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THE EFFECT OF IRON, AND OF A BACTERIAL SIDEROPHORE, ON FUNGAL INFECTION OF PLANTS

by

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A thesis submitted to the
FACULTY OF SCIENCE
LONDON UNIVERSITY
in Candidature for the degree of
DOCTOR OF PHILOSOPHY

IMPERIAL COLLEGE OF SCIENCE AND TECHNOLOGY/
EAST MALLING RESEARCH STATION

September, 1985

ABSTRACT

A siderophore (SA) purified from low iron cultures of an isolate of Pseudomonas fluorescens stimulated germination and appressorium formation, both in vitro and in vivo, by conidia of, Botrytis cinerea, Collectotrichum musae, C. lindemuthianum, and C. acutatum. The fully chelated siderophore (SAFe) and free ferric iron were inhibitory, or not stimulatory, and the stimulation by SA could not be explained on a nutritional basis.

The development of chocolate spot lesions on Vicia faba leaves, caused by B. cinerea, was stimulated by the presence of SA in the inoculum, and inhibited by SAFe. V. faba cotyledons accumulated phytoalexins in response to a droplet of heat-killed B. cinerea placed on their surface, and SA, but not SAFe, reduced wyerone accumulation when present in the inoculum. SA, or SAFe, had no effect on the development of anthracnose lesions, caused by C. lindemuthianum race β, on the resistant Phaseolus vulgaris cultivar, Kievit. SA had no effect on lesion development on the susceptible cultivar, Wade, but lesions were significantly inhibited by SAFe. Phytoalexin accumulation in response to heat-killed conidia of C. lindemuthianum was significantly greater in the resistant cultivar, but was not effected by SA or SAFe in either.

The development of blackspot lesions on strawberry stolons and leaves, caused by C. acutatum, was stimulated by the presence of SA, and inhibited by SAFe, in the inoculum. Red

fruit were too susceptible to show any significant effect of SA. Fungitoxic compounds were extracted from lesions on strawberry stolons but these were not chemically characterised or quantified.

When grown in iron-deficient media B. cinerea and C. acutatum produced fluorescent, iron-binding ligands, visible under UV light, but these were not characterised.

Neither SA or SAFe influenced the development of B. cinerea or Cylindrocarpon heteronema, lesions on wound-inoculated apple fruit.

The stimulation of disease development by iron chelating agents in some host/pathogen combinations is evidently brought about by influences on both pre- and post-infection events. The significance of this is discussed in relation to the phytoplane microflora and associated siderophores.

ACKNOWLEDGEMENT

I wish to thank Dr. T.R. Swinburne and Dr. S.A. Archer for their help, supervision and encouragement. Special thanks are due to Dr. T.R. Swinburne for suggesting the research topic and for his advice in the preparation of this thesis.

I would also like to thank all my friends at East Malling Research Station who helped me during the course of this study, and Miss P.A. Feltham who typed the thesis.

This work was undertaken during the tenure of a studentship from the Agricultural and Food Research Council.

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Chapter 1

INTRODUCTION

1.1 The importance of iron and the need for siderophores

Life processes can be described as a series of redox reactions (Hill, 1981). For most aerobic and facultatively anaerobic organisms, these processes involve iron. The evolutionary selection of iron probably arose from its high natural abundance, although its versatility of possible oxidation states, redox potential, co-ordination number, spin state, ligand type, ligand equilibria, ligand dynamics and structure, are characteristics that make iron ideally suited for its role in biological processes (Reed, 1981).

In aqueous media at physiological pH iron exists in the form of an insoluble polymer, $(Fe(OH)_3)_n$, with a solubility constant of 4×10^{-36}. Any free iron, at pH 7, in excess of 2.5×10^{-18}M would be precipitated as $Fe(OH)_3$, (Messenger & Barclay, 1983) so any living cells must use special mechanisms to solubilise and assimilate iron. The high-affinity iron uptake mechanism of microorganisms normally consists of two components; siderophores and the siderophore transport apparatus. Siderophores are defined as relatively low molecular weight, virtually ferric-specific ligands produced under low iron growth conditions for the assimilation of ferric iron (Neilands, 1983) and almost all microorganisms grown under iron-limited conditions have been shown to produce them. A variety of molecules have been characterised as siderophores; an example of each chemical family is shown in Table 1.1.

Table 1.1

Types of molecules characterised as siderophores

Family Type	Example	Synthesising Organism
Cyclic tri-catechol	Enterobactin	Escherichia coli
Linear catechol	Agrobactin	Agrobacterium tumefaciens
Ferrichrome	Ferrichrome	Penicillium spp
Rhodotorulic acid	Coprogen	Epicoccum purpurascens
Citrate hydroxamate	Arthrobactin	Arthrobacter spp
Mycobactin	Mycobactin P	Mycobacterium phlei
Fusarinine	Fusarinine	Fusarium spp
Ferrioxamine	Ferrioxamine B	Streptomyces pilosus
Pseudobactin	Pseudobactin	Pseudomonas spp

Condensed from Neilands (1981a, 1981b & 1983)

Siderophores act not only to make iron more available to the organism that synthesised them (and other organisms which can utilise them), but also to make iron unavailable to most organisms with which it may be competing. Iron sequestration mechanisms have been related to the pathogenicity of many vertebrate and plant pathogens (Weinberg, 1978; Finkelstein, et al., 1983; Leong & Neilands, 1982). For example, the non-host specific phytotoxin syringomycin, produced by plant-pathogenic Pseudomonas syringae pv syringae is essential for pathogenicity and its action has been related to its role as a ferric siderophore (Gross, 1982).

1.2 An introduction to the involvement of iron in fungal plant diseases

Iron has been shown to be involved in the germination and appressorium formation of some fungal conidia, both *in vitro* and *in vivo*. Although iron is necessary for sustained fungal growth, stimulation of germination by the presence of iron chelators in the inoculum seems to be a widespread phenomenon among phytoplane fungi. The involvement of iron chelators in fungal germination was first reported by Swinburne (1976). He demonstrated that the germination of *Colletotrichum musae* was stimulated by iron chelators, and Graham and Harper (1983) proposed that the action of chelating agents in promoting germination of *C. musae* conidia involved the formation of complexes with the inhibitory iron at a site within the conidium, so releasing the germination mechanism. There was no significant flux of iron in or out of the conidia and no major intracellular movement of iron took place. Fully ferrated iron chelators had no effect, and conidia obtained from cultures grown on low iron media had increased germination, indicating the observed stimulation was related to a reduction in iron at some active site(s) within the conidia (Swinburne, 1981).

The presence of iron chelators in the infection court has been shown to stimulate the infection process of certain fungi. A reduction in the quantity of iron in the conidia of *C. musae* diminished the ability of the host to respond to the presence of the pathogen, resulting in an apparent increase in aggressiveness. This has been related to the chelators stimulating the formation of dark appressoria, the proportional occurrenceof which is a

critical factor in disease development, and to a temporal reduction in phytoalexin production with conidia inoculated in the presence of iron chelators (Brown & Swinburne, 1980). An increase in aggressiveness was also observed with Glomerella cingulata infection of Capsicum frutescens where the symptom expression was accelerated when conidia were inoculated in solutions of iron chelators (Adikaram et al., 1982). The production of chocolate spot disease lesions on Vicia faba leaves by Botrytis cinerea was similarly stimulated by iron chelators. In the presence of the deferrichelators lesions were produced quicker, were larger, and contained lower levels of phytoalexin, than when the conidia were inoculated in water, or in the ferrated chelators (Brown & Swinburne, 1982).

1.3 Iron chelators on the phytoplane

Germinating fungal conidia may encounter a diversity of natural iron chelators from many sources. Almost all the water soluble substances present in plant cells may be leached into droplets of water, originating as rain or dew, on plant surfaces (Tukey, 1971). These provide nutrients for the phytoplane microflora which elaborate other metabolites. These chemicals may have iron chelating properties, such as polyphenols, polyphosphate, amino acids, organic acids, chitin, chitosan, alginates and other decomposition products, and secondary metabolites may be converted to iron-binding chemicals by microorganisms. 2,3-dihydroxy-benzoic acid, the most important chelator in the stimulation of germination of C. musae on banana fruit, is derived by the fungus from anthranilic acid which leaches

from the fruit (Swinburne, 1981).

The most important group of iron-binding molecules present on plant surfaces are probably the siderophores. Previous workers have used synthetic iron chelators, such as ethylene diamine tetraacetate (EDTA) or partially purified bacterial siderophores. During the course of this study, a siderophore produced by low iron cultures of Pseudomonas fluorescens, and designated SA, was tested for its effect on the germination and appressorium formation, in vitro, of various fungi. Germination and appressorium formation in vivo were studied and the effect of SA, SAFe, or free Fe^{3+} in the inoculum on infection and subsequent disease development was studied. Where possible, phytoalexin accumulation was related to the spread of necrosis and abiotic elicitors were employed to elicit phytoalexin formation to assess the effect of SA directly on the host plants defense mechanism. The importance of siderophores, and other iron chelators, in different stages of plant diseases caused by fungi is discussed.

Chapter 2

Pseudomonas sp, isolate UV3 and Colletotrichum musae; an Introduction to the effects of Siderophore A

2.1 Pseudomonads

The pseudomonadaceae have been extensively studied and a treatise of the literature on this family has been compiled in Bergey's Manual (Holt & Krieg, 1984). Pseudomonad bacteria are Gram-negative aerobic rods that often release water-soluble fluorescent pigments into their environment, especially when grown under low-iron conditions. Some are plant pathogens, such as the Pseudomonas syringae pathovars, some are facultative pathogens of mammals, such as P. aeruginosa, whilst most are saprophytes living in a variety of habitats and niches. In some habitats, pseudomonads may represent a minority of the total microflora, but under certain conditions (pH close to neutrality, organic matter in solution, temperature in the mesophilic range, good supply of dissolved oxygen) their capacity for rapid growth in the absence of complex growth factors leads to their predominance. Saprophytic fluorescent pseudomonads are very common in soils, and they also abound in plant rhizospheres where they seem to have a stimulatory effect on plant growth. This may be due, in part, to inhibition of plant pathogenic organisms by competition for iron (Kloepper et al., 1980), antibiotic production, or stimulation of members of the microflora inhibitory to the plant growth inhibiting organisms; although utilisation of the iron-siderophore complex by the plant may also be involved (Powell et al., 1980).

2.2 Pseudomonas sp isolate UV3

2.2.1 Identification

Pseudomonas sp isolate UV3, a saprophyte isolated from beetroot leaves, was first isolated by Blakeman and Parbery (1977), who demonstrated that appressorium formation by Colletotrichum acutatum was stimulated by this organism. Later McCracken and Swinburne (1979) showed that the stimulation of germination and appressorium formation of C. musae was attributable to siderophores produced by this isolate.

Pseudomonas species isolate UV3 had not been characterised in earlier studies. Identification was undertaken here using the differentiating tests set out in Bergey's Manual (Holt & Krieg, 1984). The results of the tests, comparing Pseudomonas UV3 with P. fluorescens and P. putida isolates, are shown in Figure 2.1. The tests undertaken suggest Pseudomonas species isolate UV3 is P. fluorescens biovar V. Most previously recorded P. fluorescens isolates were, however able to hydrolyse gelatin, which isolate UV3, like P. putida, is unable to do. Only Den Dooren de Jong (1926) has previously recorded a P. fluorescens isolate incapable of hydrolysing gelatin, which was the only one of 57 strains tested. The others were classified as P. putida on the basis of their incapacity to hydrolyse gelatin. However, P. fluorescens is the only species able to utilise trehalose as a carbon source, of which Pseudomonas UV3 is also capable. If isolate UV3 is P. fluorescens, unable to hydrolyse gelatin, its ability to utilise sucrose, while not forming levan, indicates it is biovar V.

FIGURE 2.1

Differentiating Tests for Pseudomonas Species

Test	Reaction of *Pseudomonas* isolate UV3	Reaction of *Pseudomonas fluorescens*	Reaction of *Pseudomonas putida*
Possess flagellae	+	+	+
Production of fluorescent pigments on low iron medium	+	+	+
Growth after 7 days at 41°C	-	-	-
Production of levan from sucrose	-	+/-	-
Possess arginine hydrolase	+	+	+
Possess oxidase (Kovac's Test)	+	+	+
Able to hydrolyse gelatin	-	+/-	-
Able to use trehalose as C source	+	+	-
Able to use sucrose as C source	+	+/-	+/-

2.2.2 Culture maintenance

Pseudomonas fluorescens biovar V, (isolate UV3) was maintained on Kings medium B (King et al., 1954) at 18°C under continuous fluorescent illumination.

2.3 Production of Siderophore A

2.3.1 Low iron water and glassware

Low iron water used to prepare low iron media, rinsing low iron glassware and for use in various experiments, was prepared by passing distilled water through a 4 x 25 cm column of chelex 100 (Na^+ form, 200-400 mesh, Biorad Laboratories Limited) previously adjusted to pH 6.8 with 0.5 M- sodium acetate buffer and washed with 10 bed volumes of distilled water. Three litre conical flasks and other glassware required, were soaked in 6M-HCl overnight, and then washed with copious quantities of deionised water to remove the acid. Culture flasks were not washed, but simply rinsed in deionised water and resterilised.

2.3.2 Low iron medium and growth of bacteria

For maximal siderophore production P. fluorescens was grown in medium containing; 7 g K_2HPO_4, 4 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, and 5 g glucose, per litre of low iron water. The medium was prepared at 10 times the final concentration, with the omission of $MgSO_4.7H_2O$, and subjected to ion exchange with chelex 100 resin (see section 2.3.1). Following dilution with deionised water, and addition of the $MgSO_4.7H_2O$, the medium was sterilised by membrane filtration (Millipore, 0.22 um) and 1.25 l dispensed into 3 litre flasks.

The medium was inoculated with bacteria scraped off Kings medium B plates with a sterile glass rod, and incubated at 21°C, in darkness, on an orbital shaker, with 3 cm eccentricity, at 150 rev. min^{-1}, for 5 days.

The iron content of the medium was not assessed, although similar media used by McCracken and Swinburne (1979), analysed by atomic absorption spectrophotometry had a residual iron content of less than 0.05 ug ml^{-1}.

2.3.3 Extraction and purification of Siderophore A

Bacteria were removed from a 5 day old culture by centrifugation at 1.1 x 10^5 rev. per min. for 20 mins and the supernatant reduced to 100 ml by rotary evaporation at 60°C. At this stage much of the dissolved salts and sugars precipitated and were discarded. The supernatant was then adjusted to pH 2 with 4 M-HCl, and extracted with 200 ml chloroform : phenol (1:1, w/w), the separated aqueous phase being discarded. One litre of ether and 100 ml low iron water were then added to the chloroform : phenol mixture and the separated organic phase discarded. The aqueous phase was then repeatedly extracted with 50 ml volumes of ether until the solution became clear orange colour, and all traces of phenol were removed. The extract was then concentrated to 30 ml by rotary evaporation at 30°C and mixed with a 100 ml aqueous slurry of Bio-beads SM2 (Bio-Rad Labs. Ltd) previously washed in methanol and rinsed in deionised water. The slurry was poured into a sintered glass funnel and washed with 5x100 ml volumes of low iron water, the eluate being discarded. The funnel was then transferred to a flask and the beads eluted with 2x100 ml volumes of analytical grade

methanol. Five ml low iron water was added to the extract which was then reduced to 2 ml by rotary evaporation at 30°C. This brown solution was applied to a 2 x 54 cm column of Bio-gel P2 (Bio-Rad Labs. Ltd) previously equilibrated with deionised water. The column was run at 0.5 ml $min.^{-1}$, and 4 ml fractions were collected. Many peaks were detected in the eluate by monitoring the uv. absorbance at 280 nm with a LKB Bromma 2238 Uvicord SII uv. monitor. A Pye-Unicam SP8-100 scanning spectrophotometer was used to scan the absorbance of each fraction from 500 nm to 200 nm, which indicated the peak eluted first from the P2 column and well-separated from the other peaks was the main siderophore-containing fraction, resembling that previously described (McCracken and Swinburne, 1979). From the absorbance spectra it appeared that this fraction was low in iron, whilst the peak eluted after it was high in iron (see section 2.3.4). The separation of a low-iron fraction from an iron-bound one was not expected since any difference in molecular size would have been too small for separation by P2 gel. The iron-bound fraction was probably retarded by binding loosely to the polyacrylamide molecules during elution. Charge effects in P2 gel are, according to the manufacturers, not expected, but this property greatly assisted separation.

The first peak eluted from the P2 gel column was designated siderophore A (SA). Most of the material, not including the first and last fractions of the peak, was bulked, concentrated by rotary evaporation at 30°C, and stored in the dark at -18°C. (Initial studies on the peaks eluted after SA from the P2 column indicated they may also have had iron-binding capacity, but were not as stimulating to fungal

The structure of Pseudobactin consisted of a linear hexapeptide, L - Lys - D - threo - B - OH - Asp - L - Ala - D - allo - Thr - L - Ala - D - N - OH - Orn, linked via an amide bond to a fluorescent quinoline derivative. The iron chelating groups consisted of a hydroxamate group derived from the N - hydroxy-ornithine, a hydroxy acid derived from B - hydroxy-aspartic acid, and an O - dihydroxy aromatic group derived from the quinoline moiety.

The extinction coefficient of Pseudobactin at 400 nm was 1.5×10^4 L mol^{-1} cm^{-1} and it had a molecular weight of 1220 g mol^{-1}. (Teintze et al., 1981).

germination as SA. These fractions were brighter green than SA and more fluorescent under 366 nm light. They may have contained a predominance of the fluorescent moiety without the iron-binding part of the complete siderophore molecule, but were not studied further.)

2.3.4 Characterisation of Siderophore A and Preparation of Ferrated Siderophore A

UV absorption spectra of SA were recorded with increasing concentrations of added iron over a pH range of 4 to 9. The absorbance maximum of the major peak was pH dependent (398 nm at pH 7) and addition of iron directly increased absorbance at 450 nm. This suggested SA was a member of the Pseudobactin (Teintze et al., 1981; Teintze & Leong, 1981) and Pyoverdine (Meyer & Hornsperger, 1978; Meyer & Abdallah, 1978) class of siderophores produced by Pseudomonas spp. Analysis of the amino acid composition of an acid hydrolysate of SA, undertaken by Dr. R.C. Hignett showed SA contained the same amino acids as the Pseudobactin characterised by Teintze et al. (1981).Λ.

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Fully chelated SA, (SAFe) was prepared by titration of SA with 0.1 M- $Fe_2(SO_4)_3$ in a spectrophotometer cuvette until the absorbance at 450 nm did not rise further and the bright yellow-green fluorescence was lost. This brown complex contained approximately equimolar SA and Fe^{3+} with no excess Fe^{3+}, as shown by titration of SA with $Fe_2(SO_4)_3$ in a Baird-Atomic Fluoripoint spectrofluorimeter, and analysis of the iron content of the complex. The molecular weight, or extinction coefficient, of SA were not accurately determined.Λ. It was assumed they were close to the figures for Pseudobactin since the siderophores in the class are similar. SA and SAFe

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concentrations were calculated using the extinction coefficient of Pseudobactin (Teintze et al., 1981), adjusted to 10^{-4}M, and stored at -18°C in darkness.

2.4 Colletotrichum musae

Stimulation of germination and appressorium formation of C. musae conidia by siderophores extracted from low-iron culture of Pseudomonas species isolate UV3 was first reported by Swinburne and his co-workers in Queens University, Belfast (see Swinburne, 1981).

Investigations into the observed effect that banana fruit leachate stimulated germination of the banana anthracnose pathogen, C. musae, revealed the involvement of iron chelating agents (Swinburne, 1976). The main active constituent of the leachate was anthranilic acid, and it was shown C. musae also responded to apple fruit leachate containing other aromatic acids (Brown & Swinburne, 1981). It was then demonstrated that anthranilic acid was rapidly converted to 2,3-dihydroxybenzoic acid (DHBA) and that this was the active compound (Harper & Swinburne, 1979; Harper et al., 1980). DHBA is a known chelating agent implicated in the active transport of iron in bacteria (Neilands, 1973 & 1974) and so a number of synthetic chelating agents were tested in germination assays with C. musae, all of which proved to some extent to be stimulatory (Harper et al., 1980).

Iron was implicated in germination auto-inhibition from two lines of research. Firstly, C. musae grown in iron-deficient medium produced conidia that germinated much more readily in water than those from iron replete medium; and germination of conidia obtained from media containing a

range of Fe^{3+} concentrations showed an inverse correlation with iron content (Harper et al., 1980). Secondly, when suspensions of washed cells of Pseudomonas UV3 were added to water droplets containing C. musae conidia an increase in germination and appressorium formation was observed (McCracken & Swinburne, 1979). An extract of the bacterial siderophore from low iron culture was shown to be stimulatory, but when iron was added to the extract no stimulation was observed.

The study reported here confirms the stimulatory effect of a siderophore from Pseudomonas UV3 on germination of C. musae conidia, and acts as an introduction to the methods used in the work with other fungi. The inhibitory action of the fully chelated siderophore (SAFe) in relation to pH is also studied, and a comparison is made between the effects of SA and nutrient solutions.

2.4.1 Culture maintenance and preparation of spore suspensions

Colletotrichum musae isolate 15, was isolated from anthracnose lesions on banana fruit obtained from Ecuador, by Dr. A.E. Brown at Queens University, Belfast. The fungus was cultured on modified Cooks No. 2 medium (Swinburne, 1976) at $18^{o}C$ under continuous illumination by fluorescent light. Conidial suspensions were prepared in sterile distilled water by removing slime spores from 14 day old cultures with a sterile nickel-chromium loop. Conidia were washed three times by centrifugation and resuspension in sterile distilled water, and diluted to a final concentration of c. $10^{5} ml^{-1}$, for use in germination assays.

In each field of view the number of germinated conidia, the number of dark appressoria, the total number of appressoria and the total number of conidia were recorded. From this data the percentage germination, percentage of germinated conidia with appressoria, percentage of germinated conidia with dark appressoria, percentage of all conidia with appressoria, percentage of appressoria that were dark, and the percentage of all conidia with dark appressoria were calculated, each figure being the average for all the fields of view recorded.

2.4.2 Preparation of acid-washed, low-iron glass slides

New clean glass slides were soaked in concentrated nitric acid for at least 12 hours. The acid was then washed off the slides with copious quantities of low-iron water, which were then transferred to 1M-sodium hydroxide for at least 2 hours to neutralise any remaining acid. The hydroxide was then washed off and the slides soaked in deionised water for at least 2 hours. After washing with 100% ethanol the slides were stored under 100% ethanol until use in germination assays. Prior to use, ethanol was removed by evaporation in a hot air stream.

2.4.3 Slide germination assay apparatus

Three 10 µl droplets of spore suspension, each containing about 5×10^3 conidia, in either water or treatment solution, were placed on each slide. The slides were placed in an horizontal slide rack, in a humid chamber, for 18 hours, at $18^{\circ}C$, in the dark. Using a Leitz Dialux 20EB microscope 6 fields of view, in 6 replicate drops were counted. Experiments were repeated at least twice and statistical analysis, to calculate the standard error of the mean, and the Least Significant Difference at P = 0.05 was performed on the bulked data.

