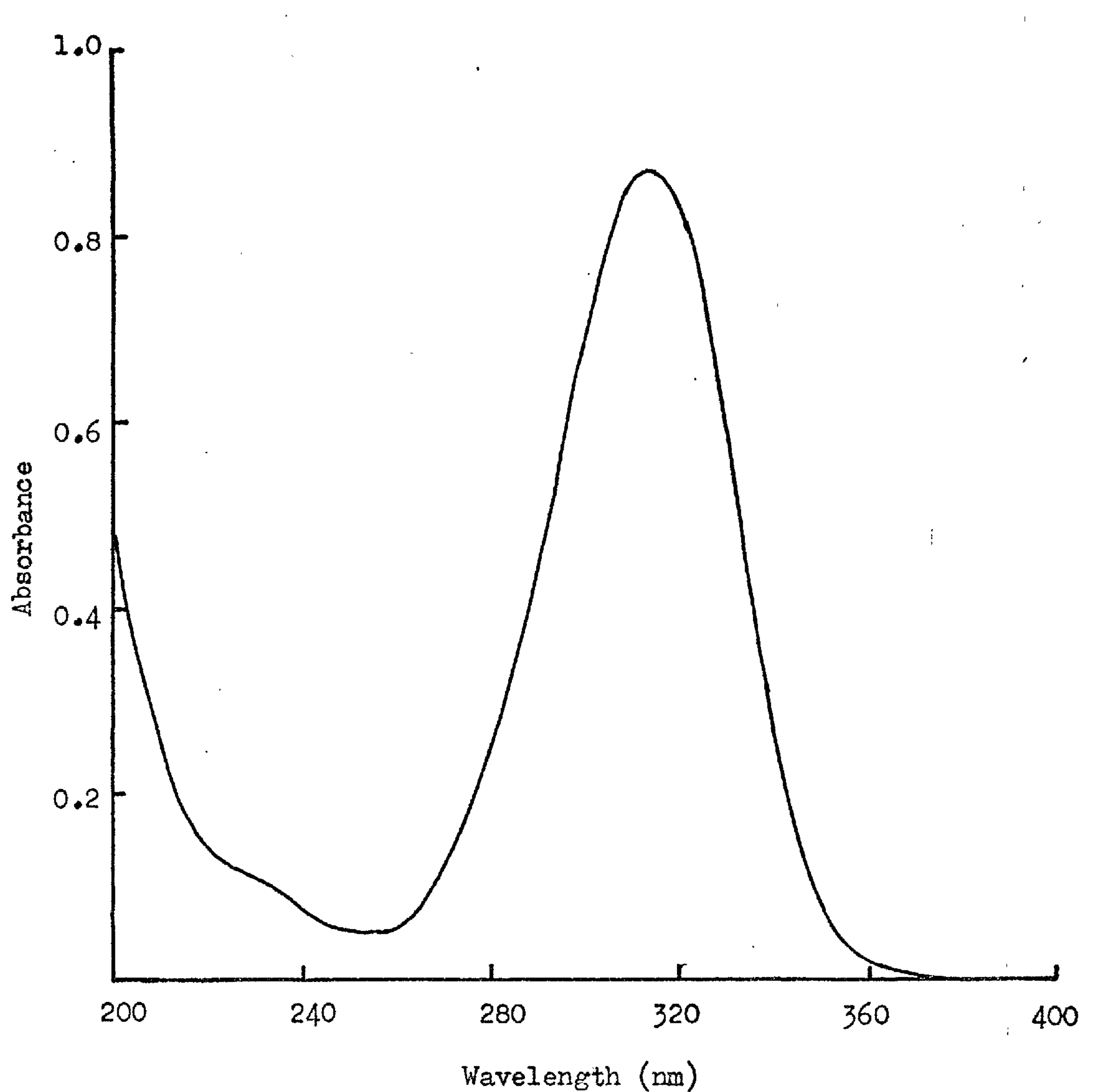
A sample of the eluate from T.L.C. was chromatographed on "Whatman" 3 MM paper in n-propanol/water. It ran to R_F 0.95 and was detected before elution as a dark U.V. absorbing band. The similar properties of U.V. absorbance and behaviour on paper suggest that 'substance I' prepared by T.L.C. was the same compound as that prepared by paper chromatography in previous experiments.

To facilitate spectroscopic analyses the eluate from T.L.C. described as 'substance I' was converted to its methyl ester by treatment with diazomethane. The behaviour of a sample of the reaction products was examined on a Merck T.L.C. 0.25 mm plate developed in ethyl acetate. The dried chromatogram was examined under U.V. The principal band separated from the reaction mixture ran to R_F 0.93. The corresponding ethanolic eluate was associated with λmax at 312 nm. This faster running fraction was considered to contain esterified 'substance I'.

Chromatographs of samples of the methylation products were developed in a range of solvents. Ethyl acetate : pet ether (3:1) separated the principal product, with λmax 312 nm (ethanol), now described as methylated 'substance I', at R_F 0.74, from unmethylated 'substance I' at R_F 0.1.

The bulk of the reaction products were separated by T.L.C. in ethyl acetate : pet ether (3:1). The U.V. absorption spectrum of methylated 'substance I' eluted in ethanol from R_F 0.78 is illustrated

Figure 49. U.V. absorption spectrum in ethanol of the methyl ester of 'Substance I' purified by TLC



in Figure 49. The methyl ester was examined by infra red, proton magnetic resonance and mass spectrometry.

In addition, the unmethylated compound was eluted from R_F 0 - 0.2 in the chromatogram and again treated with diazomethane. Methylated 'substance I' was separated from the reaction products by T.L.C. as previously described except that it was eluted from the silica gel with chloroform. The methylated sample was further purified by repeated T.L.C. developed in benzene : ether (9:1), with intervals for drying. After two runs both allowing the solvent front to reach 10 cm above the origin, methylated 'substance I' was detected as a band at R_F 0.43 which quenched the fluorescence of the silica gel. Spectroscopic analyses were repeated on the methyl ester eluted in chloroform, and this gave similar results to the first sample of methylated 'substance I' examined.

The spectroscopic analyses and interpretation of the results were carried out by Dr. D.A.Widdowson, Department of Chemistry, Imperial College.

The infra red absorption spectrum of the methyl ester had defined bands at ν max 3450 cm^{-1} broad (OH), 1722 cm^{-1} (ester C = O), 1640 cm^{-1} (C = C), 1200 cm^{-1} (C - O), 1018 cm^{-1} and 855 cm^{-1} (furan).

The proton magnetic resonance spectrum obtained for methylated 'substance I' is described in Figure 50.

The significant peaks observed in the mass spectrum, and their proposed origin are described in Table 62. Measurement of metastable peaks indicated fragmentation of M^+ 264 $\rightarrow$ $^m/_e$ 194,

Figure 50. Proton magnetic resonance spectrum of methylated substance I

Absorbance τ	2.68	3.80	3.53	3.76	4.68	5.36	6.29	7.70–8.30	9.08
No. of protons	1H	1H	1H	1H	2H	1H	3H	6H	3H
Splitting*	d	d	d	d	m	t	s	m	t
Coupling constant J(Hz)	16	16	3.5	3.5	–	6	–	–	7
Assignment to formula below	2-H	3-H	5-H	6-H	11,12-H	8-H	O-Me	9,10,13-H	14-H

0 1 2 t 3 4 5 6 7 8 . 9 10 11 ? 12 13 14

$Me\ O_2\ C \cdot CH = CH \cdot C{:}CH \cdot CH{:}C \cdot CH(OH) \cdot CH_2 \cdot CH_2 \cdot CH = CH \cdot CH_2 \cdot CH_3$

O

(* d = doublet, s = singlet, t = triplet, m = multiplet)

Table 62. Characteristic fragments in the mass spectra of methylated substance I

Significant peaks	Proposed origin
264	Molecular ion M^+
246	$M^+ - H_2O$
233	$M^+ - MeO$
231	$M^+ - Me - H_2O$
207	$^m/_e$ 233 - $HC \equiv CH$ (Furan fragmentation)
194	$M^+ - C_5H_{10}$ (side chain)
181	$M^+ - C_6H_{11}$ (side chain)
149	$^m/_e$ 181 - MeOH
121	$^m/_e$ 149 - $C \equiv O$

Figure 51. Proposed Mass spectral fragmentation of methylated 'Substance I'

I

MeOOC ... OH

M^{+} 264

↓

MeOOC ... OH

M/e 181

↓ −MeOH

M/e 149

↓

M/e 121

Figure 51. Proposed Mass spectral fragmentation of methylated 'Substance I'

II

Figure 52.

$$CH_3.CH_2.CH\overset{c}{=}CH.C\equiv C.CO.C{=}CH.CH{=}C.CH\overset{t}{=}CH.COOH$$

(O bridge between the C after CO and the C after CH=, shown below the chain)

Wyerone acid

$$CH_3.CH_2.CH\overset{?}{=}CH.CH_2.CH_2.CH(OH)C{=}CH.CH{=}C.CH\overset{t}{=}CH.COOR$$

(O bridge between the C after CH(OH) and the C after CH=, shown below the chain)

$R{=}CH_3$, Methylated Substance I

$R{=}H$, Substance I (Reduced wyerone acid)

$^m/e$ 181 → 149, and $^m/e$ 233 — 207. The proposed patterns of fragmentation of the parent ion are illustrated in Figure 51. Accurate mass measurements were made of the parent ion M.W = 264.1363 ($C_{15}H_{20}O_4$ requires 264.1362), and the fragment $^m/e$ 181, M.W = 181.0494 ($C_9H_9O_4$ requires 181.05007).

The structures determined for methylated 'substance I' and 'substance I' are compared with the structure of wyerone acid reported by Letcher et al. (1970) in Figure 52. The configuration of the side chain double bond, and stereochemistry of the assymetric centre in 'substance I' could not be determined.

The structural similarity between 'substance I' and wyerone acid provided further evidence that 'substance I' was a product of the metabolism of wyerone acid by B. fabae. 'Substance I' appears to be a reduced form of wyerone acid, having undergone reductions in the acetylenic and keto- parts of the molecule.

h) Investigation of the metabolism of wyerone acid by "cell free" preparations of diffusates from B. fabae infected pods

The possibility that extra-cellular enzymes capable of degrading wyerone acid to 'substance I' were present in diffusates from B. fabae infected pods was investigated.

"Cell free" preparations of diffusates were prepared 38 h after inoculation, the time at which it was estimated (Section 3) that the most rapid increase in 'substance I' concentration occurred in infection droplets. The prepared diffusates were enriched with 15 μg

Table 63. U.V. absorbance of ether extracts of cell free *B. fabae* pod diffusates enriched with 15µg wyerone acid/ml.

Incubation time at 25°C (h)	Absorbance/ml. at selected wavelengths*			
	Autoclaved		Unautoclaved	
	310 nm	350 nm	310	350
0	-	-	12.4	4.6
1	12.8	5.0	12.3	4.4
5	12.0	4.54	12.8	4.6
25	12.7	4.4	12.1	4.45

* Mean of two replicates

Table 64. Concentrations of wyerone acid and 'Substance I' extracted from 'cell free' *B. fabae* pod diffusates enriched with 15µg wyerone acid/ml. incubated for 25 h at 25°C.

Compound	Parameter	Concentration/ml of diffusate*	
		Autoclaved	Unautoclaved
'Substance I'	Absorbance at λ max 300 nm	6.3	6.3
Wyerone acid	Absorbance at λ max 360 nm	2.3	2.1
	µg	20.7	19.3

* Mean of two replicates

wyerone acid/ml, autoclaved and unautoclaved samples were incubated at 25°C for 1, 5 and 25 h as described in Materials and Methods. After different periods of incubation the ether solubles were extracted from the enriched diffusates and examined in ethanol by U.V. spectrophotometry. The O.D of the solutions at wavelengths 310 nm and 350 nm were measured, and are expressed per ml of diffusate in Table 63.

The ether solubles from diffusates incubated for 28 h were further separated into basic and neutral, and acidic fractions. Wyerone acid and 'substance I' were separated from other ether soluble acids by paper chromatography, and identified by their characteristic absorption spectra in aqueous ethanolic eluates. The concentrations of these compounds extracted are recorded in Table 64.

As similar concentrations of wyerone acid and the metabolite were extracted from autoclaved and unautoclaved diffusates after incubation for 25 h at 25°C it was concluded that there were no enzymes capable of metabolizing wyerone acid present in the cell free diffusate, and that the reduction of wyerone acid presumably occurred in the hyphae of <u>B. fabae</u>.

DISCUSSION

a) Development of lesions caused by *B. cinerea* in water.

Conidia of the C_3 isolate of *B. cinerea* rarely produced spreading infections when inoculated in water, and frequently caused few or no symptoms at the infection site during the first 6 days after inoculation. Therefore the C_3 strain possessed pathogenicity intermediate between the isolate used by Purkayastha and Deverall (1965a), which failed to develop spreading infections; and that used by Chou and Preece (1968) which frequently spread from infection sites.

Variation in the type of lesion produced in leaves by conidia of *B. cinerea* in water has been reported by several workers; Deverall and Wood (1961a), Wastie (1962), Purkayastha and Deverall (1965a), and, Chou and Preece (1968). In this study the use of the more precise grading system 3 revealed the range of lesion types in more detail. Thus it has been shown that several different types of limited lesion can be produced, each with a particular pattern of symptom development.

In any discussion of the cause of the observed variation it is necessary to consider the relationship between observed fungal development, and symptom development at each inoculation site. It has been shown that spreading infections are the result of the complete colonisation and blackening of leaf tissues by the fungus. The relationship between the development of the fungus, and of

symptoms at other inoculation sites is less clear.

Quantitative studies of fungal development were confined to measurements of growth on the leaf surface. There is a lack of evidence available on the extent of fungal invasion of the leaf in limited infections. Knowledge of this very important feature of infection is restricted to the qualitative observations of free hand sections of inoculation sites. These histological studies indicated that in limited infections hyphae extended no further than into a single epidermal cell. This is in accord with the finding of Purkayastha (1965), who described the limitation of fungal invasion to the production of infection pegs in the epidermal cell. However, there is no evidence available on the percentage of hyphae which penetrate the epidermis at the sites where most conidia germinate in the infection droplet. Although penetration was detected relatively infrequently here, and by Purkayastha (1965) it cannot be assumed that it only rarely takes place. The chance of cutting a section in the same plane as a small infection peg is very low and moreover the invading hypha may be masked by deposits of reaction material as described by Skipp (1971).