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2.4.4 Preparation of treatment solutions

5×10^{-5} M SA, and SAFe, were prepared as previously described in Chapter 2.3.4. Unless otherwise stated, SAFe was made with ferric iron and not pH-adjusted.

The pH of 5×10^{-5} M SA was 5.9, the same as the distilled water, while the pH of SAFe made with $Fe_2(SO_4)_3$ was 3.3. The addition of sufficient $FeSO_4$ to SA to be fully bound if it

had been Fe^{3+}, reduced the pH to 4.6, but did not quench the fluorescence or alter the colour of the SA. The siderophore, SA, is ferric iron specific. Eventually the SA solution went brown and lost the associated fluorescence as the Fe^{2+} was oxidised to Fe^{3+} and was bound by the SA. The pH of the SAFe solution made with $FeSO_4$ was altered to pH 3.3 for comparison in germination assays with the SAFe made with $Fe_2(SO_4)_3$. SAFe solutions made with $Fe_2(SO_4)_3$ and $FeSO_4$ (designated $SAFe^{3+}$ and $SAFe^{2+}$, but probably only initially binding to Fe^{3+}) were also compared at pH 5.5. Adding NaOH to a mixture of $FeSO_4$ and SA accelerated the conversion to Fe^{3+} and complexing of the iron by SA.

The effect of $Fe_2(SO_4)_3$ and $FeSO_4$ solution on germination and appressorium production was also examined at different pH. Dilute H_2SO_4 was used to lower the pH of $FeSO_4$ to 3.3 for comparison with $Fe_2(SO_4)_3$, both solutions containing 5×10^{-5} M Fe. When dilute NaOH was added to $Fe_2(SO_4)_3$ and $FeSO_4$, a yellow-brown precipitate was formed. This was $Fe(OH)_3$ as described in Chapter 1.1. A saturated solution at pH 5.5 of the salts was used.

2.5 Results

SA (5×10^{-5}M) present in the inoculum consistently stimulated the germination of C. musae and the formation of both light and dark appressoria. 5×10^{-5}M SAFe, at the unadjusted pH of 3.3, significantly inhibited germination and appressorium formation, the production of dark appressoria being almost completely inhibited (see Table 2.1). At pH 5.5 SAFe significantly inhibited germination and appressorium formation, but less than at the lower pH. Very dilute H_2SO_4,

TABLE 2.1

The effect of SA, Fe^{3+}, $SAFe^{3+}$ and $SAFe^{2+}$ at different pH, on the germination and appressorium formation of C. musae in vitro

Treatment/pH	Percentage of Germination	Percentage of Germinated Conidia With Appressoria	Percentage of Germinated Conidia With Dark Appressoria	Percentage of All Conidia With Appressoria	Percentage of Appressoria that are dark	Percentage of All Conidia with dark Appressoria
H_2O/5.9	52.4 ± 4.9[a]	83.1 ± 4.7	70.6 ± 6.2	34.9 ± 2.0	82.0 ± 5.2	28.5 ± 2.3
5 x 10^{-5}MSA/5.9	76.2 ± 2.6	68.0 ± 4.5	55.3 ± 3.4	54.4 ± 5.7	82.4 ± 2.5	43.2 ± 3.3
5x10^{-5}M $SAFe^{3+}$/5.5	32.5 ± 3.5	49.1 ± 6.1	18.8 ± 6.5	16.5 ± 3.3	25.5 ± 7.5	9.2 ± 3.5
5x10^{-5}M $SAFe^{3+}$/3.3	23.8 ± 1.8	11.8 ± 2.7	0.5 ± 0.5	3.1 ± 0.7	3.0 ± 3.0	0.1 ± 0.1
5x10^{-5}M $SAFe^{2+}$/5.5	31.2 ± 2.7	84.5 ± 4.9	69.4 ± 6.9	26.2 ± 2.2	78.5 ± 6.7	21.9 ± 2.5
5x10^{-5}M $SAFe^{2+}$/3.3	23.7 ± 2.2	7.7 ± 3.4	2.5 ± 1.9	1.5 ± 0.6	8.3 ± 6.1	0.5 ± 0.3
Dilute H_2SO_4/3.3	37.8 ± 4.0	55.8 ± 5.7	19.4 ± 5.6	18.3 ± 2.7	25.3 ± 6.4	9.4 ± 2.9
5x10^{-5}M $Fe_2(SO_4)_3$/ 5.5*	34.2 ± 3.6	60.4 ± 4.7	25.8 ± 5.6	21.4 ± 2.9	38.3 ± 7.1	12.3 ± 2.7
5x10^{-5}M $Fe_2(SO_4)_3$/ 3.5	34.3 ± 4.7	67.3 ± 5.3	20.0 ± 4.9	23.0 ± 4.0	29.5 ± 6.4	12.3 ± 3.6
L.S.D. (P = 0.05)	6.6	9.2	9.1	5.3	11.2	4.6

* : Ferric iron precipitated, saturated solution used.

a : ± Standard Error

at pH 3.3 did not significantly effect germination but did inhibit appressorium formation, especially dark appressoria. Free ferric iron at pH 3.5 reduced germination but was significantly less inhibitory than SAFe at a similar pH. The reduced amount of ferric iron available at pH 5.5 was sufficient for the effect on germination to be not significantly different from $Fe_2(SO_4)_3$ at pH 3.5.

The difference in the effect of SAFe made from $Fe_2(SO_4)_3$ and $FeSO_4$ on germination and appressorium formation is shown in Table 2.2. The SAFe, from $Fe_2(SO_4)_3$, was inhibitory to germination and appressorium production, as previously described.

The effect of $SAFe^{2+}$ and $SAFe^{3+}$ were very similar, both almost completely inhibited dark appressorium formation at pH 3.3. At the high pH, $SAFe^{2+}$ was significantly less inhibitory, the inhibition of dark appressoria, although statistically significant, being relatively small.

A comparison of the effects of Fe^{3+} and Fe^{2+} is also shown in Table 2.2. Iron sulphate was inhibitory to the germination and appressorium formation of C. musae although the pH and iron valence altered the extent of the inhibition. As previously shown, $Fe_2(SO_4)_3$ was inhibitory to both germination and appressorium formation, there being no difference in the effect at pH 3.3 and 5.5. At pH 5.5, $FeSO_4$ was not so inhibitory to germination, but had a similar effect to $Fe_2(SO_4)_3$ on dark appressoria. At the lower pH, when presumably more iron remained in the ferrous state, it was more inhibitory to both germination and appressorium formation than $Fe_2(SO_4)_3$.

TABLE 2.2

The effect of SA and, Fe^{3+} or Fe^{2+}, at different pH, on germination and appressorium formation of C. musae in vitro

Treatment/pH	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of all conidia with appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O/5.9	48.7 ± 3.9	89.0 ± 3.6	81.4 ± 4.2	43.4 ± 3.5	90.8 ± 2.5	38.5 ± 2.5
$5x10^{-5}$M SA/5.9	62.8 ± 2.2	91.2 ± 2.5	83.8 ± 3.3	54.4 ± 3.8	91.6 ± 1.9	52.4 ± 2.2
$5x10^{-5}$M Fe^{3+}/5.5*	27.2 ± 2.7	92.6 ± 3.1	81.6 ± 5.5	25.2 ± 2.4	89.1 ± 4.2	21.9 ± 2.2
$5x10^{-5}$M Fe^{3+}/3.3	28.6 ± 3.2	90.6 ± 4.0	80.2 ± 5.5	24.9 ± 2.5	88.3 ± 4.2	22.0 ± 2.4
$5x10^{-5}$M Fe^{2+}/5.5*	37.1 ± 3.6	82.2 ± 3.7	66.4 ± 4.9	20.9 ± 3.7	80.7 ± 4.6	23.5 ± 2.6
$5x10^{-5}$M Fe^{2+}/3.3	21.0 ± 1.8	70.9 ± 7.0	62.5 ± 8.3	14.6 ± 1.6	70.9 ± 9.8	11.9 ± 1.9
L.S.D. (P = 0.05)	5.7	7.8	10.4	5.7	8.9	4.5

* Ferric iron precipitated, saturated solution used.

Compared with the stimulatory action of 5×10^{-5}M SA on the germination and production of both light and dark appressoria, an equal concentration of alanine or glucose had no significant effect on C. musae conidia (see Table 2.3).

2.6 Discussion

The consistent stimulation of germination of C. musae conidia in vitro by the siderophore SA, is in agreement with earlier reports (McCracken & Swinburne, 1979). The role of siderophores, such as SA, and of other iron chelating molecules present on the phytoplane, in the development of banana anthracnose has been discussed by Swinburne (1981).

Graham and Harper (1983) proposed that the action of chelating agents in promoting germination of conidia of C. musae involves the complexing of inhibitory iron at a site(s) within the conidia, so releasing the germination mechanism. Selective accumulation of a germination stimulant (EDTA) within the organelle fraction during the induction of germination in iron replete conidia suggested that the site involved was located in that fraction, possibly on ribosomes. However, the marked differences observed between the relative dimensions of low iron and iron replete conidia also implicated the conidial wall as the site of iron complexed by chelating agents involved in the stimulation of germination.

Perhaps the most important effect of the SA in relation to C. musae in vivo, is the stimulation of dark appressoria. DHBA stimulated the formation of dark appressoria, and it would appear that the proportion of dark appressoria produced is a critical factor in the development of banana anthracnose (Brown & Swinburne, 1981). It is these appressoria which, failing to infect before the fruit ripens, do not provoke

TABLE 2.3

The effect of SA, alanine and glucose, on germination and appressorium formation of C. musae in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	13.5 ± 4.5	78.7 ± 8.8	9.5 ± 2.3	58.6 ± 16.2	80.0 ± 20.0	8.0 ± 3.0
5 x 10^{-5}M SA	32.3 ± 6.3	95.3 ± 3.9	30.6 ± 6.4	87.7 ± 8.1	88.4 ± 11.1	22.9 ± 2.5
5 x 10^{-5}M alanine	9.9 ± 2.1	97.1 ± 2.9	9.5 ± 1.9	92.1 ± 5.1	95.0 ± 5.0	8.9 ± 1.8
5 x 10^{-5}M glucose	10.1 ± 2.2	96.1 ± 2.7	9.0 ± 1.9	76.4 ± 10.1	83.3 ± 9.8	8.3 ± 1.9
L.S.D. (P = 0.05)	7.4	9.1	6.2	19.5	22.7	4.5

phytoalexin synthesis and consequently when infection does occur, preformed phytoalexins do not impede colonisation. In water more of the appressoria were of the hyaline type and produced infection threads soon after inoculation. The phytoalexins elicited retarded infection so a significantly lower number of progressive lesions developed in water than in the presence of the iron chelator. In fully chelated DHBA no spreading lesions were produced by C. musae.

5×10^{-5}M alanine and glucose had no significant effect on germination and appressorium formation by C. musae conidia in vitro. 5×10^{-5}M SA was stimulatory. Some microorganisms may be able to utilise SA as a nutrient source, but the observed stimulation of C. musae by SA cannot be accounted for on a nutritional basis. Alanine has a C:N ratio closest of the amino acids to that of SA, although it would have been better to have compared SA with nutrient solutions containing the same amount of carbon and nitrogen.

Fully chelated SAFe was inhibitory to germination, either by increasing the binding of iron to the active site of germination inhibition, by stopping the necessary removal of iron from that site prior to germination, or by forming toxic iron complexes within the conidia. Since SAFe was more inhibitory than free iron, the siderophore may have aided the uptake of iron by the conidia and/or enhanced its effect as an inhibitor. Inhibition by SAFe was more marked at lower pH, but the pH itself was not inhibitory. This may have been due to increased cell wall permeability, or a more active uptake mechanism at lower pH. However other effects may operate at low pH, notably conversion of ferric to ferrous iron.

At reduced pH ferric iron complexes, of the sort seen in pseudobactin-like siderophores, such as SA, undergo internal

redox reactions when the iron becomes reduced to the ferrous state (Hider, 1985). Germination of spores of a number of plant pathogenic fungi was inhibited by ferrous iron complexed with synthetic chelating compounds, but no such inhibition occurred with ferric complexes (Brown & Sharma, 1985). The increased inhibition of C. musae conidia by SAFe at low pH may have been due to a higher proportion of the complexed iron being in the ferrous state.

The SAFe complexes made from either ferric or ferrous sulphate had similar biological properties, suggesting that the products were similar. Ferrous iron is unstable, especially at higher pH and is rapidly oxidised to ferric, which would have been chelated by the ferric-specific SA ligand. Experiments with ferrous and ferric salts alone also gave similar results, probably because of the rapid oxidation of ferrous to ferric. The greater inhibition of germination and appressorium formation by ferrous sulphate at pH 3.3, when more iron would have remained in the ferrous state supports the proposal (Brown & Sharma, 1985) that ferrous iron is the active form in conidia.

Ungerminated spores of Phycomyes blakesleanus contained iron complexed with ferritin and as germination commenced the iron was transferred to a soluble iron pool, probably complexed with a siderophore-like molecule (David, 1974). It is possible that C. musae and probably other fungi mentioned later in this thesis, possess a mechanism which effectively solubilises ferric iron as germination proceeds, even if iron is involved in germination inhibition prior to the process of germination itself.

As well as inhibiting the derepression of the germination mechanism, iron complexes may be directly inhibitory to fungal growth. Compounds such as folic acid and riboflavin can form stable chelates with heavy metals, especially ferrous iron (Albert, 1960) and iron complexed with such important compounds may alone be detrimental to fungal growth. In yeasts such as Candida gulliermondii glycolysis was suppressed by high iron concentrations due to a low amount of active aldolase (Light & Clegg, 1974). Formation of ferrous-protein complexes may be responsible for inhibiting germination and reducing germ tube growth.

The effect of the fully chelated SAFe on appressorium formation, especially dark appressoria, may be of critical importance in vivo as described above. It is not clear if appressorium production is a necessary end-point of some germinated conidia, so the SA only actually stimulated germination or if the SA stimulated appressorium formation independently. Appressoria, and dark appressoria especially, appeared to be particularly sensitive to inhibition by SAFe at the lower pH. Fewer dark appressoria were formed in water at reduced pH, but the action of the SAFe was not due to pH alone and may have been due to the effects as described for germination inhibition; either as a result of an effect at the same time as germination was inhibited, so germinated conidia were necessarily less likely to form appressoria, or independent of germination, so germ tubes were directly inhibited from producing mature appressoria.

The inhibition of appressorium formation, especially mature appressoria, may be concomitant with germination inhibition, as described above. However, melanin biosynthesis

is a prerequisite for the maturation and penetration of appressoria of Colletotrichum lagenarium (Kubo et al., 1985) and iron or iron complexes may be involved in melanin biosynthesis. Ferrous iron-gallic acid complexes incorporated into growth media of Zygorrhynchus sp inhibited melanisation of zygospores (A. Brown, personal communication). Oxidation of iron-chelating phenolics, such as catechol, is involved in the synthesis of melanin and although iron complexed to the phenolics would not necessarily inhibit their oxidation, iron may affect the process. Gallic acid-ferrous iron complex is a strong reducing agent and would inhibit the oxidative formation of polyphenolics such as melanin. However, fungal oxidising capacity would be higher in the presence of ferric iron, the iron being involved in electron transport. Depletion of ferric iron was inhibitory to melanin formation by C. acutatum (see Chapter 6). The involvement of the various states of iron in fungal germination and appressorium formation clearly requires further research.

Chapter 3

Botrytis cinerea infection of Vicia faba

3.1 Introduction

Extensive research on the resistance of *V. faba* to *B. cinerea* (e.g. Mansfield & Deverall, 1974; Rossall & Mansfield, 1981) has shown that in general, following the application of conidial suspensions to adaxial surfaces of leaves, either no symptoms develop or limited lesions develop in which the growth of hyphae is arrested. Failure to develop symptoms was associated with failure of conidia to germinate, which Rossall and Mansfield (1980) attributed to interaction with epiphytic bacteria (also Blakeman, 1972) and phytoalexins, notably wyerone derivatives, implicated in cessation of hyphal growth in limited lesions by Hargreaves *et al.* (1976, 1977). The presence of exogenous nutrients on the leaf surface has also been implicated in *B. cinerea* infection of *V. faba* leaves (Harper & Strange, 1981; Rossall & Mansfield, 1981).

The involvement of iron chelators in the development of lesions by *B. cinerea* was first reported by Swinburne (1981). Lesions were produced in *V. faba* leaves more rapidly and many more spreading lesions developed when inocula contained the iron chelating agents ethylene diamine tetra acetate (EDTA) and 2,3-dihydroxybenzoic acid (DHBA). Pretreatment of conidia with EDTA also enhanced fungal aggression, whilst FeEDTA and FeDHBA, the ferrated forms of the chelators, reduced lesion formation. Phytoalexins accumulated in the restricted lesions caused by *B. cinerea* in water, but only low levels accumulated in the spreading lesions caused by *B. cinerea* inoculated in EDTA or DHBA, and at the site of inoculation in FeEDTA or FeDHBA which remained symptomless.

In the experiments reported here, the effect of the natural iron chelating agent, SA, on B. cinerea germination and infection of V. faba was studied and compared to the effects of similar concentrations of EDTA. The effect of SA on the toxicity of wyerone to germinating conidia in vitro was investigated, and with the use of abiotic elicitors, the action of SA on phytoalexin production by V. faba was studied.

3.2 Methods

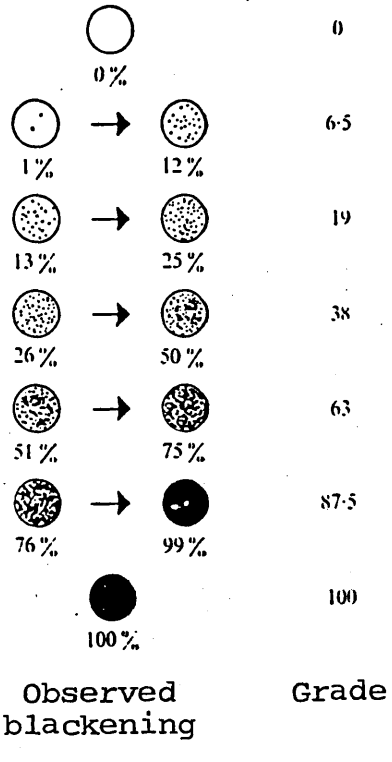
3.2.1 Vicia faba

Leaves - Bifoliate leaves of V. faba, c.v. Masterpiece Green Longpod, obtained from green-house grown plants as previously described (Brown & Swinburne, 1982) were placed in an illuminated humid chamber at 18°C with their petioles in water. Six 10 µl inoculum droplets were placed on the adaxial surface of each leaflet and lesion development was periodically assessed using the infection grade key devised by Mansfield & Deverall (1974), see Figure 3.1.

Cotyledons - Seeds of V. faba c.v. Masterpiece Green Longpod were surface-sterilised in 70% (v/v) methanol- 1% (v/v) formalin for 15 minutes, rinsed in sterile distilled water and left to soak in sterile distilled water for 18 hours. The seed coats were removed and the separated cotyledons laid flat-side-up in a humid chamber. Any malformed or contaminated cotyledons were discarded. Each cotyledon was inoculated with one or two 10 µl droplets of liquid and the phytoalexin accumulation was assessed after 10 days at 18°C under continuous fluorescent illumination.

FIGURE 3.1

Infection grade key based on the percentage blackening beneath the inoculum droplet.



From Mansfield & Deverall (1974), sites with spreading lesions were recorded as 100.

3.2.2 Culture maintenance and preparation of conidial suspensions

B. cinerea isolate 13, from Dr. A. Brown, Queens University, Belfast, was grown on autoclaved Phaseolus vulgaris c.v. Canadian Wonder leaves in test tubes at 18°C under continuous fluorescent light. Conidia were removed by shaking the culture with 5 ml of sterile distilled water and washed three times by centrifugation and resuspension in sterile distilled water. The final suspensions were adjusted to c. 1 x 10^6 conidia ml^{-1} and mixed with an equal volume of test solution or water, prior to inoculation.

3.2.3 Preparation and use of abiotic elicitors

Conidia and aerial hyphae of B. cinerea were removed from cultures on autoclaved bean leaves, suspended in sterile distilled water and coarsely ground with a mortar and pestle. The resulting suspension was autoclaved, divided into 2 ml samples and stored at $^{-}18°C$. No attempt was made to characterise the active constituents of the elicitor suspension and sufficient was prepared at one time to use in all experiments. The autoclaved fungal elicitor was mixed with equal volumes of the test solutions or water before inoculation onto cotyledons or leaves. To elicit phytoalexin formation in the absence of added exogenous material a 25 W soldering iron was used to burn a 12 mm^2 area of tissue. When fully heated the bit was held against the cotyledon surface for 2 seconds. In the absence of an exogenous elicitor, phytoalexin production would have been elicited by the charred tissue or endogenous elicitors released by the killed cells.

3.2.4. Extraction, detection and measurement of phytoalexin

A 6 mm diameter cork borer was used to harvest inoculated areas of V. faba leaves and 2 mm deep areas of cotyledon tissue. A known weight of cotyledon tissue was homogenised in diethyl ether (10 ml g^{-1}), the extracts evaporated to dryness in vacuo at 30°C as previously described (Hargreaves et al., 1977) and the residue taken up in 0.4 ml analytical grade methanol. The phytoalexin concentration in the samples was measured using either, (a) U.V. absorbance spectrophotometry, or (b) high performance liquid chromotography.

(a) U.V. absorbance spectrophotometry - The methanol extracts were applied to precoated thin layer chromotography (t.l.c.) plates (Merck, Kieselgel 60 F_{254}; 0.5 mm thick silica) and developed in hexane:acetone (2:1) followed by chloroform: petroleum spirit (60 to 80°C) (2:1). Phytoalexins were located by comparison with the Rf values given by Hargreaves et al. (1977) and the band corresponding to wyerone eluted into 0.4 ml methanol. The silica was removed by centrifugation and the U.V. absorbance spectrum of the eluant recorded using a Pye-Unicam SP8-100 spectrophotometer. Wyerone concentration was calculated as previously described (Rossall et al., 1980).

(b) High performance liquid chromatography - 20 µl samples of the crude methanol extract were loaded onto a 20 cm Magnusphere C22 column, fitted with a 2.5 cm silica precolumn. An Eldex AA-94-8 dual piston precision pump produced a flow rate of 0.9 ml min^{-1} at 2500 p.s.i. using 65% (v/v in water) H.P.L.C. grade methanol solvent. Elution was monitored at 351 nm using a Cecil CE212 variable wavelength U.V. monitor connected to a pen recorder operating at 5 mm min^{-1}.

Under these conditions wyerone produced a peak at 12.5 cm. The amount of wyerone in the 20 µl load was calculated by comparing the peak area with known standards. Pure wyerone was produced by repeated t.l.c. and its concentration calculated by spectrophotometry.