Clearly more detailed histological studies involving light or even electron microscopy of serial sections of embedded material are necessary before any direct conclusions can be drawn on the problem of penetration. However circumstantial evidence concerning the frequency of penetration was obtained from the studies of the biological activity of diffusates from leaves. Infection droplets collected from

sites which developed substantial browning ($\geqslant$ grade 19 lesions) stimulated germ tube extension by B. cinerea conidia. This shows that fungal growth was not inhibited in the droplets, on the leaf surface, and therefore implies that the majority of hyphae did penetrate the epidermis, but hyphal growth was inhibited from within the infected tissue at these sites. Conversely droplets from above sites which developed little or no symptoms during the first days after inoculation (grade 0 and 6.5) became inhibitory. It is therefore possible that growth was inhibited on the leaf surface at these latter sites and that most germ tubes here may have failed to penetrate.

The division of lesion types into two populations with more or less than grade 19 infection was also suggested from the detailed studies of the relationship between browning of the leaf surface and germ tube growth, 4 days after inoculation. These studies showed that the type of limited infection produced was related to the length of germ tubes produced on the leaf. The longer the germ tubes produced, the greater was the extent of browning beneath the infection droplet. Sites where little or no symptoms were produced were associated with short germ tubes which frequently failed to produce appressoria.

Differences between the conditions at infection sites were clearly indicated 12h after inoculation, before symptoms had developed, by the detection of both stimulation, and inhibition of fungal growth on leaves compared with that on glass slides. It is

proposed that at those sites where growth was inhibited hyphae would fail to penetrate, and therefore that little or no visible symptoms (grades 0 and 6.5) would develop during the first 6 days after inoculation. At other sites, particularly where conidial germination and germ tube growth was stimulated hyphae would penetrate the epidermis, and give rise to substantial browning beneath the infection droplet. This proposal implies that the type of limited lesion produced at each infection site was determined by the conditions in the infection droplet during the first 12h after inoculation. It is therefore necessary to consider the possible causes of the different fungal behaviour in droplets.

The failure of most conidia to germinate on the leaf at certain sites which developed no visible symptoms suggests that antifungal substances were present before inoculation. Purkayastha and Deverall (1965b) found that organic solvent extracts of leaf surfaces stimulated growth of B. cinerea conidia. It is however possible that the presence of stimulants masked the activity of inhibitory substance which may be present in small amounts but concentrated certain sites on the leaf surface. The detection of antifungal activity in droplets of water incubated on leaves in this study repeated the observation of Brown (1922) and Purkayastha and Deverall (1965a), and indicated that the presence of conidia is not essential for accumulation of inhibitors. The length of incubation (71h) required for droplets to become inhibitory makes it unlikely that the same inhibitory factors are effective during the first 12h after inoculation. However this

possibility cannot be overlooked as inhibitors may be concentrated at certain sites, and by the pooling of infection droplets the detection of antifungal activity may be obscured by dilution. Moreover stimulants may be present at other sites and in pooled diffusate they might overcome inhibitory effects.

Whether the inhibitors are present before inoculation or not it is convenient to consider their nature, origin and accumulation in this section. The greatest inhibitory activity was measured in B. cinerea infection droplets collected from sites which had developed little or no visible symptoms. This repeated the observation made by Purkayastha and Deverall (1965b) who suggested that the inhibitor which they collected from all types of infection site could be regarded as a phytoalexin. This study has clearly shown that the inhibitory principle in leaf diffusate is not the known phytoalexin from Vicia, wyerone acid, which can have no major inhibitory activity in infection droplets on leaves. Therefore the first report of phytoalexin-like activity in drops on leaves of Vicia made by Purkayastha and Deverall (1964), cannot have related to the phytoalexin subsequently discovered in studies on diffusates from pods, and on tissue extracts. Nevertheless the antifungal activity observed in diffusates from leaves may originate from the cells of the epidermis.

A second possibility is that the inhibitory substances may be the products of the microflora of the leaf surface. The detection of greater activity in droplets of conidial suspension closely

parallels the recent work by Blakeman and Fraser (1971) on the inhibition of B. cinerea conidia by bacteria on leaves of chrysanthemum. In their work the presence of conidia provided a low level of nutrients which enhanced bacterial growth, and therefore inhibition compared with that observed in water alone. A similar system may operate on the bean leaf.

In the present work it was shown that the antifungal activity of the ether solubles extracted from an inhibitory diffusate was masked by the presence of pod nutrients. Similarly, Blakeman and Fraser (1971) found that the inhibition of conidia by bacteria could be overcome by the addition of fungal nutrients. The interactions between inhibitors of whatever source and nutrients in the infection droplet may therefore be an important determinant of the observed variation in lesion production of B. cinerea.

The presence of stimulants at certain sites during the first 12h after inoculation 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 and 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 constitution and/or physiologic 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 became stimulatory to B. cinerea conidia to different degrees depending upon sites. Random variation in exosmosis from healthy tissues is therefore not unknown. Brown (1922) also found that a rapid increase in conductivity in drops containing conidia coincided with penetration of the epidermis. Therefore the stimulation of fungal growth observed 12h after inoculation may follow the successful penetration of an epidermal cell in the absence of inhibitors on the leaf, and the release of nutrients into the infection droplet. Further investigations are needed before any distinction can be drawn between these alternative proposals for the origin of stimulants in the first 12h after inoculation.

The relationship between penetration and the development of cell browning, which was the typical symptom produced by B. cinerea during the first days after inoculation requires further examination. However the results reported here suggest that penetration of the epidermis is an essential prelude to the development of extensive cellular discolouration. Although invading hyphae were shown to be restricted within single brown cells of the epidermis in all types of limited lesions examined, cellular discolouration typically extended into surrounding epidermal and palisade mesophyll cells. The variation in the amount of cell discolouration in limited infections (Grade 19 $\rightarrow$ S_1) was therefore probably a reflection of the extent of browning in advance of the restricted invading hyphae. The sites with greatest browning were associated with the longest germ tubes on the leaf surface. The correlation between fungal growth

in the infection droplet and the extent of browning suggests that phytotoxic substances which may induce cell discolouration might be produced in the droplet, and diffuse into the leaf through penetration points. The direct diffusion of substances through the cuticle is unlikely in view of observation of Deverall (1960) that droplets of highly phytotoxic B. cinerea culture filtrates placed on bean leaves, caused no discolouration unless the cuticle was damaged. An investigation of the phytotoxicity of infection droplets is necessary for confirmation of this hypothesis, however it is supported by the observations of Brown (1915, 1916), that "cytolytic principles" accumulate in infection drops containing B. cinerea on the surface of host plants.

The browning of groups of cells was the only visible symptom of infection during the first few days after inoculation; but after 15 days localized reaction material could be seen at some sites where no browning had developed during the first 4 days. The delayed appearance of localized granular reaction material was observed by Mercer (1970) in pod endocarp cells of Phaseolus vulgaris below appressoria of Colletotrichum lindemuthianum.

Localized reaction material has been observed by light microscopy in other host-parasite combinations by Young (1926 a and b), and Fraymouth (1956). These workers detected deposits around infection pegs of invading fungi. Reaction material has been similarly described from electron microscope studies discussed by Chou (1970).