H.P.L.C. was the favoured method, being easier, quicker and more accurate than the spectrophotometric method. Concentrations as low as 2.5×10^{-4} µg μl^{-1} in a 20 µl sample of crude extract could be detected by H.P.L.C.

Results from both techniques were recorded as µg of wyerone in each gram fresh weight of tissue, (µg g FW^{-1}). The experiment to calculate the effect of SA on wyerone accumulation in cotyledons elicited by heat-killed B. cinerea was repeated 8 times and other experiments were repeated 4 times. Mean values and standard errors are shown. with the Least Significant Difference at P = 0.05.

3.2.5 Use of low-iron glassware, deionised water, SA and SAFe

Low iron glassware and siderophore solutions were prepared and used as described in Chapter 2.

3.2.6 Use of EDTA and EDTAFe

Ethylene diamine tetra-acetic acid (EDTA) and its iron complex (EDTAFe) were used in a final concentration of 5×10^{-4}M in the inoculum droplets. EDTAFe was made by adding sufficient $Fe_2(SO_4)_3$ to the EDTA to be fully chelated, prior to dilution to 10^{-3}M, as previously described (Brown & Swinburne, 1982).

3.2.7 Spore germination assays

Germination assays on low-iron slides were carried out as described in Chapter 2.

3.2.8 Low-iron B. cinerea culture

Germination assays were also conducted on conidia of B. cinerea from low-iron culture. Czapek-Dox medium (Commonwealth Mycological Institute, 1968) was deferrated using Chelex 100 resin as described in Chapter 2. Acid-washed glass fibre paper was placed around the inside of an acid-washed glass jar containing the medium and inoculated with a suspension, in low-iron water, of conidia from a culture on Kings medium B. After 7 days, conidia were carefully removed by suction through a drawn-out Pasteur pipette and impaction on to a 0.45 μm millipore filter. Conidia were washed off with low-iron water for germination assays. Conidia grown in iron-replete medium, in normal glassware, were used as controls.

3.2.9 Wyerone toxicity assay

Germination assays to assess the effect of 5×10^{-5}M SA on the fungitoxicity of various concentrations of pure wyerone were done on commercial pregnancy testing slides. 10 μl droplets of pure wyerone produced by repeated t.l.c. in 100% methanol or 100% methanol alone, were placed in the wells on the slides and allowed to dry. A 10 μl droplet of B. cinerea conidia in either water or SA solution was then placed in the wells, and the slides incubated as described in Chapter 2.

3.3 Results

3.3.1 Germination and appressorium formation

The effect of 5×10^{-4}M EDTA and EDTAFe in the inoculum droplet on the germination and appressorium formation by B. cinerea isolate 13 is shown in Table 3.1. The significant

TABLE 3.1

The effect of EDTA and FeEDTA on germination and appressorium formation by B. cinerea in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria
H_2O	17.7 ± 3.6[a]	7.5 ± 4.9	1.4 ± 0.9
5×10^{-4}M EDTA	42.6 ± 3.9	19.3 ± 7.9	9.3 ± 3.9
5×10^{-4}M FeEDTA	9.8 ± 3.4	0.0 ± 0.0	0.0 ± 0.0
L.S.D. (P = 0.05)	7.2	8.4	3.2

a : Mean ± standard error

inhibition by EDTAFe is similar to that demonstrated by Brown & Swinburne (1982), but they were unable to show a significant stimulation by EDTA. This was probably due to a high germination of the fungus in water, rather than less effective EDTA treatment, since during this study it was noted that the stimulation by EDTA was statistically less significant when the control germination in water was high.

5×10^{-5}M SA significantly stimulated germination and appressorium formation *in vitro*, with more appressoria being formed than were produced in the EDTA treatment. (See Table 3.2) The addition of ferric iron to the SA, to form SAFe, completely nullified the stimulatory effect of the SA, while free Fe^{3+} had no significant effect on germination (see Table 3.3).

3.3.2 Lesion production

The formation of lesions on *V. faba* leaves by conidia inoculated in water droplets, although variable, was generally limited to a few necrotic flecks under the inoculation droplet. Conidia inoculated in a solution of EDTA produced lesions on leaves more rapidly and many more spreading lesions developed, than conidia in water. EDTAFe reduced lesion formation to a very low level and no spreading lesions formed. These effects of EDTA and EDTAFe, *in vivo*, were the same as those reported by Brown & Swinburne (1982) and no data are shown here. 5×10^{-5}M SA significantly stimulated both the speed lesions were formed and the number of spreading lesions that developed. (see Table 3.4 and Figure 3.2.). SAFe inhibited lesion production (data not shown).

TABLE 3.2

The effect of SA on germination and appressorium formation of B. cinerea in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria
H_2O	36.9 ± 12.2	5.4 ± 3.3	2.6 ± 1.6
5×10^{-5}M SA	59.5 ± 5.9	57.2 ± 4.2	33.8 ± 3.6
L.S.D. (P = 0.05)	17.9	7.4	5.1

TABLE 3.3

The effect of SA, SAFe and Fe^{3+} on the germination of B. cinerea in vitro

Treatment	Percentage Germination
H_2O	45.9 ± 4.7
5 x 10^{-5}M SA	70.6 ± 3.7
5 x 10^{-5}M SAFe	47.9 ± 7.1
5 x 10^{-5}M $Fe_2(SO_4)_3$	34.7 ± 8.3
L.S.D. (P = 0.05)	11.8

TABLE 3.4

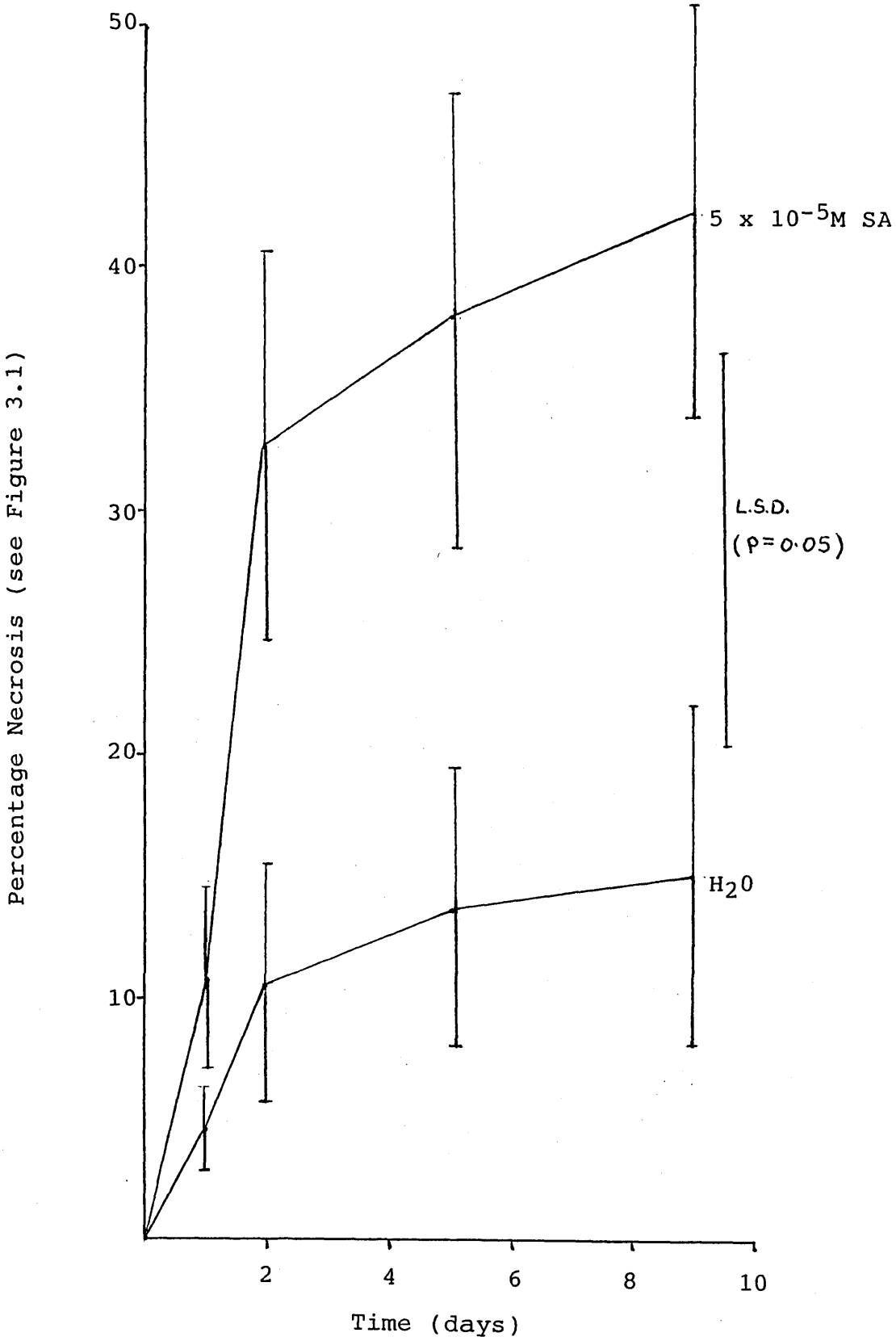
The effect of SA in the inoculum on the development of lesions on V. faba leaves

Treatment	Average % browning under inoculum droplet (± S.E.)			
	Day 1	Day 2	Day 5	Day 9
H_2O	4.9 ± 2.1[a]	10.7 ± 5.0	13.9 ± 6.3	15.3 ± 6.9
5 x 10^{-5}M SA	11.3 ± 3.8	32.9 ± 7.8	37.4 ± 8.7	41.9 ± 9.3

a : See Figure 3.1

FIGURE 3.2

The effect of SA on the development of B. cinerea lesions on V. faba leaves



3.3.3 Low-iron conidia

So few conidia were retrieved from the liquid medium cultures, only a few experiments were undertaken. 74.7$\pm$ 4.8 per cent of conidia derived from the iron replete culture germinated *in vitro* : 83.9$\pm$ 4.6 per cent from the low-iron culture. The number of germ tubes and germ tube initials produced by conidia was significantly higher when conidia were grown in low iron conditions; 2.8$\pm$ 0.3 germ tubes per conidia from low iron medium, 1.7$\pm$ 0.2 from normal medium. Germ tubes produced by low-iron conidia were considerably shorter than those produced by iron replete conidia.

3.3.4 Elicitors and phytoalexin accumulation

The presence of a droplet of crude heat-killed *B. cinerea* extract on the surface of *V. faba* cotyledons elicited the accumulation of the phytoalexin wyerone in the cells beneath the droplet, to a level about 10 times greater than that seen in the cells beneath distilled water droplets (see Table 3.5). The small amount of wyerone that accumulated under a water droplet may have been due to slight damage done in preparing the cotyledons, or the presence of a low level of contaminating micro-organisms observed on some cotyledons. The amount of wyerone that accumulated in response to the elicitor was reduced by over 40% when the suspension contained 5 x 10^{-5}M SA. The fully chelated SAFe had no significant effect on wyerone accumulation elicited by the fungal extract (see Table 3.6). Free ferric iron in the form of $Fe_2(SO_4)_3$ also had no significant effect on phytoalexin elicitation.

10 μl droplets of 5 x 10^{-5}M SAFe, or $Fe_2(SO_4)_3$ on the cotyledon surface did not elicit a significantly different

TABLE 3.5

The effect of SA on wyerone accumulation in V. faba cotyledons in response to heat-killed B. cinerea

Treatment	Mean wyerone concentration ($\mu g\ g\ FW^{-1}$)
H_2O control	25.8 ± 5.2
Elicitor/H_2O	232.1 ± 30.8
Elicitor/ 5 x 10^{-5}M SA	129.6 ± 20.4
L.S.D. (P = 0.05)	43.2

TABLE 3.6

The effect of SAFe and $Fe_2(SO_4)_3$ on wyerone accumulation in V. faba cotyledons in response to heat-killed B. cinerea; and wyerone accumulation in response to SA, SAFe and $Fe_2(SO_4)_3$ alone

Treatment	Mean wyerone concentration ($\mu g\ g\ FW^{-1}$)
H_2O control	12.4 ± 6.4
Elicitor/H_2O	178.2 ± 36.7
Elicitor/5 x 10^{-5}M SAFe	186.7 ± 60.4
Elicitor/5 x 10^{-5}M $Fe_2(SO_4)_3$	194.0 ± 82.6
5 x 10^{-5}M SA only	1.8 ± 0.3a
5 x 10^{-5}M SAFe only	15.1 ± 13.6
5 x 10^{-5}M $Fe_2(SO_4)_3$ only	24.6 ± 14.9
L.S.D. (P = 0.05)	70.6

a : Values less than 5.0 are inaccurate

amount of wyerone from that seen in the water controls. SA, however, consistently reduced the accumulation of the phytoalexin compared to the controls, although the amount was often too low to measure accurately.

Burning the surface layers of the cotyledons elicited a much greater phytoalexin accumulation than the crude elicitor suspension (see Table 3.7). The amount of wyerone in the heat-killed zone was significantly reduced when a droplet of SA, but not SAFe, was placed over the burnt area after the tissue was killed.

A spreading lesion developed on V. faba cotyledons inoculated with a 10 µl suspension of B. cinerea conidia in water. There was no visible difference in the lesions produced when SA or SAFe was present in the inoculum droplet compared to B. cinerea in water alone. The phytoalexin concentration correlated with the size of the lesion (data not shown) but was not affected by SA or SAFe in the infection court (see Table 3.8).

3.3.5 A possible siderophore produced by B. cinerea

B. cinerea isolate 13 was grown on King's medium B (see Chapter 2). After 10 days the medium through which the fungus was growing and a zone surrounding the colony became pigmented brown and slightly fluorescent under 366 nm U.V. light. No such pigmented zone was visible when the fungus was grown on malt extract medium (3% malt extract w/v, 0.5% peptone w/v), or V8 medium (20% Campbells V8 Juice, v/v).

An attempt was made to isolate the pigment for further study. After scraping the mycelium from the surface the medium was homogenised in 100% methanol in a blender. The

TABLE 3.7

The effect of SA and SAFe on wyerone accumulation in V. faba cotyledons in response to burnt surface cells

Treatment	Mean wyerone concentration ($\mu g\ g\ FW^{-1}$)
Burnt/H_2O	867.7 ± 6.6
Burnt/5 x 10^{-5}M SA	479.8 ± 84.9
Burnt/5 x 10^{-5}M SAFe	805.6 ± 76.9
L.S.D. (P = 0.05)	129.1

TABLE 3.8

The effect of SA and SAFe on wyerone accumulation in V. faba cotyledons in response to infection by B. cinerea

Treatment	Mean wyerone concentration ($\mu g\ g\ FW^{-1}$)
H_2O control	23.9 ± 15.7
B. cinerea/H_2O	911.6 ± 198.9
B. cinerea/5 x 10^{-5}M SA	972.4 ± 443.7
B. cinerea/5 x 10^{-5}M SAFe	953.4 ± 296.6
L.S.D. (P = 0.05)	549.1

extract from a number of plates was bulked, rotary evaporated at 50°C to a reduced volume, and taken into low-iron distilled water. The clear brown aqueous extract was mixed with a slurry of SM2 bio-beads (Bio Rad Labs, Ltd), prepared as described in Chapter 2. The slurry was poured into a glass column (4 cm diameter, 30 cm tall) with a scintered glass base. The beads were washed with copious quantities of low-iron water, followed by a gradient of methanol, from 50% to 100%. A phenol-sulphuric acid assay, undertaken on bulked samples of the water and methanol fractions, indicated that the aqueous sample contained large amounts of carbohydrates, while there was an insignificant amount in the methanol fractions. The aqueous fraction was discarded.

Two peaks of fluorescent brown material were eluted by the methanol gradient. The second, eluted by almost 100% methanol, was the largest and was bulked. This sample was rotary evaporated, taken into water, and applied to a 2.2 cm diameter, 45 cm long, column of P4 bio gel (Bio Rad Labs, Ltd) previously equilibrated in low-iron water. Three broad bands were eluted in water. One band absorbed to the top of the column, and had to be removed by passage of 1M- NaCl down the column. This band was discarded, but the three other bands were retained and designated BcF1, BcF2 and BcF3. Germination assays done on the fractions showed only BcF1, the major peak eluted from P4, was stimulatory to germination and appressorium formation by B. cinerea (see Table 3.9).

The three samples were all fluorescent under 366 nm U.V. light, the fluorescence being quenched by the addition of $Fe_2(SO_4)_3$ solution. An aqueous solution of BcF1 was dialysed

against low iron water. After 24 hours both the diffusable and non-diffusable fractions were yellowish in colour and fluorescent under U.V. light. Both had similar U.V. absorbtion spectra; and same as that of BcF1, with no main absorbance peaks. This indicated the BcF1 fraction probably contained a mixture of a substance polymerised to different sizes. The brown, polymerised substance may have been polyphenolic, but only produced slight colouration in response to Diazotised paranitroaniline reagent spray, followed by exposure to Na_2CO_3, probably indicating only limited phenolic composition. The mixture was, however, Ninhydrin positive, indicating the presence of amino acids and positive in Csakys test for the iron-chelating hydroxylamine moeity (Csaky, 1984).

Analysis of the cation concentration in BcF1, BcF2 and BcF3 indicated the main peak, BcF1, contained relatively more iron than the others (see Table 3.10). The iron concentration was 110 times more than the equivalent amount of Kings B medium, apparently indicating preferential accumulation of iron from the medium into BcF1, while the other cations were less concentrated in the samples than in the medium. (Kings B medium is not actually a low iron medium, although no iron is added; the iron is made unavailable to microorganisms growing on it (King et al., 1954)).

Although the evidence suggested that the complex mixture of substances released by B. cinerea growing on Kings B medium, may have been involved in iron sequestration, possibly by binding to hydroxamate or amino acid residues in a polymerised polyphenolic compound of variable molecular mass, the substance was not characterised and only preliminary investigations into its biological activity were undertaken.

TABLE 3.9

The effect of fluorescent compounds produced by B. cinerea and appressorium formation by B. cinerea in vitro

Treatment	Percentage Germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria
H_2O	73.5 a≠	5.1 a	4.3 a
1.5 gl^{-1} BcF_1	91.0 b	15.9 b	14.2 b
1.5 gl^{-1} BcF_2	79.8 a	10.0 ab	7.3 b
1.5 gl^{-1} BcF_3	70.6 a	0.8 a	0.6 c

≠ : Values with same letter in each column are not significantly different, P = 0.05

TABLE 3.10

Concentration of cations in fluorescent compounds produced by B. cinerea and in Kings B medium

Sample	Concentration (µg ml^{-1})				
	K	Ca	Mg	Na	Fe
0.4 gl^{-1} BcF_1	10	2.5	2.3	9	2.3
0.4 gl^{-1} BcF_2	39	1.0	8.4	49	2.3
0.4 gl^{-1} BcF_3	11	4.5	20.5	32	1.8
Kings B medium (23 gl^{-1})	624	11.0	143.0	348	1.2

Ca and Mg were analysed by atomic absorption spectroscopy, Na and K by atomic emission spectroscopy, and Fe by colourimetry (Ferrozine) after Kjeldahl digestion. The analysis was carried out by T.J. Samuelson, E.M.R.S., 1984.

The effect of BcF1 on the *in vitro* germination and appressorium formation of *B. cinerea* derived from two media is shown in Table 3.11. The fully replete medium, V8, was compared to the 'iron limited' Kings B. There was no significant difference between the germination and appressorium formation of conidia derived from mycelia grown on either medium. Presumably the fungus obtained sufficient iron from Kings B to produce normal, iron replete conidia. There was no significant difference between the effects of BcF1 on conidia from either medium; the BcF1 significantly stimulated germination and appressorium formation. Germination and appressorium formation by *C. musae* conidia was stimulated by the presence of BcF1 in the inoculum droplet. (see Table 3.12).

3.3.6 Wyerone toxicity

In addition to the effects of siderophores on germination, appressorium formation and phytoalexin accumulation, these compounds may also interact with the toxicity of phytoalexins, such as wyerone, to the pathogen. This possibility was examined by measuring *B. cinerea* germination and appressorium formation in different concentrations of wyerone, with or without SA in the inoculum. Concentrations were chosen to kill approximately 50% and 25% of the *B. cinerea* conidia, assuming the toxicity of the wyerone used to be the same as that reported previously (Rossall *et al.*, 1980). The concentrations, 38 μg ml^{-1} and 22 μg ml^{-1} respectively, were also chosen for their ease of preparation by spectrophotometry.

38 μg ml^{-1} wyerone considerably inhibited germination of *B. cinerea in vitro* and completely inhibited appressorium

TABLE 3.11

The effect of BcF_1, and different sources, on germination and appressorium formation by B. cinerea in vitro

Medium Source/Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria
$V8/H_2O$	75.0 a ≠	1.5 ab	1.2 a
$V8/BcF_1$ (1.5 gl^{-1})	92.3 bc	11.6 bc	10.5 bd
Kings B/H_2O	79.5 ab	3.3 ab	2.5 ab
Kings B/BcF_1 (1.5 gl^{-1})	96.6 c	21.2 c	20.1 d

≠ : Values with the same letter in each column are not significantly different P = 0.05

TABLE 3.12

The effect of BcF_1 on germination and appressorium formation of C. musae in vitro

Treatment	Percentage germination	Percentage of all conidia with appressoria	Percentage of all conidia with dark appressoria
H_2O	26.4 a[≠]	19.2 a	16.4 a
1.5 gl^{-1} BcF_1	59.6 b	50.6 b	32.7 b

≠ : Values with the same letter in each column are not significantly different at P = 0.05

formation (see Table 3.13). A reduction from 62.7% germination to 3.3% indicated an ED_{50} value for wyerone of 4.0 $\mu g\ ml^{-1}$, assuming there is a linear relationship between the number of conidia killed and wyerone concentration, as described by Rossall *et al.* (1980). In the presence of SA the reduction from 69.9% to 8.8% was comparatively less than that seen in water, and gave an ED_{50} of 6·7 $\mu g\ ml^{-1}$ based on the above assumption. The number of conidia that did not germinate when exposed to 22 $\mu g\ ml^{-1}$ was less when SA was present, in a similar way to that observed with 38 $\mu g\ ml^{-1}$. However, 22 $\mu g\ ml^{-1}$ did not completely inhibit appressorium formation, although this was apparently more susceptible to the phytoalexin than the germination. Since *B. cinerea* conidia germinate and produce appressoria more readily in the presence of SA, more germinated conidia with appressoria were produced when conidia were exposed to wyerone when SA was also present, irrespective of whether the SA affected the action of wyerone on individual conidia.