However Stanbridge, Gay and Wood (1971) have demonstrated that electron translucent deposits, termed reaction material, are formed in epidermal cells of barley leaves before penetration of the cuticle by Erysiphe graminis. Possibly electron microscopy may resolve the question of whether penetration by Botrytis is a necessary prelude to the formation of reaction material in vicia.

The causes of the frequent failure, and occasional success of invading hyphae to progress through the leaf after penetration will be discussed elsewhere.

b) Development of lesions caused by B. fabae in water.

In previous investigations of the development of lesions caused by B. fabae both Deverall and Wood (1961 a), and Purkayastha and Deverall (1965 a) found that a few infection droplets failed to produce lesions and that many of the lesions which were produced failed to spread through the leaf. In this study lesions were produced at each site inoculated with the F_3 strain and spreading lesions developed from at least 95% of inoculations. Deverall and co-workers used 1 µl infection droplets compared with the 10 µl drops used in the present investigations. The larger droplets are much less likely to dry up, and the presence of the droplet has been shown here to have a marked effect on the establishment, and spread of infection. Variation between experimental results may simply reflect the difference in inoculation technique. However, the F_3 isolate may also be more pathogenic than the strains used in previous investigations.

In contrast to B. cinerea the development of B. fabae in the leaf was typically not confined to the epidermis. One day after inoculation hyphae had penetrated into the palisade mesophyll tissues and there was considerable discolouration of the infection site, affected cells looked black rather than brown. The fungus continued to spread both intra-, and intercellularly through the infection site, and then into surrounding tissues.

The spread of lesions caused by B. fabae was delayed until the tissue beneath the infection droplet was completely blackened, and colonised by the fungus. It was considered that this delay may indicate that fungal growth is partially inhibited during the initial phase of infection. This implies either that the tissue surrounding the infection site becomes less inhibitory or that the pathogenicity of the fungus is enhanced after it is established within the leaf. In its subsequent rapid spread and the disorganization of tissues in advance of fungal hyphae, the established B. fabae lesion possesses the characteristics of a typical soft rot as described by Wood (1967).

The variation in the susceptibility of leaves of different age to B. fabae recorded in this work to some extent conflicts with the results obtained by other workers. Deverall and Wood (1961 a) and Purkayastha and Deverall (1965 a) both found that young leaves were more resistant than old to the production of lesions by B. fabae. In this investigation young leaves were clearly more susceptible to the rapid spread of the B. fabae lesion.

The greater susceptibility of younger leaves was manifested in part by the development of extensive vascular browning after infection. Young tissues may be more sensitive than old to toxic substances produced by B. fabae. Purkayastha (1965) produced evidence that phytotoxic metabolites are produced by B. fabae in vivo. The nature of the metabolites remains to be determined. Balusubramanian, Deverall and Murphy (1971) detected active pectic enzymes both in and around lesions caused by B. fabae in the bean. Pectic enzymes have been shown to cause vascular browning in tomato cuttings (Scheffer and Walker 1953, and Gothoskar et al., 1955), and young plant tissues have been reported to be most susceptible to their action (Bateman and Lumsden, 1965). It is possible that pectic enzymes are solely responsible for the discolouration and disorganisation of leaf tissues in advance of B. fabae hyphae. An examination of the effect of purified preparations of pectic enzymes extracted from B. fabae lesions on leaf tissue of the broad bean would help to elucidate this problem.

c) The effect of additives to the infection droplet on the development of B. cinerea infections in leaves.

This work has confirmed the finding of Chou and Preece (1968), that pollen extracts enhance the pathogenicity of B. cinerea toward the bean; but suggests that the activity of orange juice, which they showed to have a similar effect, may depend on the variety of orange

extracted.

Detailed studies on the effect of additives have been carried out by Purkayastha and Deverall (1965 a). None of the factors that they examined, including various nutrients added to the infection droplet such as yeast extract, casein hydrolysate and sugars, either singly or in combination, and also healthy potato and bean leaf extracts, or plant hormones (indole acetic acid, and gibberellic acid) fed via the petiole, stimulated the production of spreading infections by B. cinerea. Chou and Preece (1968) confirmed that various sugars were relatively inactive, particularly in the concentrations at which they occurred in pollen diffusate. Chou (1970) demonstrated that many sugars, amino acids, organic acids, (including abscisic acid), mineral salts and certain vitamins such as nicotinic acid and inositol, which are common constituents of pollen (Todd and Bretherick, 1942; Virtanen and Kari, 1955; Borecka and Pieniazek, 1968), either singly or in various combinations were not as active as pollen extracts in promoting spreading of infection. The apparently unique character of the pollen diffusate led Chou (1970) to propose that the activity of extracts could be assigned to an unknown principle termed "aggressin".

In view of these results it is somewhat surprising that pod nutrients also possess the ability to stimulate the development of a high percentage of spreading B. cinerea infections. Pod nutrients were found to be slightly less active than pollen extracts, and the

pattern of loss of the ability to enhance pathogenicity by dilution was different for the two stimulants. The latter suggested that different factors were active in pollen and pod nutrients. Further indication of differences between the two stimulants became apparent when their mode of action was investigated; this will be discussed later. It is therefore proposed that pod nutrients do not exactly stimulate the effect of pollen.

When considering stimulation of infectivity it is important to remember that B. cinerea in water can sometimes cause spreading infections. Therefore an exogenous 'aggressin' factor is not essential for the development of spreading infections. Moreover Chou and Preece (1968), and Chou (1970) showed that many substances can cause large increases in the percentage of inoculations which spread from the infection site. It is possible that pod nutrients provide an appropriate mixture of these individually stimulatory substances, which promotes infection without the presence of "aggressin".

Pollen grains have been reported to affect both the germination of Botrytis conidia, and subsequent infection on a variety of host tissues such as; almond petals (Ogawa and English, 1960); flowers and other parts of American holly (Bachelder and Orton, 1963) and strawberry fruits and petals (Chou and Preece, 1968; and Chou, 1970). Moreover pollen grains from a range of plants are

capable of stimulating the growth of B. cinerea in broad bean leaves (Chou, 1970).

Fungal stimulation by pollen grains is not confined to Botrytis. Fokkema (1971) has demonstrated that, in the field, the presence of pollen grains stimulates the development of epiphytic mycoflora on rye leaves, and that pollen added to inocula of Helminthosporium sativum enhances infection of rye by this fungus. Strange and Smith (1969) reported that wheat pollen contains a fungal growth stimulant which promotes head infection by Fusarium graminearum. Experimental results suggest that the stimulation of fungal pathogenicity towards rye and wheat is the effect of a pollen specific "aggressin" factor.

The identity of the active principle in pollen grains remains to be determined. Chou and Preece (1968), Strange and Smith (1970) and Fokkema (1971) all found that the principal is water soluble, and passes through "Visking" tubing, and these observations have been confirmed in the present investigation. Chou (1970), and Strange and Smith (1970), have made preliminary attempts to determine the nature of the active principle in pollen grains from the broad bean and wheat respectively. Both investigations have shown that the active fraction is basic or cationic in nature. It is possible that the same fungal stimulant is present in all pollen. The separation and identification of the active principles in pollen would facilitate further investigation of its mode of action in breaking the resistance

of plants to infection by pathogenic fungi.

d) Wyerone acid, a phytoalexin from *Vicia faba*.

The identification of wyerone acid (a monoacetylenic furanoid keto acid) as a phytoalexin in *Vicia faba* (Letcher et al., 1970) provided a further example of the characterization of an antifungal substance which accumulated after fungal infection of a member of the Leguminosae. Other phytoalexins which have been isolated from legumes have all been identified as pterocarpans i.e. pisatin from pea (Perrin and Bottomley, 1962) phaseollin from French bean (Perrin, 1964), hydroxy phaseollin from soybean (Keen et al., 1971) and medicarpin from alfalfa (Smith et al., 1971). The broad bean is therefore the only legume which has been shown to produce monoacetylenic phytoalexin.

Although phytoalexins appear to be host specific they may belong to a range of chemical families. Thus phytoalexin like activity has been attributed to the phenolics orchinol in *Orchis militaris* (Hardegger et al., 1963) and gossypol in cotton (Bell, 1967); the terpenes rishitin and phytuberin in potato tubers (Sato et al., 1968; Varns and Kuc, 1971), and ipomeamarone in sweet potato (Kubota and Matsurua, 1953); and the polyacetylene, safynol in safflower (Thomas and Allen, 1970).

In detailed studies Deverall (personal communication) has failed to detect in bean leaves, any compounds other than wyerone acid which possesses antifungal activity against the C_3 strain of *B. cinerea*. It is possible that other compounds with phytoalexin-like activity may

be produced by Vicia faba but they seem unlikely to have a role in the resistance of the bean to infection by Botrytis. In this respect wyerone, which accumulates in bean leaves infected with Botrytis (Fawcett et al., 1971), would appear to be inactive in the limitation of growth of Botrytis, although it may be very important in controlling growth of other fungi.

Cruickshank (1963) reported that an antifungal substance assigned the trivial name 'viciatin' accumulated in infection droplets containing spores of Monilinia fructicola incubated in cavities of pods of Vicia, and stated that the phytoalexin was chemically similar to pisatin. Unfortunately no quantitative data on the formation or chemical characteristics of 'viciatin' have been published. Production of the pterocarpans pisatin and phaseollin can be induced in pea and French bean pods respectively, by the presence of heavy metal ions. It would be interesting to see if a phenolic phytoalexin accumulated in pods of Vicia faba treated with heavy metals in the absence of fungal spores.

The time course of wyerone acid formation observed in this work is very similar to that of wyerone formation described by Fawcett et al., (1971). This suggests the almost simultaneous production of both compounds, and discounts the proposal put forward by Fawcett et al., (1971), that wyerone acid may be produced by a later conversion from wyerone. The pathways of biosynthesis of the acetylenes in Vicia remain to be determined.

Fawcett et al., (1969) reported that there was a considerable loss of wyerone during extraction due to polymerization caused by the U.V. component of daylight. In the present work it has been shown that wyerone acid in ethanolic solution is also lost after exposure to daylight. All extractions of wyerone acid were therefore carried out in subdued light, away from direct daylight. Although only 67% of added wyerone acid was extracted from a leaf macerate other experiments demonstrated that losses during solvent partition and paper chromatography were minimal; 95 - 100% of phytoalexin was extracted from control solution in metabolism experiments. Apparently losses during the extraction of leaf tissues were therefore not primarily due to the unstable nature of wyerone acid. Possibly wyerone acid was adsorbed onto ethanol insoluble cell walls or metabolized by enzymes in the debris.

The degradation of wyerone acid by daylight raises doubts about the possible significance of the phytoalexin to resistance under field conditions. However, in the plant, wyerone acid might be shielded from exposure to daylight by cell walls and pigments, and the detection of wyerone acid in infected leaves collected from the field is important in this respect.

e) Antifungal activity of wyerone acid against Botrytis, in pod nutrients solution.

Pod nutrients were selected as a medium in which to examine the activity of wyerone acid against Botrytis because they were

considered to closely approach the conditions within infected bean tissue, and were highly stimulatory to fungal growth. Pod nutrients comprised the water phase remaining after ether extraction of combined B. cinerea infection droplets, collected 24h after inoculation into pod seed cavities.

The differential activity of wyerone acid against conidial germination and germ tube growth by B. allii, B. cinerea, and B. fabae correlates with the pathogenicity of the fungi towards the bean. The phytoalexin was most toxic to the non-pathogen B. allii, and least active against the virulent B. fabae. A similar relationship was observed for antifungal activity against the mycelial growth of the two pathogens of bean, the avirulent B. cinerea, and virulent B. fabae. Mycelial growth by Botrytis appeared to be less sensitive than conidial germination to wyerone acid.

Though assays against conidial germination provide a simple means of estimating antifungal activity, mycelial and germ tube growth tests are of more significance to the process of infection. The failure of B. cinerea mycelium to grow even after 12 days on pod nutrients agar plus 54 µg wyerone acid/ml suggests that the fungus must be exposed to a similar concentration of phytoalexin within infected tissues if it is to be solely effective in limiting fungal growth. Concentrations per g. fresh infected tissue extracted frequently exceeded this value.

One of the major tenets of the phytoalexin theory put forward by Cruickshank (1963) is that phytoalexins are less toxic to pathogens than to non-pathogens of the parent plant. This is supported by the

differential activity of wyerone acid to species of *Botrytis*. The reason for the differential toxicity of wyerone acid and other phytoalexins is not known. It might be explained in part in terms of the ability of pathogenic fungi to degrade the phytoalexin. Some pathogens of pea have been shown to degrade pisatin *in vitro* (Nonaka, 1967; de Wit-Elshore 1969). Bailey (1971) found that mycelium of *Colletotrichum lindemuthianum*, the cause of bean anthracnose, appeared to metabolize phaseollin. Lyon (1971) confirmed the latter result, but failed to show *in vivo* metabolism of phaseollin. The present work showed that *B. fabae* may metabolize wyerone acid in pod nutrients at a faster rate than *B. cinerea*. However there is a lack of evidence that ability to metabolize a phytoalexin is directly related to fungal sensitivity.

f) Production of wyerone acid by bean leaves in response to infection by *Botrytis* in water.

Wyerone acid was produced by leaves of *Vicia* in response to infection by *Botrytis*. The concentration of wyerone acid at inoculation sites increased rapidly after tissue damage became macroscopically visible. However the phytoalexin was not confined to discoloured cells. The detection of wyerone acid in dissected apparently healthy tissues in advance of damaged cells probably implies that wyerone acid can be synthesised in green cells, but the most rapid

synthesis may take place in cells undergoing necrosis.

The production of phytoalexins not only by damaged but also by surrounding healthy cells has been suggested by results obtained in studies of other host/pathogen combinations. For example Pierre and Bateman (1967) found that phaseollin and 'substance II' were concentrated in lesions produced by _Rhizoctonia solani_ in _Phaseolus_ hypocotyls but low concentrations of the phytoalexins were present a few mm. away from lesions. Similarly orchinol has been detected in low concentrations in healthy tissues of _Orchis militaris_ surrounding the site of infection by _Rhizoctonia repens_ (Gäumann and Hohl, 1960).

Sato and Tomiyama (1969), and Bailey (1971) have proposed that rishitin and phaseollin may only be produced in cells of the potato tuber, and bean (_Phaseolus_) hypocotyl respectively which have undergone hypersensitive cell death in response to fungal infection. However Sato et al (1971) were unable to resolve the question of whether rishitin was confined to hypersensitive, or was also produced in adjacent healthy cells. The answer to the questions of the localization of phytoalexin in hypersensitive cells must be in the development of histological techniques for detecting phytoalexins _in situ_.