3.4 Discussion

SA stimulated the fungus in a similar manner to the chelator EDTA; iron was implicated in the inhibition of germination and appressorium formation by the low level of germination and appressorium production observed in SAFe and EDTAFe and by the previously described effect of pretreating conidia with iron chelators, or using low-iron conidia, which resulted in stimulated germination and appressorium formation (Brown & Swinburne, 1982). Free iron in the form of $Fe_2(SO_4)_3$, like SAFe, had no significant effect on germination *in vitro*, the stimulatory effect of SA being more marked than any

TABLE 3.13

The effect of SA on the inhibitory action of wyerone on germination of B. cinerea conidia in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria
H_2O	62.7 ± 4.6	32.8 ± 3.8	20.4 ± 2.7
5 x 10^{-5}M SA	69.9 ± 3.7	26.5 ± 2.8	18.2 ± 2.0
38 µg ml^{-1} wyerone/H_2O	3.3 ± 1.6	0.0 ± 0.0	0.0 ± 0.0
38 µg ml^{-1} wyerone/5 x 10^{-5}M SA	8.8 ± 2.8	0.0 ± 0.0	0.0 ± 0.0
22 µg ml^{-1} wyerone/H_2O	12.9 ± 3.1	3.1 ± 3.1	0.6 ± 0.6
22 µg ml^{-1} wyerone/5 x 10^{-5}M SA	28.5 ± 5.0	6.5 ± 3.4	1.7 ± 0.8
L.S.D. (P = 0.05)	6.9	6.5	3.1

inhibitory effect of SAFe, unlike that observed with C. musae (see Chapter 2).

Although SA and EDTA stimulated both germination and appressorium formation, the stimulation of appressoria may be of greater importance in lesion development. B. cinerea can infect through germ tube apices and infection cushions (Emmett & Parbery, 1975), but more commonly infects through appressoria produced on germ tubes, as seen in this study. SA significantly stimulated appressorium formation, thus raising the infection potential of a droplet of conidial suspension. The increased infection potential observed in the presence of iron chelators enabled the fungus to overcome the potentially inhibitory effects of factors associated with the leaf surface environment. This inhibition has been related to competition from members of the epiphytic microflora (Blakeman & Brodie, 1977; and Rossall & Mansfield, 1980), inhibitory substances associated with leaf surface wax (Rossall & Mansfield, 1980) and variability in the amount of nutrients leached into the inoculum droplets from the leaf (Rossall & Mansfield, 1981).

The presence of SA in the inoculum allowed both, accelerated development of B. cinerea lesions on V. faba leaves, and an increase in the number of inoculation sites that gave rise to progressive lesions. An increase in the speed of germination and appressorium formation and an increase in the total number of infection sites produced by a droplet of spore suspension probably enabled the development of lesions before the plant was able to respond. Phytoalexins accumulated to fungitoxic concentrations in B. cinerea lesions on V. faba (Rossall et al., 1980); the SA may have stimulated

the fungus at the early stage of the infection process allowing the spread of the lesion and rapid kill of host cells ahead of the plants resistance response.

Direct involvement of the SA in an effect on the plants defence mechanism has also been implicated by this study. The results implied the effect of the SA was to alter, either the elicitation of phytoalexins in V. faba cotyledons at the stage when phytoalexin elicitors released by the host cells are produced (assuming the heat-killed fungus acted in a similar way to burning the cotyledons, by releasing such 'endogenous' elicitors), or to alter the biosynthesis of the phytoalexins directly. Many important enzymes contain iron (e.g. catalase, peroxidase, NADH dehydrogenase and succinate dehydrogenase) and others need iron as a cofactor (e.g. aconitase) (Jacobs & Worwood, 1974). EDTA specifically inhibits certain enzymes due to its action as a chelator (Lehninger, 1970), but it is not known if any of the enzymes involved in wyerone synthesis are affected by a siderophore in a similar way. That naturally occurring iron chelators, such as those found in humic acid, can affect plant biosynthetic pathways, has been demonstrated by Vaughn (1974) and Vaughn & Ord (1983). They showed the cessation of cell expansion growth, due to the incorporation of hydroxyproline into cell walls, was inhibited by such chelators. A similar result was reported when a brown fungal extract (possibly like BcF1) was used (Vaughn, 1969). Other natural iron chelators such as microbial metabolites and phytotoxins have been shown to effect host iron metabolism (Gross, 1982; and Hayashi et al., 1982). The inhibition of phytoalexin accumulation in host plants by substances released by microbial pathogens has been reported before; a substance released by Mycosphaerella pinodes conidia

inhibited pisatin formation in Pea and a metabolite released by Phytophthora infestans zoospores prolonged the time to hypersensitive cell death and phytoalexin accumulation in potato tubers, (Oku et al., 1977), phaseotoxin released by Pseudomonas phaseolicola suppressed phytoalexin synthesis in bean (Gnanamanickam & Patil, 1977), and metabolites of Alternaria carthami suppressed phytoalexin accumulation in Safflower (Tietjen & Matern, 1984). The action of the phytoalexin suppressors mentioned, has not been related to iron chelation, but a comparable method of action to the inhibition of wyerone accumulation by SA may be involved.

The concept proposed by Oku et al., that pathogenicity is related to the ability of a pathogen to suppress the host's defence mechanism, implies a specific suppressor mechanism. The reduction of phytoalexin accumulation by siderophores from saprophytic bacteria would not be specific although it may be advantageous to the pathogen. Siderophores produced by pathogens in vivo, may be of more importance in this instance (see later).

In the low concentration used here, SA had no significant effect on the amount of wyerone in the spreading lesions produced by live B. cinerea. The phytoalexin, wyerone, accumulated to a greater extent in such spreading lesions and there may have been insufficient SA to inhibit its production, although the high levels of phytoalexin in the limited lesions of burnt tissue were reduced by the siderophore. Since SA stimulated germination, appressorium formation and infection, the observed reduction of wyerone accumulation may have been masked by the increased phytoalexin production resulting from stimulated fungal growth. Although the lesions

produced by B. cinerea in different treatments appeared to be similar in size (the V. faba cotyledons were so susceptible the fungus was able to produce lesions in all treatments, unlike on leaves) if the amount of phytoalexin was related to the actual amount of fungus in the lesion, a relationship between phytoalexin accumulation and the presence of SA may have been observed. The inhibition of host defences may partly explain the previously observed stimulation of disease development of C. musae, G. cingulata and B. cinerea (Brown & Swinburne, 1981; Adikaram et al., 1982; Brown & Swinburne, 1982) by various chelating agents. However, the increase in disease observed when conidia were pretreated with iron chelators, but without such chelators present in the inoculum, could not be accounted for by the inhibition of phytoalexin production by iron chelators. Pretreatment may have 'primed' the fungus to produce its own siderophores, in a similar way the virulence of some bacterial pathogens is enhanced by treatment with iron chelators before inoculation into their mammalian hosts (Weinberg 1978).

It must be remembered that V. faba produces more than one phytoalexin (Bailey & Mansfield, 1982) and that only the accumulation of wyerone was recorded in this study. It is unlikely an iron chelating agent such as SA would specifically inhibit the formation of one phytoalexin in particular, but the effect of SA on the elicitation and accumulation of the other phytoalexins produced by V. faba cotyledons was not investigated.

During the preparation of the cotyledons it was noted that the most common contaminating microorganism was a

pseudomonad bacterium. Fluorescence, clearly visible under 366 nm UV light, after contaminated cotyledons were incubated at 18°C for two days, was probably due to bacterial siderophores, which may have stimulated bacterial growth, not only by making iron available to the bacterium, but by reducing the plants phytoalexin defence response, as mentioned earlier.

It seems unlikely that any iron chelating agents, such as SA, present in an inoculum droplet in which conidia are exposed to inhibitory concentrations of a phytoalexin, could significantly affect the tolerance of the conidia to the phytoalexin. Wyerone consistently inhibited B. cinerea conidia to a smaller extent in the presence of SA than in water, but this inhibition was still sufficient to have been effective at inhibiting lesion formation in vivo (Hargreaves et al., 1977). Since SA stimulated germination and appressorium formation and wyerone only inhibited a certain percentage of conidia, the inoculum had a higher infection potential in the presence of SA, than without it.

The involvement of a complex mixture of substances, produced by B. cinerea on Kings medium B, was implicated in the iron sequestration process of the fungus. Further study is necessary to ascertain if any such siderophore-like substances are involved in diseases caused by the fungus. Since SA inhibits the plants defence mechanism, it is not unreasonable to propose chelating agents may be produced in the disease situation and not only be involved in symptom expression, but in determining pathogenicity. The compound, BcF1, stimulated the in vitro germination and appressorium formation of B. cinerea and C. musae. This stimulation may

be attributed to iron chelation by BcF1 in a similar way to EDTA and SA, but the structure was not elucidated and the actual iron-binding capacity of the BcF1 used was not determined. Although a comparison of the effects of BcF1 with those of nutrients was not undertaken, the BcF1 was probably not acting only as a stimulatory nutrient source, since appressorium formation, as well as germination, was stimulated. The formation of appressoria by B. cinerea and C. musae is not stimulated in the presence of nutrient solutions, see Chapter 2.

The limited results gained with low-iron B. cinerea conidia indicate the involvement of iron in conidial germination repression. They are also in accord with the idea that the iron inhibitory to germination may be associated with the conidial cell wall (Graham & Harper, 1983) since a weakened cell wall would have allowed the observed emergence of more than the usual number of germ tubes.

Chocolate spot of V. faba caused by B. cinerea in the field is often associated with prolonged wet periods and pollen production (Békési, 1979). These conditions may favour the growth of siderophore-producing elements of the phytoplane microflora, which may affect B. cinerea infection, both by stimulating the infection process of the fungus and by reducing the capacity of the plant to respond. Chocolate spot lesions are often associated with contaminating bacteria which may be acting in this way. The involvement of iron-chelating substances produced by B. cinerea is also uncertain.

Chapter 4

Colletotrichum lindemuthianum races, infection of Phaseolus vulgaris cultivars

4.1 Introduction

The effect of iron chelators on the development of certain fungal diseases has already been referred to. Preliminary work has indicated that pretreatment of conidia with iron-chelators can alter the response of a differential host to a race specific pathogen (Swinburne, 1981). Phaseolus vulgaris cultivars vary in their susceptibility to the different races of Colletotrichum lindemuthianum; the cultivar Kievit is resistant to the race β, whilst cultivar Wade is susceptible to this race of the fungus.

The effect of SA, present in the inoculum, on the infection of resistant and susceptible P. vulgaris cultivars by C. lindemuthianum race β is reported in this chapter. The germination and appressorium formation were also studied in vivo and in vitro, and the accumulation of phytoalexins in hypocotyl lesions monitored under different conditions.

4.2 Methods

4.2.1 Plants. P. vulgaris plants, cultivars Wade and Kievit, were grown in moist, loam-based compost, at 20°C for 7 days, in complete darkness. Etiolated hypocotyls were cut 1 cm below the cotyledonary node and 1 cm, or more, above the compost surface. The cut ends were sealed with petroleum jelly to stop the hypocotyls drying out. They were placed in a humid chamber under constant

fluorescent illumination at 18°C, and inoculated with 10 µl droplets of conidial suspension or test solutions, placed 3 cm apart. Lesion development was recorded periodically, up to 10 days, using the infection grade key shown in figure 4.1. The phytoalexin concentration in the lesioned hypocotyls was assessed after 10 days.

FIGURE 4.1

Infection grade key, based on the amount of necrosis* under the inoculum droplet and its subsequent spread

Grade	Necrosis visible by eye
0	None
1	Few necrotic flecks
2	Discrete necrotic flecks over whole area
3	Necrotic flecks coalesced, necrotic zone
4	Necrosis spread beyond inoculated area, brown exudation from lesions.

* *C. lindemuthianum* infection of etiolated *P. vulgaris* hypocotyls.

4.2.2 Culture maintenance and preparation of conidial suspensions. *C. lindemuthianum* race β, from the culture collection at Imperial College of Science and Technology, was grown on Cooks No. 2 medium as described for *C. musae* in chapter 2. Conidia were removed from an 8 day old culture, washed and diluted to 1 x $10^6 ml^{-1}$, as previously described, and mixed with equal volumes of test solutions or water for inoculation.

4.2.3 Formation and use of an abiotic elicitor. Conidia and aerial hyphae of *C. lindemuthianum* race β were scraped off the growth medium, suspended in sterile distilled water and ground coarsely with a mortar and pestle. The resulting

suspension was autoclaved, divided into 2 ml samples and stored at -18°C. No attempt was made to characterise the active phytoalexin-eliciting component of the crude elicitor and sufficient was made at one time to use in all experiments. The elicitor suspension was mixed with equal volumes of water or test solutions before inoculation.

4.2.4 Extraction, detection and measurement of phaseollin. Segments of hypocotyl tissue, 3 mm deep, were excised from areas corresponding to inoculation sites, and frozen until extraction of the phytoalexins. Extracts were made by homogenising weighed samples in ethanol (10 ml g^{-1}), filtering through gauze and evaporation to dryness at 30°C *in vacuo*. Phaseollin content of the samples was analysed by (a) uv spectrophotometry, and (b) high performance liquid chromatography.

(a) A similar system to that described in chapter 3 was used except an extra purification step was employed before loading on to the t.l.c. plate. The extract was mixed with an equal volume of distilled water, reduced in volume by rotary evaporation, partitioned three times with equal volumes of ethyl acetate, taken to dryness *in vacuo*, and then dissolved in 0.4 ml h.p.l.c. grade methanol. The t.l.c. solvent system was chloroform:ethanol (33:1) and the concentration of phaseollin in the band eluted from the t.l.c. plate was calculated as described in chapter 3.

(b) The same equipment was used to analyse the crude hypocotyl extracts as was used for the analysis of *V. faba* extracts in chapter 3. However, 75% h.p.l.c. grade methanol pumping at 0.8 ml min^{-1} under a pressure of

2800 p.s.i. was used, and the eluate absorbance monitored at 280 nm. The peak corresponding to phaseollin was at 4.5 cm when the chart paper was moving at 5 mm min^{-1}. Phaseollin concentration in the 20 µl load was calculated from the area of the peak produced, compared with the area produced by a known concentration of phaseollin. Experiments were repeated three times. Results are shown as the concentration of phaseollin that accumulated per gram fresh weight of tissue. Statistical analysis was undertaken as described in chapter 3.

4.2.5 Low iron water, low iron glassware and siderophore solutions. These were prepared as described in chapter 2.

4.2.6 Spore germination assays. Germination assays were conducted on glass slides as described for C. musae in chapter 2. The germination and appressorium formation of C. lindemuthianum after 18 hours, in 10 µl droplets on P. vulgaris hypocotyls, was assessed by examining microscopically thin longitudinal sections of inoculated areas stained with cotton blue in lactophenol. Statistical analysis was performed as described in chapter 2.

4.3 Results

4.3.1 Germination and appressorium formation. Germination and appressorium formation was stimulated by the presence of 5×10^{-5} M SA in vitro. (See table 4.1.) Germination and appressorium formation in water was very poor. The reasons for this are not clear, but may have been similar to those described in chapter 6. Germination and appressorium formation was so low in water, that no significant inhibition by SAFe or Fe^{3+} could be shown.

TABLE 4.1

The effect of SA, SAFe, and Fe^{3+} on germination and appressorium formation by C. lindemuthianum race β in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	2.0 ± 0.8	12.5 ± 8.5	0.9 ± 0.7	9.4 ± 6.8	79.4 ± 6.8	0.7 ± 0.5
$5x10^{-5}$M SA	10.3 ± 1.8	86.9 ± 6.9	9.1 ± 1.5	82.3 ± 7.5	87.3 ± 6.9	8.1 ± 1.2
$5x10^{-5}$M SAFe	2.0 ± 0.7	6.3 ± 6.3	0.3 ± 0.3	6.3 ± 6.3	100.0 ± 100.0	0.3 ± 0.3
$5x10^{-5}$M $Fe_2(SO_4)_3$	4.4 ± 0.9	21.9 ± 10.2	1.1 ± 0.5	12.5 ± 7.2	55.6 ± 8.8	0.6 ± 0.3
L.S.D.(P = 0.05)	2.1	15.9	1.5	13.9	161.3	1.1

The germination of C. lindemuthianum race β in water on either P. vulgaris cultivar tested, was greater than that observed in vitro (see tables 4.2 and 4.3). There was no significant difference in germination and appressorium formation in water on the two cultivars. 5×10^{-5}M SA stimulated germination and appressorium formation on both cultivars to a similar degree. Germination of the fungus on cv. Kievit was significantly inhibited by the presence of SAFe in the inoculum droplet, but free Fe^{3+} had no effect, and on cv. Wade neither SAFe or Fe^{3+} had a significant effect on germination. Appressorium formation on both cultivars was significantly inhibited by SAFe, but only on cv. Wade did free Fe^{3+} inhibit appressorium formation.

4.3.2 Lesion production. The effect of SA and SAFe in the inoculum on C. lindemuthianum race B infection of P. vulgaris cultivars is summarised in figure 4.2. The fungus produced spreading lesions on etiolated hypocotyls of cv. Wade within 10 days of inoculation, and while initial development on cv. Kievit was similar, lesions became limited in size and the fungus failed to sporulate. See table 4.4 and figure 4.3. The presence of 5×10^{-5}M SA in the inoculum droplet had no significant effect on lesion production on either cultivar, although the development of lesions resulting from conidia inoculated in SA solution was consistently more advanced than those resulting from inoculation in water. Conidia inoculated in 5×10^{-5}M SAFe resulted in significantly smaller, less advanced, lesions on cv. Wade compared to

TABLE 4.2

The effect of SA, SAFe and $Fe_2(SO_4)_3$ on germination and appressorium formation by C. lindemuthianum race β on etiolated hypocotyls of P. vulgaris cv. Wade

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	32.5 ± 3.5	51.3 ± 6.2	14.9 ± 2.7	17.7 ± 5.5	33.9 ± 10.3	6.2 ± 2.1
$5x10^{-5}$M SA	54.3 ± 4.3	57.3 ± 4.2	29.5 ± 1.9	35.7 ± 5.3	61.1 ± 8.8	17.6 ± 2.4
$5x10^{-5}$M SAFe	28.2 ± 2.9	27.2 ± 8.3	8.2 ± 3.2	4.9 ± 3.1	8.6 ± 3.8	0.7 ± 0.3
$5x10^{-5}$M $Fe_2(SO_4)_3$	24.9 ± 3.0	35.5 ± 10.6	6.9 ± 1.9	14.8 ± 8.1	23.6 ± 8.9	2.4 ± 0.7
L.S.D.(P = 0.05)	6.8	14.5	4.8	10.9	15.7	2.7

TABLE 4.3

The effect of SA, SAFe and $Fe_2(SO_4)_3$ on germination and appressorium formation by C. lindemuthianum race β on etiolated hypocotyls of P. vulgaris cv. Kievit

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	40.9 ± 3.2	43.8 ± 3.6	17.6 ± 2.2	14.5 ± 3.8	27.6 ± 6.8	5.3 ± 1.4
$5x10^{-5}$M SA	50.5 ± 2.8	71.7 ± 5.8	35.3 ± 2.6	48.2 ± 7.9	63.5 ± 8.3	22.6 ± 3.2
$5x10^{-5}$M SAFe	27.1 ± 3.8	33.6 ± 8.4	9.2 ± 2.4	7.9 ± 4.8	13.9 ± 8.7	1.3 ± 0.7
$5x10^{-5}$M $Fe_2(SO_4)_3$	35.4 ± 4.4	52.7 ± 7.3	19.0 ± 3.8	22.3 ± 5.3	35.6 ± 8.9	9.3 ± 2.8
L.S.D.(P = 0.05)	7.0	12.4	5.4	10.9	16.1	4.0

FIGURE 4.2

Effect of SA and SAFe on disease development of C. lindemuthianum on P. vulgaris cultivars

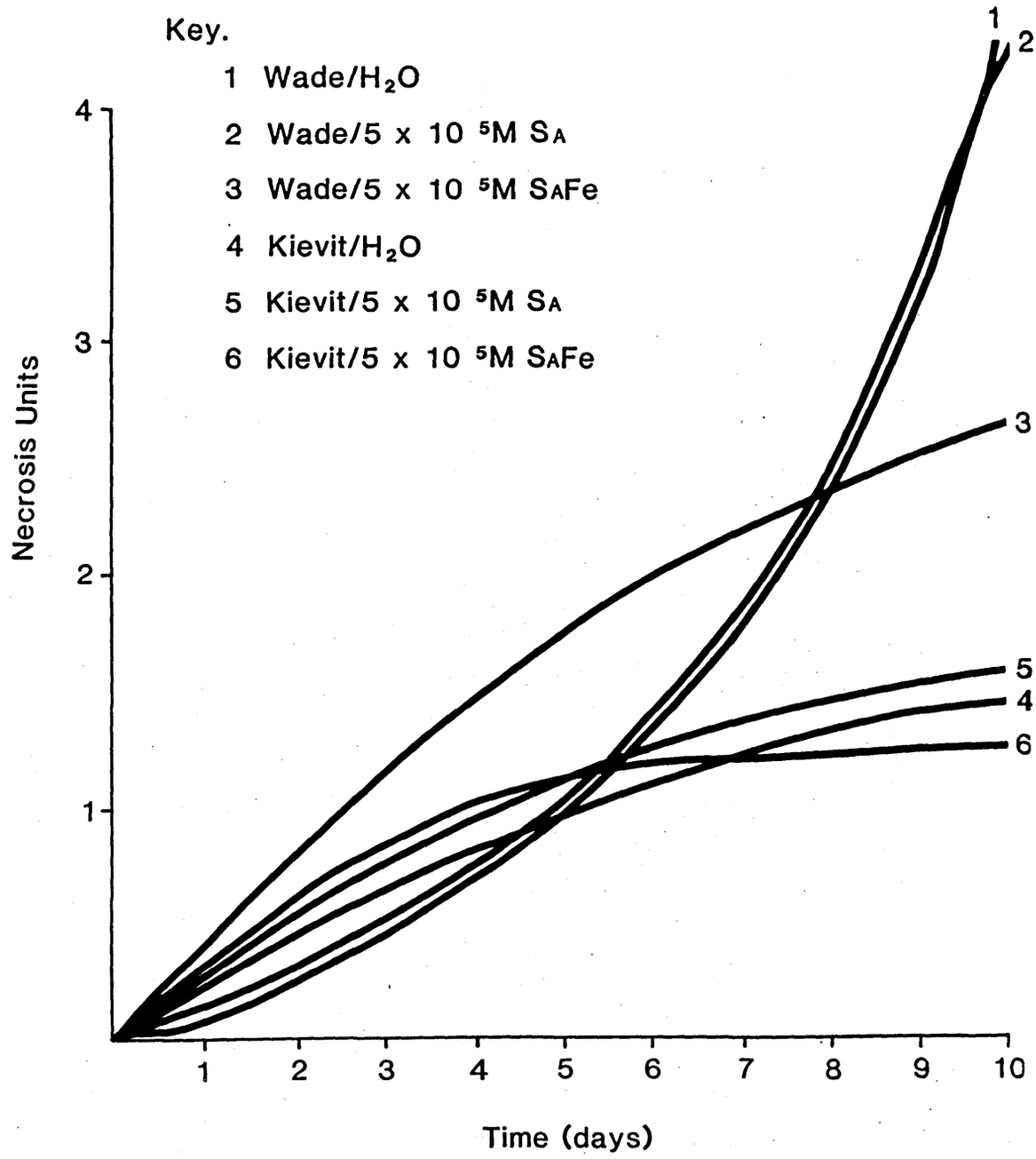


TABLE 4.4

The effect of SA and SAFe on the development of C. lindemuthianum race β lesions on P. vulgaris cultivars