Inherent in any consideration of the site of production of phytoalexin is the question of the factor which induces phytoalexin synthesis. Early investigations of phytoalexin metabolism indicated that culture filtrates of both non-pathogenic and pathogenic fungi induced the formation of antifungal compounds in the host to which

they were applied (Uehara 1959; Cruickshank and Perrin, 1963; Klarmann and Gerdemann, 1963; Nüesch, 1963). In addition phytoalexins may be induced by heavy metal ions (Perrin and Cruickshank, 1965; Cruickshank and Perrin, 1971), and compounds such as organic acids (Bailey, 1968), amino acids (Cruickshank and Perrin 1965), antibiotics for example patulin (Bailey 1969), and gliotoxin (Schwochau and Hadwiger, 1968), and ethylene (Chalutz and Stahmann, 1969a; Chalutz et al., 1969b). Cruickshank and Perrin (1968), and Frank and Paxton (1971) have isolated polypeptides from culture filtrates which are very active inducers of phaseollin and PA_k phytoalexin, in French bean and soybean respectively. Thus these results show that a wide range of substances effectively induce phytoalexin production. The proposal put forward by Cruickshank and Perrin (1968) that specific inducing compounds are involved in the initiation of phytoalexin synthesis in infected host plants seems unlikely. It is difficult to understand why a fungus should evolve a special compound to cause the plant to react in a detrimental way towards the fungus. It is more probable that in any disease situation several factors may affect phytoalexin synthesis.

In the case of wyerone acid production by the bean after Botrytis infection, fungal induced cell damage would appear to be the trigger for rapid phytoalexin synthesis in damaged and neighbouring cells. The penetration of the cell wall by invading hyphae may act as the initial inducer of wyerone acid synthesis in epidermal cells. In order to account for phytoalexin production in advance of fungal

hyphae it is necessary to propose that diffusible inducing substances may also initiate wyerone acid synthesis. Effective inducers may be the same principle which at sufficiently high concentrations causes cell browning, or substances which diffuse out of damaged cells.

In view of the inactivity of wyerone acid in diffusates from leaves, the phytoalexin can only contribute to the limitation of Botrytis cinerea infection after hyphae have penetrated into the leaf, into an environment in which wyerone acid is probably highly antifungal.

Thus wyerone acid does not have a major role in limiting B. cinerea infections where the majority of hyphae probably fail to penetrate, and little or no symptoms are produced (Grades 0 and 6.5), by six days after inoculation. The trace amounts of wyerone acid sometimes detected at these inoculation sites after 6 days, may have been produced by the leaf in response to infrequent penetration and cell damage. In this respect it is important that though wyerone acid was sometimes recovered from sites without macroscopically visible symptoms; microscopic observations frequently revealed the presence of isolated brown cells below certain germ tubes.

At inoculation sites which developed extensive browning (grade 19 or more infections) after 6 days, but where infections failed to spread, growth of B. cinerea was inhibited within the leaf. The concentrations of wyerone acid extracted from these sites 6 days after inoculation were greater than that which inhibited all mycelial

growth by B. cinerea in pod nutrients agar. Wyerone acid therefore probably inhibited fungal invasion of the leaf beyond the epidermis.

Detailed studies were made of germ tube growth on the leaf surface, and wyerone acid production by the subtending tissues at inoculation sites which developed grade 19 infections after 6 days. These investigations showed that the major increase in wyerone acid production occurred when mean germ tube extension ceased on the leaf surface; that is when the majority of hyphae penetrated the epidermis. This suggests that wyerone acid accumulated rapidly after penetration, and was therefore probably responsible for inhibition of growth of invading hyphae.

The loss of wyerone acid from infection droplets above developing grade 100 infections may be explained by metabolism of the phytoalexin by the mesh of hyphae which developed in droplets at certain sites. The failure of hyphae to grow in the leaf was probably due to the high concentration of wyerone acid present in the infected tissues.

The importance of wyerone acid to the limitation of B. cinerea in the leaf is further illustrated by the very low concentration of phytoalexin extracted from spreading B. cinerea infections. It appears that in the comparative absence of wyerone acid, B. cinerea is able to spread from the infection site. The development of a spreading infection may therefore relfect the failure of the tissues at the inoculation site to produce wyerone acid, the presence of particular conditions which enable the fungus to degrade the acid as it is produced,

or more probably, a combination of these effects.

The accumulation of wyerone acid at B. fabae inoculation sites after tissue damage became visible, and subsequent loss as the lesions spread show that B. fabae is able to degrade wyerone acid in vivo. These results are in accord with the loss of antifungal activity of leaf extracts observed by Deverall and Vessey (1969). The low yields of wyerone acid recovered from tissue completely blackened and colonised by B. fabae, and from infection droplets on leaves, and pods, indicate that by its ability to metabolize wyerone acid B. fabae prevents the accumulation of the phytoalexin in the tissues it has invaded. The metabolism of wyerone acid is therefore probably of primary significance to the pathogenicity of B. fabae towards the broad bean.

The recovery of relatively high yields of wyerone acid from the partially infected tissues at the infection site suggests that the phytoalexin was produced by apparently healthy non-infected cells. Deverall and Vessey (1969) proposed that the phytoalexin from Vicia faba can be produced in advance of developing lesions caused by B. fabae. This has been confirmed for wyerone acid, which was detected in healthy peripheral tissues around the infection site. However, even when peripheral tissues became partly necrotic they yielded less wyerone acid than comparable infection sites.

The failure to detect high concentrations of wyerone acid around the infection site may be due to several factors; 1) the phytoalexin may be degraded by extracellular fungal enzymes as it is produced in

advance of B. fabae hyphae. 2) The initial massive response of the tissue at the infection site may utilize most of the available precursor for wyerone acid synthesis, or similarly the supply of simple plant metabolites in the leaf may be exhausted. 3) Though initiated the production of wyerone acid may be subsequently inhibited.

The first possibility is unlikely as it was shown that no extracellular enzymes capable of degrading wyerone acid were present in diffusates from B. fabae infected pod seed cavities. It is impossible to differentiate between the other two proposals, however the former is considered to be less feasible as even in leaves naturally infected by B. fabae on whole plants, and therefore unlikely to be exhausted of metabolites or precursors, the peripheral tissues surrounding the large spreading lesions produced by B. fabae contained little wyerone acid.

Whatever the cause, the failure of peripheral tissues to produce large amounts of wyerone acid may contribute to the rapid spread of lesions through the leaf once the infection site is completely blackened and colonised by Botrytis. Conversely the delay in the spread of infection from the infection site is therefore probably due to the partial inhibition of B. fabae by the wyerone acid produced in the newly infected tissue.

It is interesting to speculate on the development of the B. fabae lesion which would take place if the peripheral tissues were capable of producing the same amounts of wyerone acid as the tissues at the infection site. The fungus would be constantly challenged by the highest

levels of wyerone acid which the cells could produce. The infection would undoubtedly have a slower rate of spread.

g) Metabolism of wyerone acid by *Botrytis*.

The experiments carried out on metabolism of wyerone acid by *Botrytis* were limited to a comparison of loss from pod nutrients and dialysed pollen diffusate. It was demonstrated that extension of germ tubes by conidia of *B. fabae* or *B. cinerea* in pod nutrients, was associated with disappearance of wyerone acid. In contrast similar fungal growth in dialysed pollen diffusate was not associated with substantial loss of phytoalexin. Metabolism of wyerone acid by *Botrytis* was clearly influenced by the composition of the solution in which it was assayed. The effect of pollen on the interaction between *Botrytis* and wyerone acid is discussed elsewhere. This section will be confined to discussion of the loss of wyerone acid from pod nutrients.

Deverall and Vessey (1969) showed that both species of *Botrytis* removed phytoalexin from solution in pod nutrients, but that per conidium *B. fabae* did so at a faster rate than *B. cinerea.* These observations have been repeated for wyerone acid, and it has been shown that loss of the phytoalexin is due to its metabolism by the fungi rather than absorption onto fungal cell walls as described for pisatin by Cruickshank and Perrin (1965). *B. fabae* and *B. cinerea* were able to metabolize wyerone acid following germination of conidia. This suggests that lower toxicity of wyerone acid to conidial germination by *B. fabae*

than B. cinerea is due to the differential sensitivity of the two fungi to the phytoalexin and not an expression of the greater ability of B. fabae to degrade wyerone acid. In consequence the difference in the ability of the two fungi to metabolize the phytoalexin observed in this, and earlier work, may be more apparent than real, for it may merely reflect a delay in germ tube growth by B. cinerea, and therefore the initiation of wyerone acid metabolism. More detailed studies are necessary before any firm conclusions can be drawn on the rates at which the two fungi degrade wyerone acid.

Though differences between the rate of disappearance of wyerone acid from solutions incubated with B. cinerea or B. fabae conidia remain in doubt, there are clear differences between the metabolic fates of the acid in the two fungi. B. fabae converted wyerone acid to a relatively stable reduced product, in vitro and in vivo, but this compound could not be detected in association with B. cinerea. B. cinerea may be unable to carry out this reduction or it may metabolize the product, substance I, as it is formed.

The conversion of wyerone acid by the saturation of the acetylenic bond, and the reduction of the keto group to an alcohol renders it much less active. This implies that the keto-acetylenic moiety may account for the antifungal activity of wyerone acid. This is in accord with the proposal put forward by Fawcett et al (1969) that the sequence of groups which account for the toxicity of wyerone, (Fawcett et al 1968) and capillin (Imai, 1956) can be generalized to the R-keto-acetylenic moiety where R is a group possessing aromatic character. This view is

supported by the fungistatic activity shown by a series of synthetic R-keto-acetylenic compounds in which R was furanyl-2- or 5-bromofuryl-2- (Hillers *et al*., 1966).

The structural similarity between 'substance I' and wyerone acid suggests that conversion may be effected in a single step. However whether 'substance I' is the first or indeed final product in the metabolism of wyerone acid by *B. fabae* remains to be determined. The decrease in concentration of the metabolite in infection droplets after prolonged incubation on pod seed savities suggests that it may be further decomposed.

The metabolism of medicarpin (Higgins and Millar, 1969), and pisatin (Christensen, 1969; de-wit Elshore and Fuchs, 1971) by fungi pathogenic to alfalfa and pea respectively has been studied using radioactive phytoalexins to confirm that other compounds, including CO_2, which appear are products of the metabolism of phytoalexin. Similar investigations of the metabolism of labelled wyerone acid *in vitro*, and *in vivo* would both help to confirm the identity of 'substance I' as the product of fungal reduction of wyerone acid, and also enable the detection of any further metabolites.

h) Mode of action of pollen extracts and pod nutrients in breaking the resistance of the bean to infection by *B. cinerea.*

The ability of pollen extracts and pod nutrients solution to enhance pathogenicity of *B. cinerea* towards bean may be explained in

terms of their effect on the interaction between fungus, plant, and phytoalexin.

Experimental results have shown that *in vitro* 1) pod nutrients stimulate growth of *B. cinerea*, and metabolism of wyerone acid by the fungus. 2) Dialysed pollen diffusate stimulates growth of *B. cinerea*, renders the fungus insensitive to wyerone acid, and allows growth without metabolism of the phytoalexin. *In vivo* wyerone acid accumulated in lesions produced after inoculation with *Botrytis* in either stimulant. The development of spreading lesions after inoculation of *B. cinerea* in pod nutrients was associated with a loss of wyerone acid from the infection site. In contrast high yields of wyerone acid were extracted from spreading infections after inoculation in pollen extract. However, eventually the phytoalexin disappeared from large spreading lesions caused by *Botrytis* in pollen diffusate.

Some caution must be called for in the interpretation of *in vivo* results on the basis of conclusions drawn from *in vitro* experiments. However, on the basis of studies made *in vitro* it is possible to explain *in vivo* changes in wyerone acid concentration, and the development of spreading infections.

Thus pod nutrients may also stimulate the ability of *B. cinerea* to metabolize wyerone acid *in vivo*. Therefore the fungus may prevent the accumulation of wyerone acid to toxic levels, and thus be able to spread from the infection site. This would explain the similar levels of wyerone acid associated with developing *B. fabae* in water, and *B. cinerea* in pod nutrients infections.

The presence of pollen extract may have the same effect on the insensitivity of *Botrytis* to wyerone acid in the leaf as in the test tube. The fungus might therefore be rendered insensitive to the phytoalexin and consequently be able to spread uninhibited through the leaf. The presence of a high concentration of wyerone acid in blackened completely colonised leaf tissues suggests that pollen extracts may allow the growth of *B. cinerea* without degradation of the phytoalexin *in vivo* as well as *in vitro.* It is probable that in time the active principle in the pollen extract becomes diluted within infected tissues to such an extent that fungus may gradually become more sensitive to wyerone acid. This would result in the limitation of the infection but for the apparent ability of the established mycelium within the leaf to metabolize accumulated wyerone acid, so that in time spreading lesions are associated with low levels of phytoalexin.

Experimental work shows that the spread of lesions caused by *B. cinerea* in either stimulant is ultimately associated with metabolism of wyerone acid by the fungus. However the effect of pod nutrients and pollen extracts would appear to differ in the initial stages of establishment of infection. Pod nutrients may enhance the ability of *B. cinerea* to degrade wyerone acid, whereas pollen extracts may have a more direct effect on the sensitivity of the fungus to the phytoalexin.

Although this work has described certain features of the effect of pod nutrients and pollen extracts on host parasite relations in the bean, their primary mode of action remains unknown. It is particularly of

interest to consider how pollen extracts overcome the inhibitory activity of wyerone acid. The ability of both B. cinerea and B. fabae to grow in dialysed pollen diffusate without metabolizing wyerone acid in vitro suggests that the presence of some principle in pollen may prevent the uptake of phytoalexin by the fungi. Thus without uptake there is neither inhibition nor induction of wyerone acid degrading enzymes. The isolation of the active principle in pollen extract would facilitate further experiments on its mode of action.

It is concluded that the effects of pod nutrients and pollen extract are to modify the interaction between B. cinerea, Vicia faba and wyerone acid so that the activity of the phytoalexin, and therefore the resistance of the plant to infection, is overcome by the fungus.

i) Biological activity of diffusates from different bean tissues.

The most striking feature of investigations on the biological activity of diffusates was the difference between diffusates from leaves and pod seed cavities. The observed activity of all infection droplets was an expression of the interaction of the water and ether soluble components in the diffusates. The ether soluble inhibitor, active in water, but not when added to pod nutrients, and detected in diffusates from healthy leaves and infections with little or no visible symptoms has already been discussed. This discussion will be confined to changes in water phase of diffusates and their interaction with endogenous and added wyerone acid.