Treatment	Mean necrosis rating[a]		
	4 days	6 days	10 days
WADE			
H_2O	0.49 ± 0.07	0.76 ± 0.14	3.89 ± 0.11
$5x10^{-5}$M SA	0.65 ± 0.09	1.05 ± 0.22	3.83 ± 0.09
$5x10^{-5}$M SAFe	1.67 ± 0.74	1.74 ± 0.42	2.52 ± 0.51
KIEVIT			
H_2O	0.38 ± 0.28	0.81 ± 0.21	1.34 ± 0.20
$5x10^{-5}$M SA	0.50 ± 0.32	1.08 ± 0.31	1.57 ± 0.24
$5x10^{-5}$M SAFe	0.78 ± 0.35	1.20 ± 0.30	1.08 ± 0.45
L.S.D.(P = 0.05)	0.68	0.61	0.61

[a] see figure 4.1

TABLE 4.5

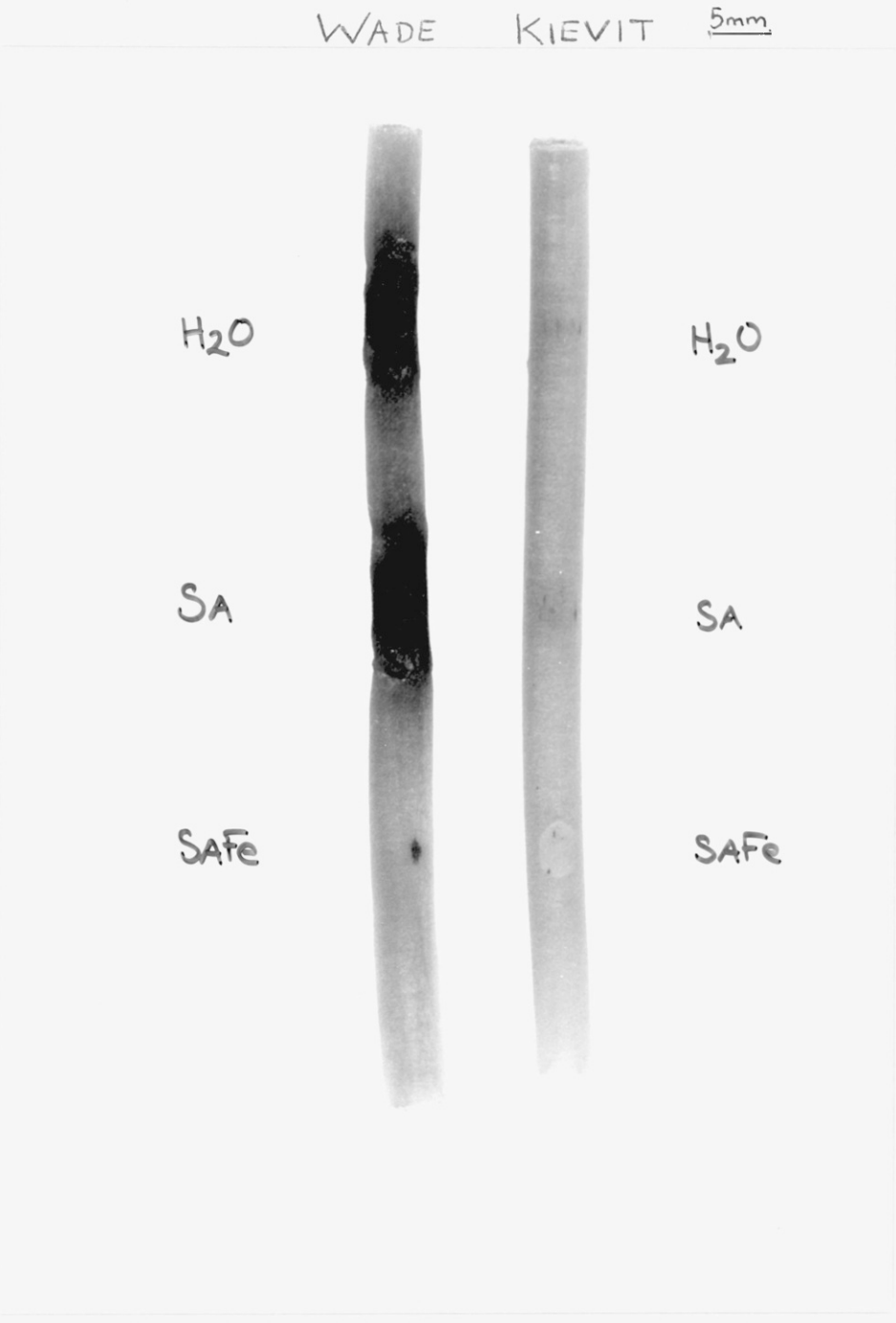
The effect of SA and SAFe droplets on P. vulgaris cultivars after 10 days

Treatment	Mean necrosis rating[a]
WADE	
H_2O	0.13 ± 0.06
$5x10^{-5}$M SA	0.24 ± 0.01
$5x10^{-5}$M SAFe	0.67 ± 0.26
KIEVIT	
H_2O	0.12 ± 0.05
$5x10^{-5}$M SA	0.23 ± 0.08
$5x10^{-5}$M SAFe	0.36 ± 0.21
L.S.D.(P = 0.05)	0.22

[a] see figure 4.1

FIGURE 4.3

The effect of SA and SAFe in the inoculum on lesion formation by C. lindemuthianum race β on hypocotyls of P. vulgaris cultivars



conidia in water, although the initial development of such limited lesions was significantly stimulated. The presence of SAFe in the inoculum had no significant effect on the infection of cv. Kievit, although initially lesion development was marginally greater.

After 10 days a droplet of water, or SA, resulted in a low level of necrosis on both cultivars (see table 4.5). On cv. Kievit the level of necrosis resulting from a droplet of SAFe was not significantly different from that due to water, or SA, but cv. Wade produced significantly more necrosis in response to SAFe.

4.3.3 Elicitors and phytoalexin accumulation. The concentration of the phytoalexin phaseollin that accumulated in the spreading lesions on cv. Wade, after 10 days, was consistently higher than that produced in the limited lesions on cv. Kievit (see table 4.6). The concentration of phaseollin in the necrotic flecks on cv. Kievit may have been higher than the value given, since the tissue extracted included healthy areas. In general, the spreading lesions extracted from cv. Wade were entirely necrotic. The presence of 5×10^{-5}M SA, or SAFe, in the inoculum, had no significant effect on the concentration of phaseollin that accumulated in the lesions after 10 days, on either cultivar.

On both cultivars SA, or SAFe, had no significant effect on the phytoalexin accumulation per unit necrosis (see table 4.7) even though on cv. Kievit the average amount of phaseollin per unit necrosis was much higher when conidia were inoculated in the presence of SAFe.

TABLE 4.6

The effect of SA, and SAFe, on phaseollin accummulation in C. lindemuthianum race β lesions on P. vulgaris cultivars after 10 days

Treatment	Mean phaseollin concentration ($\mu g\ g\ FW^{-1}$)
WADE	
H_2O	427.0 ± 263.9
$5x10^{-5}M$ SA	621.7 ± 469.7
$5x10^{-5}M$ SAFe	318.3 ± 163.5
KIEVIT	
H_2O	140.3 ± 64.2
$5x10^{-5}M$ SA	118.3 ± 63.5
$5x10^{-5}M$ SAFe	141.7 ± 77.9
L.S.D.(P = 0.05)	367.6

TABLE 4.7

The effect of SA, and SAFe, on phaseollin accummulation per unit necrosis[a] in C. lindemuthianum race β lesions on P. vulgaris cultivars after 10 days

Treatment	Mean phaseollin concentration per unit of necrosis[a] ($\mu g\ g\ FW^{-1}\ N^{-1}$)
WADE	
H_2O	116.8 ± 76.1
$5x10^{-5}M$ SA	163.4 ± 129.3
$5x10^{-5}M$ SAFe	149.5 ± 66.6
KIEVIT	
H_2O	127.5 ± 71.1
$5x10^{-5}M$ SA	100.8 ± 69.9
$5x10^{-5}M$ SAFe	251.6 ± 211.6

[a]. see figure 4.1

L.S.D.(P = 0.05) 239.3

In the limited lesions produced in response to heat-killed fungus, cv. Kievit accumulated significantly more phaseollin than cv. Wade (table 4.8). The reduction of phytoalexin accumulation due to the presence of SA, and the increase due to SAFe, was not significant on either cultivar.

4.4 Discussion

The consistent stimulation of germination by 5×10^{-5}M SA of *C. lindemuthianum* race β conidia on glass slides was similar to that described for *C. musae*, and discussed in chapter 2. SAFe, and free ferric iron, had no significant effect *in vitro*, unlike that previously described with *C. musae*, but similar to the effect observed with *B. cinerea* (chapter 3). The insensitivity to SAFe and Fe^{3+} *in vitro* may have been due to either less uptake by the conidia, or to a reduced effect on the germination processes. The SA stimulated germination *in vitro* to about 5 times the value seen in water. This was larger than was observed with other fungi and may have been due to the poor germination of *C. lindemuthianum* race β in water on glass slides. With so few germinated conidia in water, SAFe, and Fe^{3+}, appressoria were formed on less than 1% of the total, and while SA markedly stimulated both dark and light appressoria, their production in SA was still relatively low.

Germination and appressorium formation by *C. lindemuthianum* race β *in vivo* was much higher than that seen *in vitro*. A variety of factors (discussed in chapter 7) present in the phytoplane environment, but not

TABLE 4.8

The effect of SA, and SAFe, on the accummulation of phaseollin in P. vulgaris cultivars hypocotyls in response to heat-killed C. lindemuthianum race β.

Treatment	Mean phaseollin concentration ($\mu g\ g\ FW^{-1}$)
WADE	
Elicitor/H_2O	41.2 ± 14.1
Elicitor/$5x10^{-5}$M SA	27.8 ± 5.3
Elicitor/$5x10^{-5}$M SAFe	69.3 ± 56.7
KIEVIT	
Elicitor/H_2O	165.7 ± 78.6
Elicitor/$5x10^{-5}$M SA	105.4 ± 42.9
Elicitor/$5x10^{-5}$M SAFe	219.4 ± 153.1
L.S.D.(P = 0.05)	121.2

on glass slides, could have influenced the conidial suspension inoculated onto the hypocotyls, resulting in higher germination and greater appressorium formation *in vivo*. It seems reasonable that a fungus should have evolved to germinate well and produce infection structures when landing on a plant surface which it may be able to infect, but not when landing on a barren surface, on which it would not be able to grow, and from which it may be redispersed.

The resistance of the resistant cultivar, Kievit, was not expressed at the germination and appressorium formation stage of the fungal infection since this was the same on both cultivars. SA significantly stimulated the formation of mature appressoria on both cultivars and SAFe significantly inhibited it. Free Fe^{3+} had no significant effect on conidia on cv. Kievit, but was slightly inhibitory on cv. Wade. The germination of the fungus in the presence of Fe^{3+} was also unaffected on cv. Kievit, but slightly inhibited on cv. Wade. Cultivar Wade is susceptible, cv. Kievit is resistant, but the relevance of the specific inhibition by Fe^{3+}, if it is not an anomalous result, is not easily understood.

The reaction of cv. Kievit to infection by *C. lindemuthianum* race β was unaffected by the presence of SA or SAFe in the inoculum. Lesion production on the susceptible cultivar, Wade, was unaffected by SA, but the presence of SAFe significantly inhibited it. The differential susceptibility expressed by *P. vulgaris*

cultivars is probably expressed when cellular contact between host and pathogen takes place (Bailey, 1974). The involvement of the phytoalexin phaseollin in P. vulgaris infections has been reported (Bailey & Deverall, 1971) but Bailey (1974) demonstrated that all races of the fungus are capable of metabolising the phytoalexin and there is no differential toxicity. Any effect of SA on the accumulation of phaseollin in reponse to C. lindemuthianum may have been masked by the large variability of the phytoalexin content of lesions seen in this study. The accumulation of phaseollin in the limited lesions formed in response to heat-killed fungus was consistently reduced by SA, in a similar way to that described for V. faba (chapter 3) but due to the large variation this reduction was not significant.

The variation in the amount of phaseollin that accumulated in response to each treatment in replicated experiments is not readily explained, but may have been due to small differences in plant vigour, or age, contamination, or small variation in spore age. Slight differences in the efficiency of the replicated extraction procedure may have also been involved.

It is interesting to note that in response to the heat-killed fungus, the resistant cultivar Kievit accumulated phaseollin to a level approximately 4 times higher than that elicited in cv. Wade. Perhaps this situation is more akin to that in the initial stages of infection by a live fungus; the resistant cultivar being able to recognise and respond to the elicitor from

the fungus more readily and produce a higher localised concentration of phytoalexins. Anderson (1978) postulated the existence of a factor that prevents elicitor recognition in susceptible reactions. Her purified elicitor did not contain such a fraction, but the crude mixture used in this study may have done so. It is not known if siderophores affect the initial contact reaction between host and pathogen, the recognition process, or phytoalexin biosynthesis. Iron-requiring enzymes involved in these processes may be disturbed by the presence of a strong chelator. However, the presence of 5×10^{-5}M SA in the inoculum in this study did not alter the outcome of the host plants reaction; resistant plants were not made susceptible by the presence of SA in the infection court.

A droplet of SAFe elicited a slightly higher necrotic response on cv. Wade, after 10 days, than that due to SA, or water, alone. The concentration of phaseollin that accumulated in the small lesions due to these treatments was, however, too small to measure. There was no significant difference between the necrotic response to the treatments by cv. Kievit. The inhibition of the fungus on cv. Wade in the presence of SAFe, may have been due to phytoalexins elicited by the phytotoxicity of SAFe, as well as the direct inhibition of fungal growth by the SAFe.

The differential resistance of P. vulgaris cultivars cannot be accounted for by the differences in the

germination of the fungus on the plant surface or in the formation of appressoria. However, the production of infection pegs, and actual infection, were not compared in different treatments or in different cultivars, and may have differed correspondingly. Although the presence of SA in the inoculum stimulated the formation of almost 5 times as many mature appressoria than were formed in water alone, and the SA may have depressed the accumulation of phaseollin in the host, the SA was not able to make the resistant cultivar, Kievit, susceptible to the fungus. The inhibition of germination, and more importantly appressorium formation, by SAFe, on the susceptible cultivar, Wade, was able to make it resistant. Although phytoalexins elicited in response to SAFe may have been involved, the large reduction in infection potential probably accounted for the observed inhibition of the disease. SAFe may also have been involved in heightening the plants response, since in the presence of SAFe, heat-killed fungus elicited more phytoalexin than in water, taking into account the slight phytotoxicity observed, although this was not statistically significant.

Chapter 5

Colletotrichum acutatum infection of strawberry

5.1 Introduction

Blakeman and Parbery (1977) demonstrated that appressorium formation by C. acutatum, an anthracnose-causing pathogen of strawberry, was greatly enhanced by the presence of washed cells of Pseudomonas spp. in the inoculum drop. They attributed this phenomenon to nutrient competition, implicating in particular, competition for amino acids. Blakeman and Brodie (1977), Blakeman and Parbery (1977) and Brodie and Blakeman (1976) concluded that appressoria were formed in response to nutrient stress and drew an analogy with the formation of resting structures such as chlamydospores.

Although nutrient competition from phytoplane micro-organisms is almost certainly an important factor in the stimulation of fungal germination and appressorium formation, the observed effect of Pseudomonas bacteria on C. acutatum may have been due to the action of bacterial siderophores, in a similar way to that described in chapter 2. In this chapter the effect of SA on germination and appressorium formation, in vitro, and in vivo, of C. acutatum conidia is reported, and a comparison with nutrients at similar concentrations is made. Iron chelators have been implicated in the alteration of disease development, (see chapter 2) and so the effect of SA in the inoculum on disease development of C. acutatum on strawberries was studied. Since phytoalexin accumulation is involved in the development of many diseases

(Bailey & Mansfield, 1982) an attempt was made to extract and quantify phytoalexins produced in anthracnose lesions formed under different conditions, and to study any relationship between phytoalexin production and disease resistance.

5.2 Methods

5.2.1 Culture maintenance and preparation of spore suspensions. C. acutatum Simmonds, was isolated from black-rot symptoms on strawberry fruit, cultivar Brighton, which had been grown at East Malling Research Station from runners imported from the U.S.A. The fungus was cultured on modified Cook's No. 2 medium (Swinburne, 1976) at 18°C under continuous illumination by fluorescent light. Conidial suspensions were prepared in sterile distilled water, as described in chapter 2, and diluted to give a final concentration of spores of about 5×10^5 per ml (unless otherwise stated) for use in germination assays and plant inoculations.

Suspensions of P. fluorescens, taken with a sterile loop from the edge of a three day old colony, were prepared in sterile distilled water. They were washed by centrifugation and resuspension in sterile distilled water three times, and diluted to a final concentration of about 10^7 bacteria per ml to be mixed with an equal volume of conidial suspensions for fungal germination assays.

5.2.2 Glassware, water and siderophore solutions. Low iron glassware, low iron water, and SA solutions were prepared and used as described in chapter 2.

5.2.3 Low iron conidia. C. acutatum was grown in a liquid medium, depleted of iron by passage down a column of Chelex 100 resin (Na^+ form 200-400 mesh, BioRad Labs.), as previously described (Brown & Swinburne, 1981). Although the medium used in this study was not analysed, atomic absorption spectrophotometry of the medium used by Brown and Swinburne showed an iron content of less than 12 ng ml^{-1}. To deter bacterial contamination 0.02% chloramphenicol was added to the medium which was inoculated with 2 ml of a conidial suspension (10^6 ml^{-1}) of C. acutatum from a culture on Cook's No. 2 medium. Initially an attempt was made to reduce the contamination of the low-iron medium with iron present in, or on, conidia from iron replete medium; subsequent cultures were inoculated with conidia from the previous low-iron medium. However, this practice was abandoned since conidia repeatedly sub-cultured from liquid medium developed a yeast-like morphology, budded, and did not produce appressoria.

5.2.4 Spore germination assays. Germination assays on acid-washed glass slides were undertaken as described in chapter 2.

Stolons from healthy, mature plants of field grown cv. Cambridge Favourite were removed, cut into 10 cm lengths and suspended in a humid chamber. These were inoculated with 10 µl drops of conidial suspensions, one or more, per stolon piece. Germination and appressorium formation was assessed by examining thin longitudinal sections of stolons stained with cotton blue in lactophenol using a

light microscope. Electron micrographs were also taken of the germinating conidia using a Cambridge Stereoscan S100 scanning electronmicroscope and Hexland Cryo unit. Statistical analysis was done as described in chapter 2.

5.2.5 Stolon, leaf and fruit infection. Unless otherwise stated, stolons, leaves and fruit were taken from healthy, field-grown, mature cv. Cambridge Favourite plants.

Stolons were inoculated in the way described for *in vivo* germination assays and lesion development was recorded periodically during a 14 day incubation period using the infection grade key shown in figure 5.1.

Leaves were placed in a humid chamber with their petioles under water to reduce wilting. Abaxial, or adaxial, surfaces of leaves were inoculated with one, or more, 10 µl droplets of spore suspensions in treatment solutions or water. The development of black rot symptoms was followed for 14 days. Green, or red fruit, still attached to the truss, were inoculated with a 10 µl inoculum droplet. To ensure infection, the fruits were, initially, placed in a humid chamber to stop the inoculum drop evaporating. However, the high humidity encouraged contamination, especially by *Botrytis* sp., so a method was employed to keep the relative humidity high in the vicinity of the inoculum droplet but lower elsewhere. A circular plastic vial, 2 cm deep and 1 cm wide, with damp cotton wool stuck inside it, was placed over the droplet and sealed with vaseline. Lesion development was followed for 14 days. To ensure the normal development of green fruit and to reduce wilting, the pedicel of each

truss was placed in a solution of 4% sucrose and 0.1M KH_2PO_4. 0.5% 100 vol. H_2O_2 and 0.01% w/v streptomycin were added to stop microbial contamination of the solution.

FIGURE 5.1

Infection grade key, based on the amount of necrosis and reddening under the inoculum droplet[a] and its subsequent spread

Grade	Visible symptoms
0	None
1	Slight reddening
2	Marked reddening, some black necrotic flecks
3	Dark red/black, necrotic
4	As 3, symptoms spreading beyond area under the inoculum droplet.

a *C. acutatum* infection of strawberry stolons.

5.2.6 Strawberry fruit washings. Since chemicals leaching from banana fruit were shown to stimulate germination of *C. musae* conidia (Swinburne, 1981), the effect of any substances eluted from the surface of green and red fruit on *C. acutatum* conidia, *in vitro*, was studied.

40g of green or red fruit were brushed clean of loose particulate matter, and 50 ml distilled water washed over them. The water was not allowed to wash the calyx or pedicel. The eluate was then reduced to 10 ml at 50°C *in vacuo* and sterilised by passage through a 0.22 um millipore filter. The mass of the dissolved solids was calculated from droplets dried under a heat lamp, and samples were diluted to 0.5 mg ml^{-1} for germination assays. Mixed with the treatment solutions or conidial suspensions, a final concentration of 0.25 mg ml^{-1} was

used. (5 x 10^{-5}M SA is equivalent to 0.061 mg ml^{-1} from the assumptions in chapter 2). General chemical analysis was carried out on the eluates.

5.2.7 Extraction of phytoalexins from lesions on strawberry stolons. Inoculated stolon tissue was macerated in ether and the extract taken to dryness in vacuo at 40°C. This extract was then separated by thin-layer chromotography (t.l.c.) on Merck Kieselgel $60F_{254}$ 0.5 mm silica plates using a mixture of ethyl acetate:benzene:50% ethanol (4:1:1) as solvent. The chromatogram was then dried and over-sprayed with a spore suspension of Cladosporium herbarum in nutrient medium containing 3% malt extract and 0.5% mycological peptone (w/v). After 4 days in a humid chamber at 18°C, 5 fungistatic/fungitoxic bands became visible at R.f.s of 0.65, 0.61, 0.56, 0.52 and 0.43. No inhibitory zones were visible when extracts from healthy strawberry stolon tissue were chromatographed.