The principle known difference between diffusates from leaves and

pods is the higher nutrient status of pod diffusates expressed in terms of the stimulatory activity of their constituent water phases to *B. cinerea*. This can probably be attributed to the absence of a cuticle in pod seed cavities, which permits greater diffusion of nutrients into the infection droplet than from the cuticularized epidermal cells in the leaf. Thus water droplets incubated in pod seed cavities for only 12h became coloured and stimulatory to *B. cinerea*; in contrast the water phase of diffusates from healthy leaves were colourless and inactive even 3 days after inoculation. Infection with *Botrytis* caused the release of further stimulatory water soluble substances into infection droplets in pod seed cavities. The observation of enhanced stimulation by "infected" water phases is in accord with the results obtained by Deverall (1967), who demonstrated that further, and different sugars and amino acids were released into pod diffusates after infection by *Botrytis*. Similarly the water phase of diffusates from brown lesions caused by *B. cinerea* in leaves became stimulatory. However the stimulatory activity of the water phases of infection droplets on leaves was only equivalent to that observed in water droplets incubated for 12h on healthy pod endocarp. This indicates that despite visible cell damage within the leaf the majority of the cuticle covering the infection site remained undamaged. This suggests that conditions within the tissues of the leaf are quite different from those in the infection droplet, and that conversely diffusates from infected pod seed cavities more closely approximate to the intracellular environment in which the

limitation of B. cinerea takes place in both pods and leaves.

A second major difference between diffusates from leaves and pods was their pH value. Comparable diffusates from leaves always had a higher pH than those from pods. The presence of visible fungal damage below infection droplets was reflected by a decrease in pH of diffusates.

In recent experiments Deverall (personal communication) has shown that pH has a major effect on the activity of wyerone acid. For example in 2% sucrose at pH 6.0 wyerone acid at 100 µg/ml was inactive against B. cinerea conidia. However it was very active at pH 4 or 4.5. Differences in pH may help to explain the differential activity of wyerone acid in leaf and pod diffusates. However the importance of other factors is suggested by the different activities of wyerone acid in water droplets incubated for 24h and 86h in pod seed cavities, despite their similar pH value.

The results obtained here suggest that nutrient status may also be an important factor in controlling the activity of wyerone acid. Thus wyerone acid possessed no inhibitory activity in sterile distilled water or in water droplets incubated on leaves. The phytoalexin was only slightly active as a growth retardant in diffusates from brown lesions in leaves or from healthy pods during the first 3 days after inoculation; but was inhibitory in the highly stimulatory diffusates from infected pod seed cavities. It is perhaps significant that the increase in stimulatory activity of water droplets incubated in pod seed cavities was directly related to an increase in the inhibitory activity of wyerone

acid.

Further detailed studies of the activity of wyerone acid in diffusates and defined media are necessary before the relative significance of pH and the component of water phases to the observed phenomenon of differential antifungal activity can be assessed.

The inactivity of wyerone acid in diffusates from leaves raises some doubts about the role of the phytoalexin in limiting B. cinerea infection in leaves. However the conditions within infected tissues would be of high nutrient status, and probably low pH due to the release of galacturonic acid by pectolytic enzymes; and these are the conditions in which wyerone acid would appear to be most active. Therefore the inactivity of the phytoalexin in leaf diffusates cannot be considered to be a sound argument against the hypothesis that wyerone acid has a major role in limiting Botrytis infections in leaves. Indeed the proposed differential activity of wyerone acid in infection droplets on leaves, and in infected tissue helps to explain the phenomenon of the development of a mesh of B. cinerea hyphae at some inoculation sites, but the failure of the fungus to invade the tissues of the leaf.

These observations demonstrate that the failure to detect antifungal activity in infection droplets incubated on leaves of any host may not indicate the absence of a phytoalexin-like response within the host tissues. They also emphasize the importance of the selection of a medium for bioassays which is similar to the conditions expected within infected tissues.

j) General discussion

This research has demonstrated that the rapid accumulation of wyerone acid in leaf tissues probably limits the growth of invading B. cinerea hyphae. B. fabae infections are able to spread through the leaf because the fungus is able to reduce wyerone acid in vivo and therefore prevent its accumulation to fungitoxic concentrations in infected tissues.

It has been proposed (Figure 1) that the development of Botrytis infection can be considered as the result of a balance between the production of phytoalexin by the bean and its degradation by Botrytis. The response of the bean to infections which cause visible damage by either fungus is to produce wyerone acid. The balance is therefore dependent upon the activity of the fungi. Though they may exist, no inherent differences between the ability of the Botrytis species to degrade wyerone acid have been demonstrated in this work, but it has been shown that B. fabae is much less sensitive to wyerone acid than B. cinerea. It is possible that, unlike B. cinerea, B. fabae hyphae are not inhibited by the initial exposure to a relatively low concentration of wyerone acid in epidermal cells and are therefore able to initiate the degradation of the phytoalexin. Thus the balance may be tipped against B. cinerea by its greater sensitivity to wyerone acid.

A similar 'balance' hypothesis has been put forward by Higgins and Millar (1969) to explain the different pathogenicities of the virulent Stemphylium botryosum, and avirulent S. loti to alfalfa. Both fungi are equally sensitive to fungitoxic concentrations of

medicarpin, the phytoalexin from alfalfa, but S. botryosum is able to metabolize medicarpin in dilute solution at a faster rate than S. loti. In consequence S. botryosum prevents the accumulation of fungitoxic concentrations of the phytoalexin and is able to spread through the leaf. Though the comparisons between Stemphylium infections of alfalfa, and Botrytis infections of the broad bean are incomplete they demonstrate two host/pathogen combinations where differentiation between the virulent and avirulent infection appears to take place at the level of the fungus ⇌ phytoalexin interaction. Discrimination at the same level has also been suggested to operate between Ascochyta pisi and Mycosphaerella pinodes which behaved as avirulent, and virulent pathogens of the pea, respectively, in experiments reported by Heath and Wood (1971).

Where the role of phytoalexin in the differential resistance of varieties of a host to physiologic races of a pathogen has been investigated results suggest that discrimination takes place at the level of phytoalexin production by the plant. In the resistant host infection is followed by the rapid accumulation of phytoalexin, whereas in the susceptible host phytoalexin is either not produced, produced at a much slower rate, or production is delayed compared with the incompatible response. Examples of host/pathogen combinations where varietal resistance has been investigated in detail are the soybean/Phytopthora megasperma var. sojae (Frank and Paxton, 1971)*; French bean/Colletotrichum lindemuthianum (Bailey, 1971); and potato tuber/P. infestans (Sato et al., 1971).* It seems that in these combinations either the

* Most recent publication concerning this host/pathogen combination.

susceptible response is the result of the suppression of phytoalexin production, or the resistant response is the result of induction of rapid synthesis of phytoalexin. The mechanisms involved in the control of phytoalexin synthesis are as yet unknown.

These observations illustrate the need for some modification of the theory of phytoalexins proposed by Müller and Borger (1940). Nevertheless recent investigations described in this thesis, and elsewhere have contributed to the consolidation of the basic concept that phytoalexins are major determinants of the fungal disease reaction in plants.

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