Better separation of the fungistatic bands was obtained using a solvent system containing Ethyl acetate: Benzene:50% ethanol (4:4:1) when the R.f. of the most inhibitory band to C. herbarum growth was 0.54. UV absorption spectra of the bands eluted into 100% methanol were not useful due to a lot of background absorbance. The only band with a distinct absorbance peak, at 278 nm, was that with R.f. 0.54. High performance liquid chromatography (h.p.l.c.) was then done on the fractions using the apparatus described in chapter 3. Only the R.f. 0.54 sample clearly contained a peak not

present in the control. (The control was a fraction of part of the t.l.c. plate without any bands, eluted in methanol.) The solvent, flow speed and pressure, and absorbance wavelength were altered to give optimum separation, and finally 50% methanol, 1 ml min^{-1} at 2400 psi, and 278 nm were used. The unique peak in the R.f. 0.54 sample was at 3.8 c.m. on the recorder paper moving at 5 mm min^{-1}. It was, however, difficult to discern the peak when crude ether extract was loaded, so further purification of the original extract, prior to t.l.c. or h.p.l.c., was attempted. The method previously used by Mussell and Staples (1971) using 5% NaOH was employed. Less contamination was observed and 5 fungistatic bands were well separated by t.l.c., with R.fs of 0.87, 0.74, 0.63, 0.41 and 0.23. The bands with R.fs of 0.74 and 0.41 were most inhibitory to C. herbarum, the latter corresponding to the R.f 0.54 sample previously extracted. H.p.l.c. showed that this band contained the unique peak, which was collected from repeated loadings of the band, and bulked. This sample contained fewer contaminants, seen by h.p.l.c. or t.l.c., and was very toxic to C. herbarum on t.l.c. plates. It did not, however, have a very clear UV absorbance spectrum.

It was not possible to obtain sufficient quantities of the fungitoxic compound, with the resources available, to identify any of its chemical properties. Consequently, it was not possible to measure the quantity of the compound within the lesions.

5.3 Results

5.3.1 Germination and appressorium formation in vitro.

The germination of C. acutatum conidia in distilled water, in vitro, ranged between 14% and 26%, averaging 19 ± 3%. In 5 x 10^{-5}M SA germination ranged between 37% and 93% with a mean of 57 ± 12%. The presence of live P. fluorescens cells stimulated germination but only half as much as by SA (see table 5.1). This differs from previous results in which bacteria marginally inhibited germination (Blakeman & Parbery, 1977). Appressoria production was stimulated in a manner similar to that described earlier, except that dark (mature, melanised) appressoria were not produced on glass in that study. This may have been due to the particularly clean glass surfaces used during this investigation, since it was noted that fewer appressoria were formed on new slides than on those that had been acid washed. The percentage of germinated conidia that produced appressoria was not significantly different between the SA and UV3 bacteria treatments, whilst the percentage of germinated conidia with dark appressoria was greater in the presence of the bacterial cells. The percentage of all conidia which produced appressoria was lower in the Pseudomonas treatment because of lower germination in the presence of the bacteria. Nevertheless, the percentage of conidia with dark appressoria did not differ significantly between the SA and P. fluorescens treatments due to a greater proportion of dark appressoria in the bacteria treatment.

Germination was equally inhibited by 5 x 10^{-5}M-

TABLE 5.1

The effect of SA and Pseudomonas fluorescens on germination and appressorium formation by C. acutatum in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	13.5 ± 4.8	54.7 ± 20.7	8.9 ± 4.3	34.9 ± 18.3	46.8 ± 21.1	6.9 ± 3.7
$5x10^{-5}$M SA	93.2 ± 2.6	97.2 ± 1.7	90.6 ± 3.1	55.9 ± 11.0	57.1 ± 10.4	52.6 ± 11.4
10^7 ml^{-1} Pseudomonas sp.	47.3 ± 10.9	98.7 ± 0.9	46.3 ± 10.2	94.5 ± 4.1	95.5 ± 3.5	42.1 ± 7.8
L.S.D.(P = 0.05)	12.2	15.5	11.7	22.3	23.3	15.3

$Fe_2(SO_4)_3$ and SAFe (see table 5.2). Few appressoria were formed in the presence of free Fe^{3+}, and even less in SAFe.

A comparison of SA with equal concentrations of glucose, glutamine, and alanine showed the amino acids significantly increased germination; glutamine to an extent comparable to SA (table 5.3). Percentage appressorium formation was only marginally greater in the presence of nutrients than in water and, because fewer appressoria produced were dark, the total number of conidia producing dark appressoria was not significantly different from that in water. There was no significant difference in germination or appressorium formation between 5×10^{-5}M glucose and water.

5.3.2 Low iron conidia. C. acutatum conidia taken from liquid cultures were washed by repeated centrifugation and resuspension in distilled, or low iron, water to remove all traces of nutrients, hyphal fragments and germinating conidia, for use in germination assays. Conidia from iron depleted culture germinated in water, in vitro, significantly better than conidia from iron replete culture, see table 5.4. There was some evidence, (not shown) that suggested conidia from iron replete liquid culture germinated better than conidia derived from equivalent solid medium. Appressorium formation by conidia from iron-depleted media was significantly greater than by conidia from iron replete media. The stimulation of germination and appressorium formation of low iron conidia was similar to that shown by iron replete conidia in the presence of SA, see table 5.1, indicating again the involvement of iron in stimulation by SA.

TABLE 5.2

The effect of SA, SAFe and Fe^{3+} on germination and appressorium formation by C. acutatum in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	25.9 ± 2.4	46.9 ± 6.5	9.5 ± 1.5	33.1 ± 5.9	38.8 ± 6.4	5.9 ± 1.0
$5x10^{-5}$M SA	50.0 ± 2.7	67.8 ± 3.6	35.1 ± 2.6	40.3 ± 4.0	54.1 ± 4.4	18.1 ± 1.7
$5x10^{-5}$M SAFe	11.2 ± 1.9	5.1 ± 2.7	0.8 ± 0.4	0.9 ± 0.6	4.8 ± 2.9	0.3 ± 0.2
$5x10^{-5}$M $Fe_2(SO_4)_3$	12.6 ± 2.3	33.9 ± 6.7	5.0 ± 1.1	20.2 ± 5.1	25.1 ± 6.0	3.1 ± 0.8
L.S.D.(P = 0.05)	4.7	9.8	2.8	7.8	9.9	1.9

TABLE 5.3

The effect of SA, glucose, glutamine, and alanine on germination and appressorium formation by C. acutatum in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	15.9 ± 1.9	90.2 ± 4.1	13.9 ± 1.6	86.7 ± 4.4	94.4 ± 3.2	12.8 ± 1.4
$5x10^{-5}$M SA	37.1 ± 2.9	74.6 ± 6.3	26.2 ± 6.9	64.2 ± 6.9	84.4 ± 4.5	24.5 ± 3.7
$5x10^{-5}$M glucose	11.8 ± 1.3	81.8 ± 8.5	10.6 ± 1.4	75.5 ± 8.9	42.7 ± 4.0	9.5 ± 1.3
$5x10^{-5}$M glutamine	36.9 ± 4.3	57.2 ± 8.1	17.9 ± 2.2	44.5 ± 8.8	68.9 ± 8.4	13.8 ± 2.3
$5x10^{-5}$M alanine	27.9 ± 2.6	60.1 ± 3.2	15.4 ± 1.8	44.9 ± 8.7	67.5 ± 8.6	11.8 ± 1.9
L.S.D. (P = 0.05)	5.2	12.1	5.6	15.1	11.5	4.2

TABLE 5.4

Effect of iron depletion of the growth medium on conidia of C. acutatum and the effect of SA, and SAFe on iron-depleted conidia in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
Iron replete conidia/H_2O	48.9 ± 5.7	23.8 ± 3.9	10.3 ± 1.2	16.1 ± 4.7	64.6 ± 15.6	6.8 ± 1.6
Iron depleted conidia/H_2O	75.3 ± 5.8	48.7 ± 5.7	34.8 ± 4.6	20.3 ± 5.5	35.3 ± 7.5	12.8 ± 3.3
Iron depleted conidia/ $5x10^{-5}$M SA	81.3 ± 3.2	48.0 ± 5.5	37.9 ± 3.3	22.8 ± 3.8	44.8 ± 4.9	17.3 ± 2.8
Iron depleted conidia/ $5x10^{-5}$M SAFe	25.9 ± 2.3	23.3 ± 5.9	6.1 ± 1.6	3.6 ± 2.1	9.4 ± 5.4	1.0 ± 0.6
L.S.D. (P = 0.05)	8.5	10.5	5.4	8.1	16.7	4.5

5×10^{-5}M SA consistently stimulated germination and appressorium formation of iron depleted conidia, but this was not statistically significant. Presumably a population of conidia, mostly low in iron, and without a need for an iron chelating agent to release the germination mechanism was stimulated to a smaller degree by SA than an iron replete population. SAFe inhibited germination and appressorium formation to a level less than that seen in conidia from iron replete medium. The inhibitory effect of 5×10^{-5}M SAFe was greater than the stimulatory effect of iron-depleted growth.

After two days growth in the iron deficient medium, the fungus had produced about 4×10^{6} conidia ml^{-1}. The iron replete culture had only produced 1×10^{4} ml^{-1}, but had formed larger mycelial balls in the medium. After 6 days both cultures had produced about 5×10^{6} conidia ml^{-1} and the cultures differed in colour. The iron replete culture was a grey-green colour typical of mycelium on solid medium, while the low-iron culture was a tan-brown colour. The iron deficient culture probably produced less melanin due to a lower oxidising capacity. A similar amount of fungal growth had occurred in the two media after 7 days; 0.44 g 100 ml^{-1} dry weight in low iron medium, and 0.41 g 100 ml^{-1} in replete medium. At this stage 8.8 $\pm$ 1.9% of conidia appeared to be germinating (may have been budding; see later) in the low-iron medium, and 2.9 $\pm$ 1.5% in replete medium, but these conidia were removed before germination assays were done. Conidia in the low iron medium were rounded, often

pear-shaped, or dumb-bell shaped, while those in replete medium, although being more rounded than those from solid medium were similar to the normal shape described for this species (Parbery & Blakeman, 1978). Conidia from the iron depleted medium were also larger; 20.9 ± 0.5 µm long, compared to 17.6 ± 0.5 µm in replete medium.

After a week, C. acutatum cultures in the low iron liquid medium, and, to a lesser extent, the iron replete medium, became browner in colour, and an attempt was made to extract germination stimulants, possibly iron chelating agents, from the culture filtrate. The liquid was reduced in volume, and extracted with chloroform/phenol-ether/water as described in chapter 2. The yellow, fluorescent, mixture was then purified with SM2 bio-beads and separated by P2 bio-gel as previously described for the preparation of SA. Four peaks were obtained from the P2 column, the first being the largest, the other three being broader. The molecular weight of the first substance was about 900, although this may have been a low estimate. Germination assays were undertaken using the four substances off P2, designated CaS_1, CaS_2, CaS_3 and CaS_4. CaS_1, CaS_3 and CaS_4 were significantly stimulatory to germination of C. acutatum in vitro, and all significantly stimulated appressorium formation. The most stimulatory was CaS_1, see table 5.5.

5.3.3. Germination and appressorium formation in vivo. Percentage germination was significantly higher on strawberry stolons than on glass slides. (See table 5.6 and figure 5.2.) SA had a marked stimulatory effect on

TABLE 5.5

Effect of CaS_1 on the germination and appressorium formation of C. acutatum in vitro

Treatment	Percentage Germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	55.4 ± 6.1	8.9 ± 3.2	4.6 ± 1.4	7.8 ± 3.3	31.7 ± 12.1	4.2 ± 1.8
0.1 gl^{-1} CaS_1	78.9 ± 3.3	28.9 ± 4.8	20.8 ± 3.5	18.5 ± 5.3	47.5 ± 11.4	13.6 ± 3.7
L.S.D (P = 0.05)	9.8	8.0	4.9	8.6	24.5	5.5

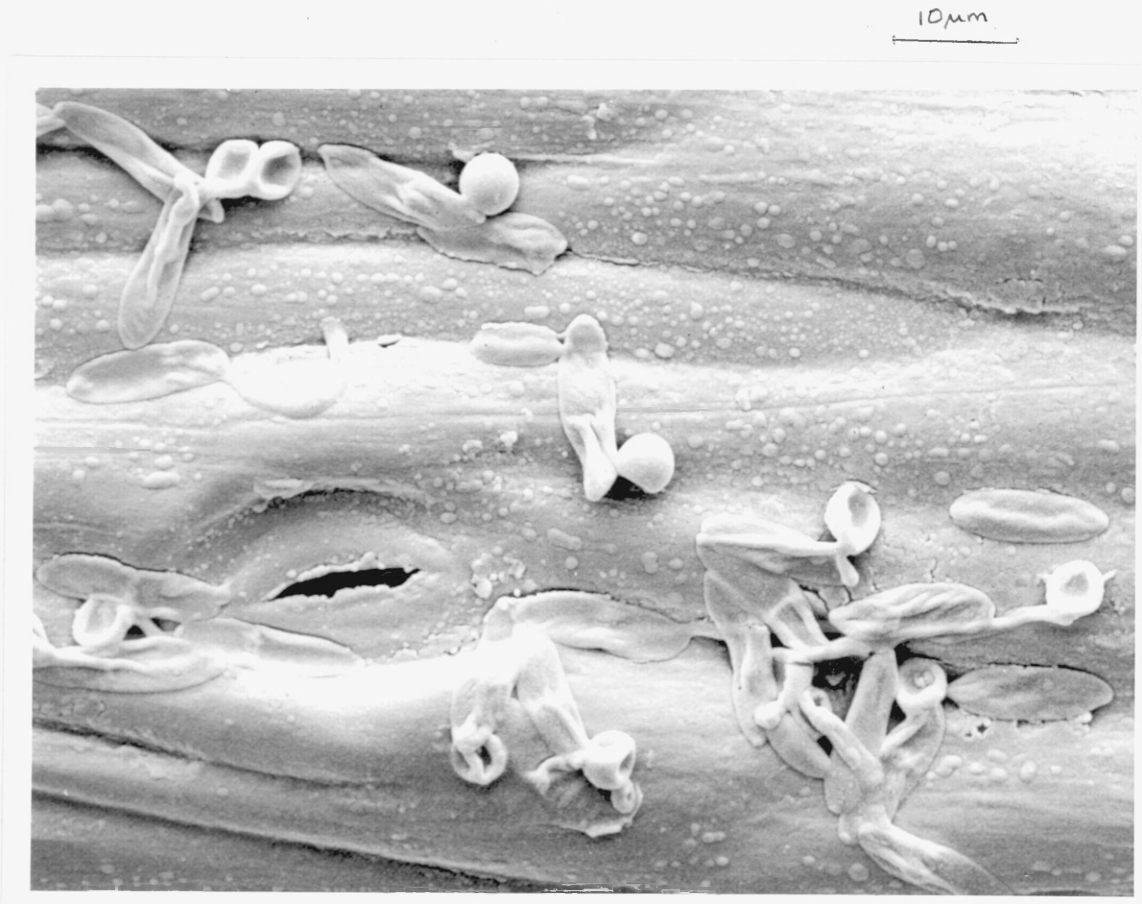
TABLE 5.6

The effect of SA, SAFe, Fe^{3+}, Pseudomonas fluorescens and the bacteria plus Fe^{3+} on germination and appressorium formation of C. acutatum on strawberry stolons

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	58.4 ± 4.7	66.4 ± 5.3	38.2 ± 4.1	49.7 ± 5.5	74.7 ± 5.1	27.9 ± 3.6
$5x10^{-5}$M SA	89.3 ± 1.7	86.8 ± 2.1	77.6 ± 2.6	72.9 ± 3.6	84.2 ± 3.9	65.6 ± 4.1
$5x10^{-5}$M SAFe	73.4 ± 2.7	51.5 ± 4.7	36.9 ± 2.9	19.3 ± 4.1	37.9 ± 6.7	13.2 ± 2.8
$5x10^{-5}$M $Fe_2(SO_4)_3$	49.7 ± 6.4	42.9 ± 6.4	24.1 ± 4.3	16.1 ± 3.4	34.8 ± 7.8	9.5 ± 2.2
Pseudomonas sp. 10^7 cells ml^{-1}	63.9 ± 3.7	74.8 ± 5.2	47.2 ± 4.3	69.8 ± 4.8	95.4 ± 1.9	44.4 ± 4.2
Pseudomonas sp. 10^7 cells ml^{-1} in $5x10^{-5}$M $Fe_2(SO_4)_3$	69.3 ± 2.9	79.5 ± 4.2	55.1 ± 4.1	61.2 ± 6.3	77.9 ± 6.2	43.2 ± 5.2
L.S.D.(P = 0.05)	8.5	9.3	7.4	9.2	10.5	7.4

FIGURE 5.2

Electronmicrograph of C. acutatum conidia germinating on a strawberry stolon



germination but the chelated siderophore was not inhibitory. The stimulation of germination by cells of P. fluorescens was diminished compared with the stimulation in vitro, resulting from a higher control value rather than a lowered efficacy of the bacterial treatment. Free Fe^{3+} in the inoculum droplet had no significant effect on germination, either in the presence or absence of added bacteria. SA increased the percentage of conidia which formed appressoria, but SAFe was not inhibitory, although it had none of the stimulatory effects of the deferri form. Free Fe^{3+} reduced the number of appressoria formed, and P. fluorescens slightly increased it, but addition of iron to the bacteria had no effect on the stimulatory properties of the latter. SAFe and Fe^{3+} markedly reduced the percentage of dark appressoria produced by germinated conidia, while SA and UV3 bacteria stimulated their formation compared with the control. The proportion of appressoria that were dark was significantly lowered by the presence of SAFe or Fe^{3+}, and raised by the SA and bacteria treatments, but there was no significant difference between the water control and the bacteria with iron. The total number of conidia which produced dark appressoria in vivo was stimulated by the deferri siderophore and inhibited by the ferri form and by free Fe^{3+}. P. fluorescens in water, and in $Fe_2(SO_4)_3$ solution increased the number of dark appressoria, but significantly less than did SA.

5.3.4 Germination and appressorium formation in fruit eluates. The substances washed off green strawberry

fruit stimulated germination to a similar degree as 5×10^{-5}M SA, whilst the eluate from red fruit was less stimulatory (see table 5.7). SAFe was inhibitory as previously described. Total appressorium production and the formation of dark appressoria were not significantly affected by the presence of washings from either green or red fruit, although there was more appressoria formed in washings from red fruit. SA significantly stimulated, and SAFe significantly inhibited, dark appressorium formation as described earlier.

5.3.5 Fruit eluate analysis. The washings from green and red fruit reacted in a similar manner to the tests undertaken. A test for the presence of amino acids using ninhydrin reagent was negative. A weak positive reaction in the P-amino benzoic acid test, but negative with $KMnO_4$ indicated the washings contained small amounts of reducing sugars. The conductivity was too low to measure (< 50 μ mho) so the eluates did not contain appreciable amounts of salts in solution. An analysis of the cations and anions present in the concentrated eluates is shown in table 5.8.

5.3.6 Lesion production on stolons. The development of C. acutatum infection of strawberry stolons is shown in table 5.9 and figure 5.3. After 3 days necrotic flecks on the epidermis were clearly visible beneath droplets containing conidia in water or 5×10^{-5}M SA, and just visible under all the sites containing SAFe. In the presence of 5×10^{-5}M SAFe, few sites developed clear flecking and none became red or black all over, or produced

TABLE 5.7

The effect of SA and SAFe, and washings from green and red strawberry fruit on the germination and appressorium formation of C. acutatum in vitro

Treatment	Percentage germination	Percentage of germinated conidia with appressoria	Percentage of all conidia with appressoria	Percentage of germinated conidia with dark appressoria	Percentage of appressoria that are dark	Percentage of all conidia with dark appressoria
H_2O	20.8 ± 4.1	32.7 ± 11.1	8.1 ± 2.7	22.1 ± 11.1	38.1 ± 15.6	5.8 ± 2.5
$5x10^{-5}$M SA	48.1 ± 6.3	53.9 ± 6.3	27.2 ± 5.2	42.9 ± 6.5	77.1 ± 7.9	21.9 ± 4.7
$5x10^{-5}$M SAFe	2.4 ± 1.2	16.7 ± 11.8	1.5 ± 1.2	16.7 ± 11.8	22.2 ± 14.7	1.5 ± 1.2
Washings from green fruit	42.7 ± 9.3	26.0 ± 13.1	9.4 ± 4.4	9.6 ± 5.7	17.0 ± 9.8	3.7 ± 2.3
Washings from red fruit	34.4 ± 9.5	32.4 ± 11.1	13.2 ± 4.4	18.9 ± 7.9	31.7 ± 12.4	7.6 ± 3.2
L.S.D. (P = 0.05)	12.2	21.4	7.2	17.2	24.2	5.6

TABLE 5.8

Minerals in eluates of strawberry fruit

Cations (quantitative)

	Element ($\mu g\ ml^{-1}$)				
	K*	Ca	Mg	Na	Fe
Red fruit	4.2	3.1	0.3	7.2	0.2
Green fruit	1.9	2.2	0.4	1.3	0.2

Anions detected (qualitative)

Anion	Present in eluate	Test; production of:-
PO_4^{3-}	No	Ammonium phosphomolybdate
Cl^-	No	Silver chloride
SO_4^{2-}	No	Barium sulphate
NO_3^-	No	Nitrogen dioxide

* For method of analysis, see table 3.10

TABLE 5.9

The effect of SA and SAFe on the development of C. acutatum lesions on strawberry stolons

Treatment	Average necrosis at site of inoculation‡ Day 3	Day 9	Day 11	Percentage of sites with infection grade ≥ 3 at day 11
H_2O	1.81 ± 0.19	2.09 ± 0.29	2.25 ± 0.27	31.3%
5×10^{-5}M SA	1.94 ± 0.21	2.31 ± 0.27	2.44 ± 0.20	50.0%
5×10^{-5}M SAFe	1.00 ± 0.00	1.06 ± 0.06	1.31 ± 0.12	0.0%
L.S.D.(P = 0.05)	0.31	0.48	0.45	

‡ See Section 5.2.5 for explanation of infection grades

FIGURE 5.3

The effect of SA and SAFe on C. acutatum lesions on strawberry stolons after 15 days

spreading lesions. Lesions that developed from conidia inoculated in SA solution were consistently more advanced than those in water, but there was no statistical difference. See figure 5.3. Although eventually most sites where conidia were inoculated in SA, or water, produced spreading lesions, after 11 days 50% of inoculated sites in the presence of SA had developed to, at least, stage 3 (red all over), while only 31.3% of inoculations made in water had reached that stage.

The effect of SA, and of spore concentration on infection of Hapil and Cambridge Favourite stolons is shown in table 5.10. Black spreading lesions were formed after 15 days when 8 µl droplets containing 5×10^6 conidia per ml were placed on Hapil stolons. No lesions were formed when the concentration of conidia was less than 8×10^4 ml^{-1} in water, but in SA 10% of inoculated sites produced reddened lesions at this concentration. Cambridge Favourite was apparently more susceptible; lesions were not formed in water when the concentration was 9.8×10^3 ml^{-1} (c. 78 conidia per inoculum droplet). In SA lesions were formed at this concentration, but were not formed at 1.23×10^3 ml^{-1} (9 conidia per droplet).

5.3.7 Lesion production on fruit. The germination of C. acutatum conidia on the surface of a red strawberry fruit is shown in figure 5.4. A lesion produced after four days showing the huge number of conidia produced by acervuli on the fruit surface is shown in figures 5.5 and 5.6.

The average surface area of C. acutatum lesions on

TABLE 5.10

The effect of SA on lesion production by different concentrations of C. acutatum conidia on strawberry stolons

Conidial concentration (spores ml^{-1}) (8 µl droplets)	Percentage of different infection grades attained after 15 days on different strawberry cultivars			
	cultivar Hapil		cultivar Cambridge Favourite	
	H_2O	$5x10^{-5}$M SA	H_2O	$5x10^{-5}$M SA
$5x10^{6}$	100%, 4	100%, 4	20%, 4; 80%, 3	100%, 4
6.25×10^{5}	100%, 3	25%, 4; 75%, 3	100%, 3	50%, 4; 50%, 3
7.81×10^{4}	100%, 0	10%, 2; 90%, 0	60%, 3; 40%, 0	100%, 3
9.8×10^{3}		100%, 0	100%, 0	10%, 3; 90%, 0
1.23×10^{3}				100%, 0

FIGURE 5.4

Electronmicrograph of C. acutatum conidia germinating on a red strawberry fruit

10µm

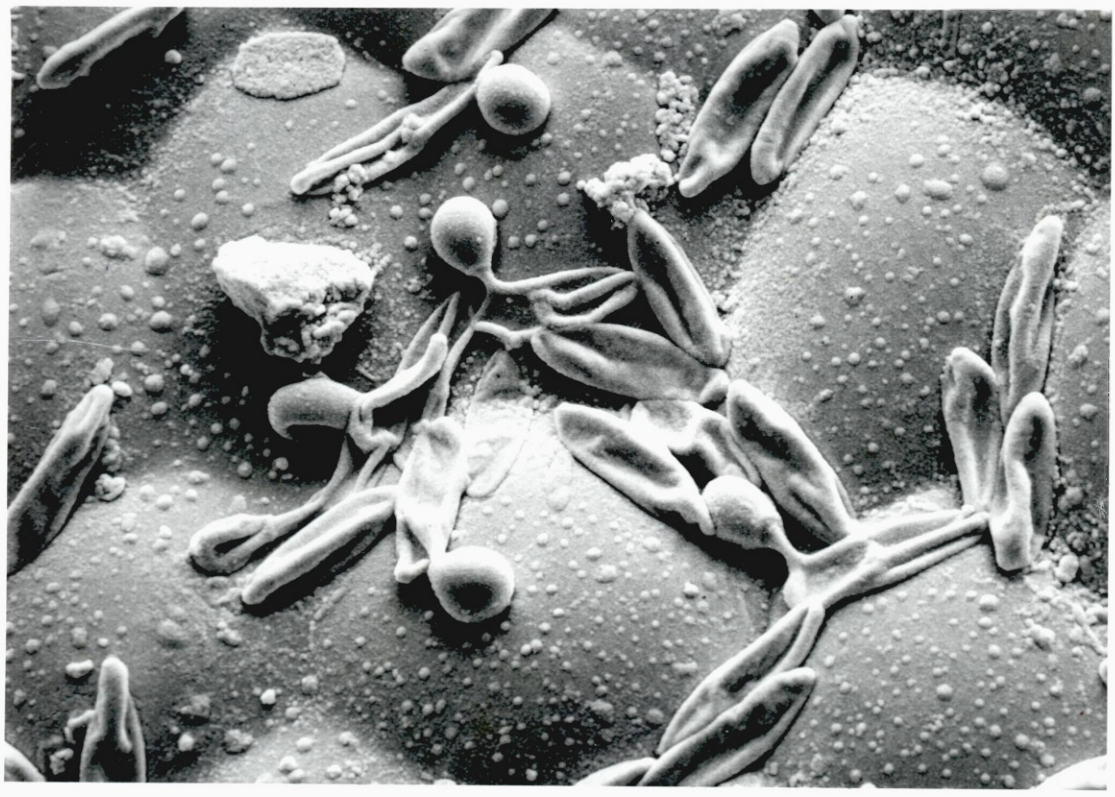


FIGURE 5.5

Electronmicrograph of C. acutatum lesion on red strawberry fruit in the region of an achene showing acervuli

200µm

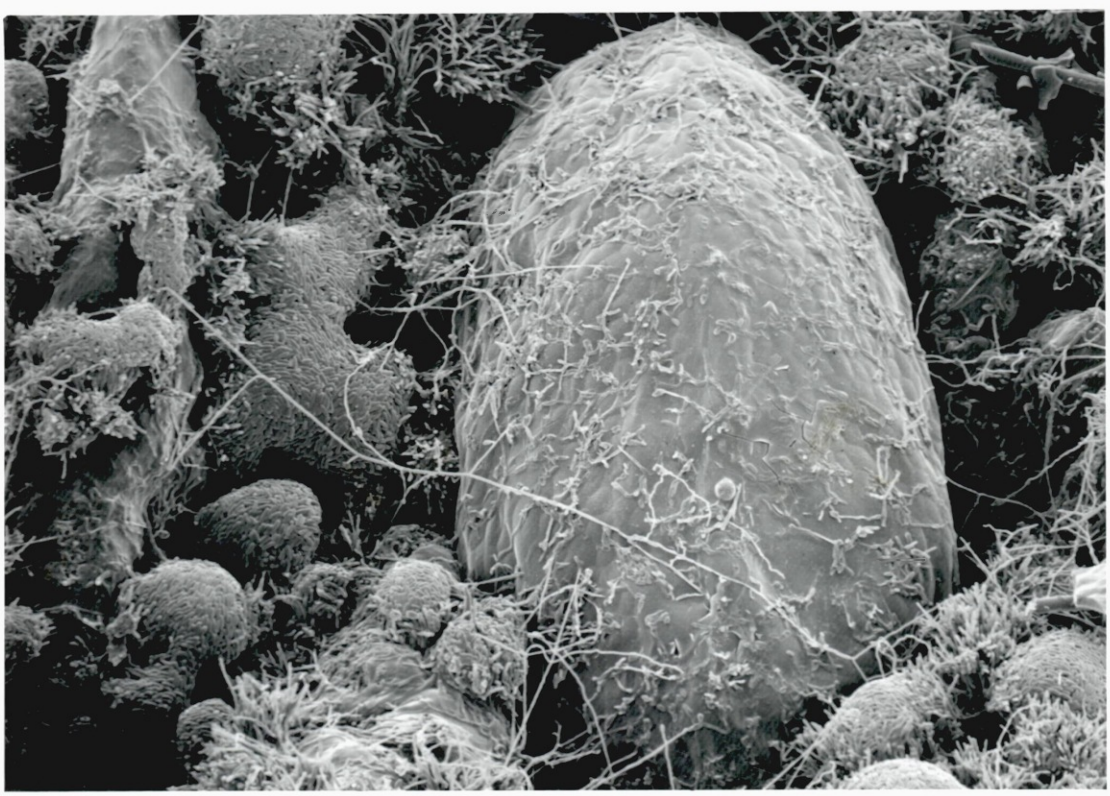


FIGURE 5.6

Electronmicrograph of a four-day-old C. acutatum lesion on a red strawberry fruit

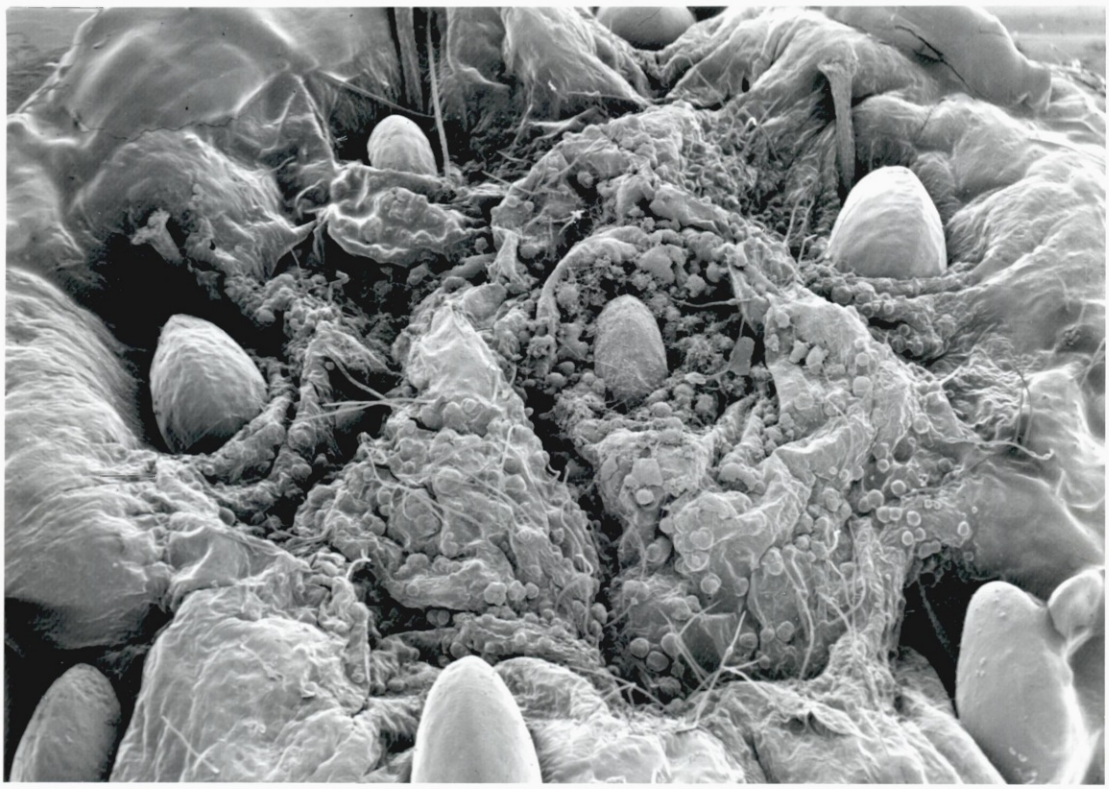
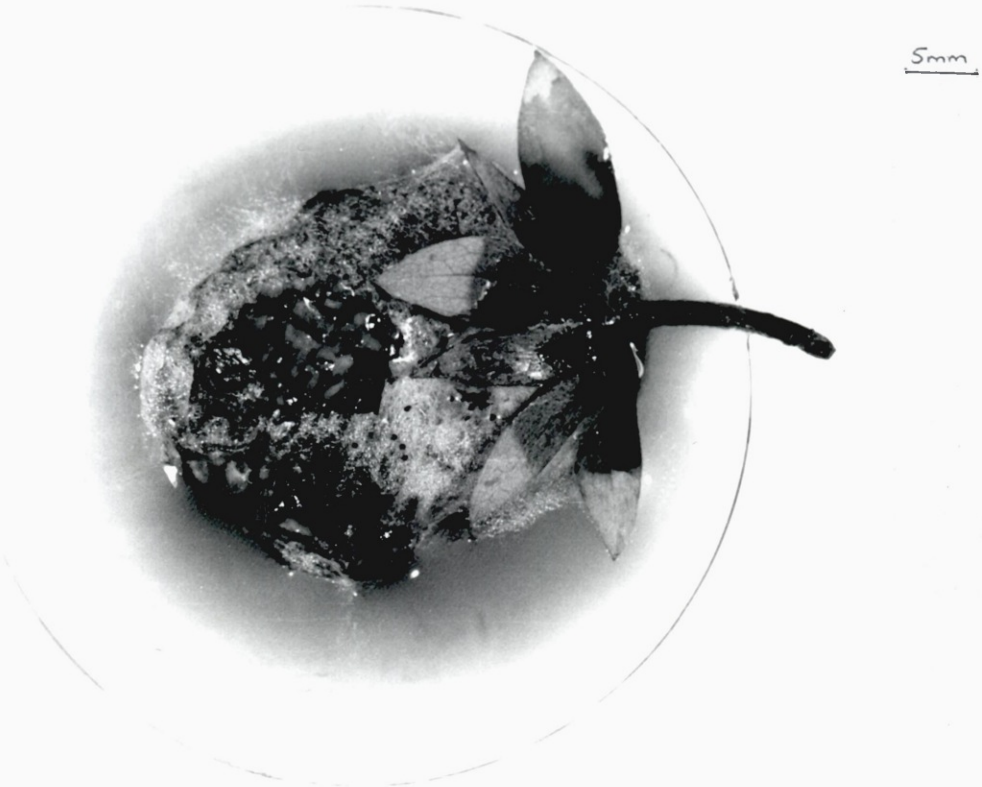


FIGURE 5.7

Red strawberry fruit completely rotted by C. acutatum



red strawberry fruit (mixed cultivars) after 10 days is shown in table 5.11. SA (5×10^{-5}M) in the inoculum droplet significantly stimulated the size of the lesion produced after 10 days. Eventually, however, all inoculated fruit became completely rotten, as shown in figure 5.7.

5×10^{-5}M SA in the inoculum significantly stimulated the average diameter and depth of lesions formed after 9 days in red Cambridge Favourite fruit, compared to that produced by a 10 µl droplet of 1×10^{6} conidia per ml in water (see table 5.12). 5×10^{-5}M SAFe significantly inhibited both diameter and depth. A 5×10^{6} ml^{-1} suspension in water produced a lesion with only slightly smaller diameter and no significant difference in depth, than 1×10^{6} ml^{-1} conidia in SA solution. Eventually all the red strawberries inoculated rotted.

After 9 days only conidia inoculated in the presence of SA produced lesions on green strawberry fruit. (Data not shown.) Green fruit cut in half axially were inoculated on the cut surface with conidia in either water, SA, or SAFe. These were more susceptible to infection than whole fruit, and spreading lesions were produced in all treatments within 4 days. Some evidence of stimulation by SA, and inhibition by SAFe, was observed but no statistically significant results were recorded.

5.3.8 Lesion production on leaves. Strawberry leaves were more difficult to infect than fruit and stolons. The waxy upper surface and hairy lower surface of leaves

TABLE 5.11

The effect of SA on the average surface area of C. acutatum lesions on red strawberry fruit after 10 days

Treatment	Surface area
H_2O	59.1 ± 9.3 mm^2
$5x10^{-5}$M SA	75.4 ± 1.6 mm^2
L.S.D.(P = 0.05)	13.3

TABLE 5.12

The effect of SA, SAFe, and conidial concentration on C. acutatum lesions formed on red strawberry fruit after 9 days

Treatment	Mean lesion size (mm ± SE)	
	Diameter	Depth
H_2O ($1x10^6$ conidia ml^{-1})	6.9 ± 0.3	3.9 ± 0.1
H_2O ($5x10^6$ conidia ml^{-1})	9.0 ± 0.4	5.8 ± 0.7
$5x10^{-5}$M SA ($1x10^6$ conidia ml^{-1})	10.9 ± 0.3	6.0 ± 0.2
$5x10^{-5}$M SAFe ($1x10^6$ conidia ml^{-1})	4.7 ± 1.7	1.5 ± 0.5
L.S.D.(P = 0.05)	1.4	0.8

resulted in inoculum droplets becoming easily dislodged. The lower surface of leaves appeared to be more susceptible to infection, than the upper, once the inoculum droplet had adhered. Initial results indicated that on the upper surface of Hapil leaves, spreading lesions developed from 20% of inoculation sites in water, or in the presence of 5×10^{-5}M-$Fe_2(SO_4)_3$, and 50% of sites containing 5×10^{-5}M SA. SAFe completely inhibited the formation of spreading lesions on Hapil leaves (see figure 5.8).

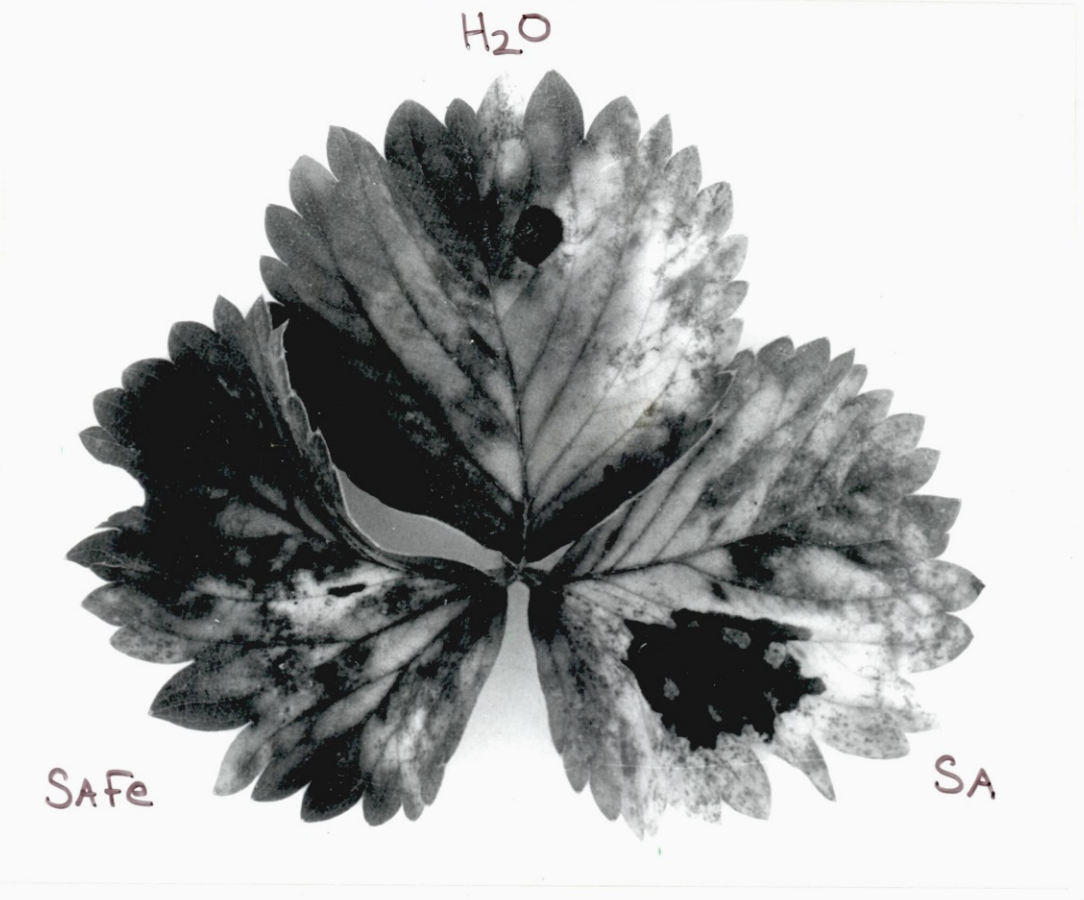
5.4 Discussion

SA (5×10^{-5}M) consistently stimulated the germination and appressorium formation of C. acutatum in water droplets on glass slides, although the extent of this stimulation was variable. The variability was not readily explained, but may have been due to small differences in spore age and absolute slide cleanliness between the experiments. Certain variable factors may have favoured the action of the SA, although there was no evidence to suggest what these may have been, as all experiments were done under the same specified conditions.

Pseudomonas fluorescens, the bacterium that produced SA, was not as stimulatory to the germination of C. acutatum as pure SA. This may have been because the washed bacterial cells did not produce a sufficiently high enough concentration of SA, or quickly enough, in the droplet to stimulate the conidia to a similar degree as 5×10^{-5}M SA. Also, the bacteria may have released antibiotics, or other fungistatic substances, into the droplet.

FIGURE 5.8

The effect of SA and SAFe on the development of C. acutatum on a strawberry leaf after 14 days



The fully chelated siderophore, SAFe, and free ferric iron was inhibitory to germination and appressorium formation of C. acutatum in vitro, in a similar way to that described for C. musae in chapter 2.

SA stimulated appressorium formation of C. acutatum in vitro, while at similar concentrations, the nutrients tested had no significant effect. SA stimulated germination to a significantly greater degree than alanine, while glucose, at the concentration tested, had no significant effect. Blakeman and Parbery (1977) found nutrients to inhibit appressorium formation, using concentrations higher than those tested here; they found germination in water was 100% and could not be stimulated. To simulate competition from bacteria they induced nutrient deprivation by leaching conidia between polycarbonate and cellulose acetate membranes. Such conidia germinated well and produced mostly dark appressoria. However, leaching may have also reduced the conidial iron content (or moved it from its site of action) which would have given rise to the observed effects in a similar way to that described here, and similar also to conidia grown under low iron conditions (Graham & Harper, 1983). However, nutrients must be involved in part, since leaching with a nutrient solution, which, unless contaminated by iron, would also lower the iron content, did not stimulate appressorium formation. Excess exogenous nutrients induce saprophytic growth with conidia producing long ramifying germ tubes and few appressoria (Van Burgh, 1950; Skoropad, 1967; see also chapter 2). Although some microorganisms may be

able to utilise siderophores as a nutrient source, the stimulation of C. acutatum by SA cannot be explained on a nutritional basis. The stimulatory effect of SA on germination and appressorium formation in vitro was much greater than that exhibited by equivalent concentrations of the nutrients tested.

Harper, et al., (1980) found conidia of C. musae germinated freely without added chelators if they were derived from an iron depleted culture. Germination of conidia obtained from media containing a range of iron concentrations showed an inverse correlation with iron content, where germination in the presence of an iron chelating agent was virtually constant irrespective of iron content. Results obtained using C. acutatum are in accord with the work of Harper, et al., and their conclusion that conidial iron is involved in inhibition of germination, and that this inhibition can be relieved non-specifically by a variety of iron chelating agents.

C. acutatum appeared to grow normally in liquid medium depleted of iron, but formed large rounded conidia. This may have been due to iron depletion in the cell walls of the conidia, making them less rigid, which may indicate the involvement of iron inhibitory to germination in cell wall strengthening. Perhaps germ tubes can emerge more readily after iron is removed from cell walls, generally, or from specific emergence sites, (conidia were often pear-shaped indicating a weakening at one end) as proposed by Graham and Harper (1983). Iron within the cell walls could be complexed with chitin

(Muzzarelli, 1973) and it is not known if exogenous iron chelating agents remove iron bound in this way. However, after a time in liquid culture the conidia began to grow bigger, and multicellular budding structures were formed in a yeast-like growth phase. Young conidia of this type may have been confused with 'normal' pear-shaped conidia.

No attempt was made to analyse CaS_1, the yellow fluorescent material from low iron cultures of C. acutatum. At a concentration of 0.1 gl^{-1} (1.1×10^{-4}M if the molecular weight is 900) it was stimulatory to both germination and appressorium formation of C. acutatum in vitro, in a similar way to SA. Siderophore production by fungi is well documented (Neilands, 1981b) and it would be not surprising if CaS_1 was an iron-binding ligand released during iron stress. Further work is necessary to determine if this substance has a role in the low iron growth, or pathogenesis of the fungus in vivo.

The complex situation on the stolon surface altered the observed effect on germination and appressorium formation from that seen in vitro. The germination in water was higher, the SAFe and Fe^{3+} less inhibitory to germination, and the bacteria less stimulatory, although the SA was significantly stimulatory to both germination and appressorium formation.

The greater germination of C. acutatum in vivo than in vitro is not readily explained, without further study, but it does seem reasonable that the fungus would germinate better on a surface which it would be likely to

infect, than on an inanimate object. Many factors are present in the complex interaction on the stolon surface and absent from the assays undertaken on glass slides (see chapter 7).

Although the reaction to infection was variable (never 100% infection) strawberry cv. Cambridge Favourite stolons were generally susceptible to infection by C. acutatum. The presence of SA in the inoculum droplet consistently stimulated the infection process, as measured by the infection stage reached after a certain time, but this was not statistically significant. After 11 days more inoculation sites on the stolons had become blackened in the presence of SA than water alone. However, a stolon with even only one spreading black lesion would eventually have become completely rotten, so the stimulation by SA was only relative and temporal. The presence of SAFe in the inoculum significantly reduced lesion formation so no spreading lesions developed and no stolons were completely killed by an infection resulting from an inoculum containg 5×10^{-5}M SAFe. The necrotic flecking resulting from such inoculation (and in the early stages of infection after inoculation in water, or SA) was characteristic of a hypersensitive response. Phytoalexin accumulation, although detected, was not measured quantitatively and any relationship in the early stages of infection between phytoalexin production and disease resistance was not observed.

In vivo, the presence of SAFe did not completely inhibit appressorium formation by C. acutatum, but did

significantly reduce it. With both cv. Hapil and cv. Cambridge Favourite, at least 80 conidia per inoculum droplet were needed to produce signs of infection visible by eye. Inhibition of infection by SAFe may have been due to a reduction in the number of appressorial infection sites thus decreasing the infection potential. When large numbers of conidia were used, as in table 5.9, SA did not increase lesion formation. With low numbers of conidia (table 5.10), the presence of SA seemed to increase the infection potential, presumably by increasing the number of appressoria relative to those formed in water.

After 10 days the average surface area of lesions on cv. Cambridge Favourite fruit caused by C. acutatum in the presence of SA were significantly bigger than those inoculated in water alone. However, red fruit were so susceptible that eventually all fruit inoculated became completely rotten. Using a higher concentration of conidia in the water inoculum resulted in lesions being formed as quickly as a lower concentration of conidia in the presence of SA would have formed them. This also indicated that the observed acceleration of disease development in the presence of SA was probably mainly due to an increased infection potential. SAFe significantly inhibited lesion production, but all red fruit eventually became rotten.

It is interesting to note that all cv. Bogota and cv. Cambridge Favourite fruit inoculated while still firm and green did not become at all rotten unless SA was

The production of dark appressoria may be of most importance in field infections if the fruit become infected while still green. Lesions may only develop, when the fruit ripen, if iron chelating agents were present at the initial infection site.

present in the inoculum. After 9 days green fruit had mostly become ripe and red, but had developed no signs of infection, other than a few discreet necrotic flecks, if they were inoculated with conidia in water or SAFe. Relatively little is known about this disease, and further work may show that this situation is similar to the infection process of C. musae on bananas (see chapter 2) and other diseases involving latent infection. Since strawberry fruit are not normally stored unripe and it appears blackrot caused by C. acutatum does not possess a latent phase of appreciable duration on red fruit, the proportional formation of dark appressoria would be less important than in the development of banana anthracnose caused by C. musae. See opposite page

Infection of stolons and leaves may also be unrelated to dark appressoria in particular, since lesion production occurred almost immediately the tissue was inoculated. In the case of low inoculum density the stimulation by SA of all appressoria, light plus dark, thus raising the infection potential, would have been important. Dark appressoria may be of particular importance on runners, if such appressoria act as resting structures (Emmett & Parbery, 1975). Distribution of runners, even after cold storage, may be important in the dispersal of the disease world-wide. Dark appressoria may also allow the fungus to overwinter, or survive adverse conditions.

Although fruit leachates have been related to the infection processes of certain fungi (Swinburne, 1981) there was no evidence to suggest that the small amount of

material found in the green, and red, fruit eluate played any part in the infection of strawberry fruit by *C. acutatum*, since *in vitro* it did not significantly effect appressorium formation. It did, however, stimulate germination and the low concentration of sugars present may have been used as a nutrient source by the fungus. Stimulation of germination and germ tube growth, but not appressoria, are characteristic of nutrients (see chapter 2). The eluates contained an insufficient concentration of inorganic ions to affect germination or appressorium formation. (The concentration of iron in the eluate from either fruit would be insufficient to suppress siderophore production by saprophytic bacteria such as *Pseudomonas fluorescens*; the iron content of an equivalent volume of the eluate was 5 times lower than that in the medium used to grow *Pseudomonas* UV3 for SA production.)

Initial results indicated that SA stimulated, and SAFe inhibited *C. acutatum* black spot lesions on strawberry leaves in a similar way to that observed on stolons. Even with SA in the inoculum, a lesion rarely encompassed the whole leaf area, but if more than one lesion developed on a leaf, the whole surface area became blackened. Lesions appeared to spread quickly along the veins and back towards the petiole. When the petiole became necrotic, the leaf quickly wilted and died.

Thus the presence of iron chelating agents on the surface of strawberry plants in the field, may stimulate the accelerated development of black spot and anthracnose lesions caused by *C. acutatum*.

Chapter 6

Botrytis cinerea and Cylindrocarpon heteronema infection of stored apple fruit

6.1 Introduction

B. cinerea and *C. heteronema* cause post-harvest rots of stored apple fruit (Edney, 1983). Iron chelators, such as bacterial siderophores, have been implicated in the stimulation of some fungi causing latent infections of fruit. Quiescent infections of *C. musae* on banana, *G. cingulata* on peppers, and *Diaporthe perniciosa* on apple were stimulated by the presence of iron chelators (Swinburne, 1983; Swinburne & Brown, 1983) but the effect of an iron chelator on *B. cinerea* and *C. heteronema* rot of apple has not previously been investigated.

B. cinerea is a wound pathogen and can cause heavy losses of apples and pears, although it is probably more important on the latter. *C. heteronema* causes lenticel rotting and attacks late in the storage period. In the orchard both fungi may infect wounds on fruit to cause a dry eye-rot which develops into spreading soft brown rots in the store. The fully developed ripe fruit used in this study were resistant to both fungi, even when a droplet of spore suspension was placed over a lenticel. In the work reported here both organisms were wound-infecting pathogens, the subsequent lesion developing after storage.

The effect of the presence of SA in the inoculum droplet on disease development of *B. cinerea* on stored Golden Delicious fruit, and freshly picked Bramley's

Seedling was investigated. Lesion development of *C. heteronema* infection of stored Bramley's Seedling, inoculated after picking, was also studied.

6.2 Methods

6.2.1 Fruit. Golden Delicious apples grown at East Malling Research Station, previously stored at 0°C for 6 months, and freshly picked (unripe) Bramley's Seedling were cut in half axially and placed, cut face downwards, in hot embedding wax in a shallow glass dish. Fruit embedded in this way enabled inoculations to be done on both sides so fewer fruit could be used. The arrangement was also stable and easily transported. Whole fruit, placed on standard cardboard trays were used for long-term studies. The fruit were placed in sealed containers at 18°C in the dark. Lesion diameter was recorded periodically and lesion volume and mass recorded at the end of each experiment.

6.2.2 Inoculations. 6 µl droplets of conidial suspensions of either fungus were placed over 5 mm deep puncture wounds made with a sterilised stainless steel pin embedded in a glass tube. The pin protruded from the tube and enabled a 5 mm deep wound to be replicated.

6.2.3 Culture maintenance and preparation of spore suspensions. *B. cinerea* was grown on autoclaved bean leaves, as described in chapter 3 and a suspension containing 2×10^5 conidia per ml was used for inoculation. *C. heteronema* was grown on medium containing; Malt extract (30 gl^{-1}), Mycological Peptone (5 gl^{-1}) and agar (15 gl^{-1}). Conidial droplets from 12 day old cultures were removed

with a sterile loop, washed by centrifugation and resuspension in sterile distilled water, and used at a concentration of 2×10^5 spores per ml.

Spore suspensions of both fungi were mixed with equal volumes of water, or test solutions, prior to inoculation.

6.3 Results

The presence of 5×10^{-5}M SA, or SAFe, in the inoculum had no significant effect on the production of B. cinerea lesions on wound-inoculated aged Golden Delicious apples (see table 6.1). The average mass of the lesions was not significantly affected by SA, or SAFe, in the inoculum. Although the percentage mass of the fruit that rotted was consistently greater in the presence of SA, and less in SAFe, the average mass of the lesions, as a percentage of the whole fruit, was not significantly different between the treatments.

The development of B. cinerea lesions on freshly picked, wound-inoculated Bramley's Seedling apples is shown in table 6.2. Although consistent, the stimulation of lesions by SA, and inhibition by SAFe, was not statistically significant. The average lesion mass (with or without the inoculated sites that did not develop), recorded after 16 days, was not significantly, but was consistently, greater when SA was present in the inoculum (table 6.3). The reduction of lesion mass due to SAFe was statistically significant, and the percentage of the fruit rotted was significantly reduced by SAFe, but unaffected by SA, in the inoculum.

TABLE 6.1

The effect of SA and SAFe in the inoculum on lesions caused by B. cinerea on stored Golden Delicious after 120 hours

Treatment	Average lesion Diameter (mm ± S.E)	Average lesion mass of all inoculated sites (g ± S.E)	Average lesion mass (g ± S.E)	Lesion mass as percentage of affected fruit (% ± S.E)
H_2O	7.5 ± 4.2	1.3 ± 1.0	4.4 ± 0.4	4.8 ± 0.1
$5x10^{-5}$M SA	8.2 ± 4.9	1.7 ± 0.7	5.8 ± 4.5	7.2 ± 6.5
$5x10^{-5}$M SAFe	10.3 ± 3.4	1.2 ± 0.6	1.7 ± 0.6	1.8 ± 0.1
L.S.D.(P = 0.05)	8.3	1.5	3.7	4.5

TABLE 6.2

The effect of SA and SAFe on the development of B. cinerea lesions on freshly picked Bramley's Seedling apple fruit

Treatment	Lesion diameter (mm ± S.E) 8 days	13 days	16 days
H_2O	4.0 ± 0.8	4.5 ± 0.8	4.7 ± 0.8
$5x10^{-5}$M SA	5.8 ± 0.9	6.3 ± 0.9	6.4 ± 0.9
$5x10^{-5}$M SAFe	2.7 ± 0.9	3.1 ± 0.9	3.2 ± 1.0
L.S.D.(P = 0.05)	1.7	1.7	1.8

TABLE 6.3

The effect of SA and SAFe on lesions caused by B. cinerea on freshly picked Bramley's Seedling fruit after 16 days

Treatment	Average lesion mass of all inoculated sites (mg ± S.E)	Average lesion mass (mg ± S.E)	Lesion mass as percentage of affected fruit (% ± S.E)
H_2O	57.6 ± 18.9	74.1 ± 20.1	0.09 ± 0.03
$5x10^{-5}$M SA	70.0 ± 12.8	85.5 ± 10.7	0.10 ± 0.02
$5x10^{-5}$M SAFe	21.4 ± 9.0	48.3 ± 7.8	0.04 ± 0.02
L.S.D.(P = 0.05)	27.1	25.8	0.05

The presence of SA, or SAFe, in the infection court had no significant effect on the development of C. heteronema lesions in wound-inoculated freshly-picked Bramley's Seedling apple fruit up to 14 days (see table 6.4). After 48 days there was still no significant difference in the lesions produced with SA, or SAFe, in the inoculum (see table 6.5). There was no significant difference in the lesion diameter, or mass, or the number of large, or small, lesions that developed in each treatment.

5×10^{-5}M SA had no significant effect on the germination of C. heteronema in vitro (table 6.6). The intrinsic germination may have been too high to detect effects of SA.

6.4 Discussion

The presence of SA in the inoculum droplet of B. cinerea on V. faba leaves was stimulatory to the development of the disease (see chapter 3), and SAFe inhibited B. cinerea on V. faba leaves. Lesion development was not stimulated by SA, or inhibited by SAFe, on V. faba cotyledons which were much more susceptible than the leaves to attack by B. cinerea. It would appear Golden Delicious apple fruit behaved more like V. faba cotyledons, since the presence of SA, or SAFe, in the inoculum had no significant effect on disease development. Apple fruit, not puncture-wounded, were completely resistant to infection by B. cinerea conidia in an inoculum droplet placed on their surface, but when wounded they were susceptible to B. cinerea and the treatments had no significant effect.

TABLE 6.4

The effect of SA and SAFe on lesion development of C. heteronema on freshly picked Bramley fruit

Treatment	Lesion diameter (mm ± S.E) 4 DAYS	6 DAYS	10 DAYS	14 DAYS	Lesion volume 14 days (mm^3 ± S.E)
H_2O	2.2 ± 0.2	4.2 ± 0.3	5.4 ± 0.1	7.4 ± 0.2	61.4 ± 2.2
$5x10^{-5}$M SA	2.5 ± 0.2	4.5 ± 0.2	5.8 ± 0.2	7.9 ± 0.1	62.8 ± 1.9
$5x10^{-5}$M SAFe	1.9 ± 0.2	4.0 ± 0.2	5.3 ± 0.3	7.2 ± 0.3	57.9 ± 2.9
L.S.D.(P = 0.05)	0.4	0.5	0.4	0.4	4.7

TABLE 6.5

The effect of SA and SAFe on lesions caused by C. heteronema on freshly picked Bramley fruit after 48 days

Treatment	Mean lesion diameter (mm ± S.E)	Mean lesion mass (g ± S.E)	Average number of lesions with diameter: >20mm	<10 mm
H_2O	23.4 ± 3.1	7.3 ± 2.1	10	3
$5x10^{-5}$M SA	21.1 ± 2.9	5.0 ± 0.9	9	5
$5x10^{-5}$M SAFe	22.2 ± 2.4	5.3 ± 1.0	14	4
L.S.D.(P = 0.05)	5.6	2.7		

TABLE 6.6

The effect of SA on the germination of C. heteronema in vitro

Treatment	Mean germination % (± S.E)
H_2O	84.2 ± 2.2
$5x10^{-5}$M SA	87.9 ± 1.9
L.S.D.(P = 0.05)	4.7

Old Golden Delicious fruit were more susceptible to B. cinerea than freshly-picked Bramley's Seedling fruit. The main antifungal compound in Bramley's Seedling fruit was shown by Brown and Swinburne (1971) to be Benzoic acid, and sufficient quantities were found in diseased tissue to account for all the antifungal activity, at the pH of the tissue, in arrested lesions of Nectria galligena. B. cinerea produced larger lesions on the Bramley's Seedling fruit when SA was present in the inoculum, SAFe being inhibitory, but neither treatment was statistically significant after 16 days. SAFe significantly inhibited the average mass of lesions produced, if inoculation sites which did not develop, were accounted for. However, the lesions produced by B. cinerea on Bramley's Seedling fruit during the time-course of the experiments were all so small that the results were not very meaningful in terms of field infections or commercial storage conditions.

The lesions produced by C. heteronema on Bramley's Seedling, which initially became limited but eventually expanded into progressive rots, were not affected by the presence of SA, or SAFe, in the inoculum. The initial delimitation of lesions was similar to that described previously by Brown and Swinburne (1971) and Edney (1983). The size of lesions eventually produced, and the number of spreading lesions that resulted, were not affected by SA, or SAFe, present in the infection court.

Germination of C. heteronema in water in vitro was high, and was not significantly affected by the presence of SA. A lower control germination may have been affected

by SA, but since fungal germination is normally greater *in vivo* than *in vitro* this is not relevant. *C. heteronema* does not produce appressoria but infects directly from germ tubes or hyphae. The pin-prick wounds used in this study were comparable to insect or bird wounds, open lenticels or other sites through which the fungus would have infected in the field.

Although the concentration of SA and SAFe may have been too small to have overcome the action of such factors as excess nutrient leakage from the wound, or benzoic acid production in the cells around the lesion, it seems unlikely that siderophores affect lesion production by such wound-infecting fungi.

Chapter 7 GENERAL DISCUSSION AND CONCLUSIONS

7.1 Phytoplane factors affecting infection development

7.1.1 General.

When propagules of a potential fungal pathogen land on a plant surface they encounter a diverse range of abiotic, and biotic, factors, which may have an effect on the development of the fungal infection. Such factors have been widely studied and will be mentioned here only briefly. Consider, for example, species of Colletotrichum, the conidia of which generally germinate more readily and produce more appressoria in vivo than in vitro. (Emmett & Parbery, 1975). One factor commonly related to fungal germination in vivo is nutrient availability. The presence of nutrients stimulate germ tube growth, but not appressoria formation, for some Colletotrichum species (Skoropad, 1967; Van Burgh, 1950). Formation of appressoria may be the natural end-point to germination, provided the external environment of the phytoplane remains conducive to appressorium formation. Excess exogenous nutrients induce saprophytic growth, not requiring infection structures such as appressoria, so conidia germinate well and produce long ramifying germ tubes without appressoria (Skoropad, 1967; Van Burgh, 1950). Nutrients exuded from pepper fruit promoted appressoria formation by C. piperatum (Preece & Dickinson, 1971; Grover, 1971) and banana leachates stimulated germination and appressorium formation by C. musae (Swinburne, 1976), although the latter may be attributed to effects other than

nutrition. Germ tubes of C. acutatum (Blakeman & Brodie, 1977), C. pomoides (Van Burgh, 1950), C. musae (Swinburne, 1981), C. dermatium (Duke, 1928) and C. graminicola (Skoropad, 1967) continue to elongate and branch in the presence of sufficient exogenous nutrients. Starvation has been proposed as the factor initiating appressoria formation in C. dematium (Emmett & Parbery, 1975), C. gloeosporioides (Biraghi, 1934) and C. acutatum (Blakeman & Parbery, 1977), as a response to the exhaustion of endogenous and exogenous nutrient reserves.

Temperature affected appressorium formation in C. lagenarium (Ishida & Akai, 1969), C. trifolii (Miehle & Lukezic, 1972) and C. lindemuthianum (Rahe & Kúc, 1970), and light intensity and temperature combined to influence C. graminicola (Skoropad 1967, 1972). Spore age (Swinburne, 1976) and host-parasite compatibility (Bailey & Deverall, 1971) may also affect conidial germination and appressorium formation. The wetness of the plant surface and relative humidity in the micro-environment around the inoculum also affects fungal germination and appressorium formation. Nearly all Colletotrichum species are water-dispersed and infect from an inoculum droplet. Surface tension phenomena were important in the development of Alfalfa anthracnose caused by C. trifoli (Miehle & Lukezic, 1972). Thigmotropic effects, and the physical characteristics of a plant's surface, also affect the frequency and location of the germination and appressorium formation of conidia. The number of appressoria produced by C. pomoides was directly

proportional to the surface hardness of the substrate (Van Burgh, 1950) and C. lindemuthianum produced appressoria more frequently over veins and cell junctions on the upper surface of leaves, but randomly on the lower surface (Preece et al., 1967; Preece & Dickinson, 1971).

Almost all the water soluble nutrients in plant tissues may be leached into droplets of rainwater or dew, on plant surfaces (Tukey, 1971). These substances allow the growth of a variety of saprophytic and facultatively saprophytic bacteria, yeasts and filamentous fungi (Blakeman, 1971, 1973), which may affect, both directly and indirectly, the development of conidia of pathogenic fungi landing on the phytoplane. Although many bacteria are commensals, such as the epiphytic bacteria that develop in inoculum droplets of C. lagenarium conidia on cucumber leaves (Leben & Daft, 1967), some may affect spores of fungi landing in their vicinity. Bacteria isolated from lemon leaves were shown to stimulate appressorium formation by C. gloeosporioides (Lenné & Parbery, 1976). The effect was independent of nutrients and was attributed to antagonism by the production of lytic enzymes. However, the bacteria were not identified and may have been siderophore-producing species. The production of appressoria by C. dematium f.sp. spiniciae on glass and on beetroot leaves was stimulated by pseudomonad bacteria, and this was also attributed to nutrient competition (Blakeman & Brodie, 1977).

A single factor operating alone is unlikely to be the sole stimulus of germination and appressorium formation

by conidia *in vivo*; a number of different factors interacting at the plant surface is likely to influence fungal development. The effect of such factors on species of *Colletotrichum* has been mentioned, but of course, there are many reports of such effects on other genera. Although the stimuli may vary in importance between species and from one time and place to another, there would probably be more than one acting in any specific situation. One factor not outlined above but made clear from the results reported here, and by the earlier work of Swinburne and his co-workers (Swinburne, 1981) is the involvement of iron and iron chelating agents. Unlike most of the factors mentioned above, these may influence not only germination, appressorium formation and infection, but also post-infection development of the fungus.

7.1.2 *Iron chelating agents*

Even before a fungal conidium lands on a plant surface iron may have had a predisposing effect on the virulence of the spore. Conidia derived from a mycelium growing in a low iron environment (and conidia 'pretreated' with iron chelating agents, but without such chelators present in the infection court) have a higher percentage germination (Harper *et al.*, 1980) and are more likely to produce appressoria, and they may also be 'primed' to produce pathogenesis-affecting siderophores. The raised infection potential of such an inoculum may allow the inhibitory factors associated with the plant's defence mechanism to be overcome or avoided, thus increasing the likelihood of an infection developing. Also, plants grown under iron

deficient conditions may be more susceptible.

On the plant's surface, fungal conidia may come into contact with iron chelating agents from diverse sources. Natural chelating polymers and other chemicals, such as those associated with humic acid, may be present, (Vaughn & Ord, 1983) and certain plant exudates may either have chelating properties or be converted by the pathogen to iron chelating agents. (Harper & Swinburne, 1979) However, the main source of naturally-occurring, specifically-iron chelating agents present on the phytoplane is the epiphytic microflora. Using nutrients on the plant's surface such microorganisms may elaborate siderophores which are then released into their environment.

These iron chelating agents stimulate germination of the fungal propagules by complexing iron in the conidia associated with germination repression. (Swinburne, 1981) Conidia derived from low iron substrate, or iron replete conidia in the presence of iron chelating agents, are free to germinate if other factors in the microenvironment are suitable. The presence of iron chelating agents in the infection court stimulates germinated conidia to produce appressoria, especially mature appressoria. It is not known if the processes are coupled so that conidia stimulated to germinate necessarily produce appressoria, or if iron chelating agents stimulate appressorium formation independently. The stimulation of appressoria is particularly important for many fungi since it is through these structures that the fungus infects. (Emmett & Parbery, 1975)

The raised infection potential resulting from the presence of iron chelating agents in the infection court

may be the main factor allowing the development of spreading lesions. In such situations the fungus may be able to quickly kill many host cells and rapidly grow away from the infection site before the host responds by, for example, producing phytoalexins. Ligands fully chelated with iron inhibit fungal germination and appressorium formation and inhibit lesion formation.

There is evidence to suggest iron chelating agents present in the infection court may stimulate lesion formation by directly inhibiting the plant's defence mechanisms. It is likely a strong iron chelating agent present at the site of initial wounding would inhibit iron-requiring enzymes involved in phytoalexin biosynthesis (Lehninger, 1970), or other biochemical processes such as lignification (Milgrom, 1985), which are part of the plant's defence mechanisms. It is also possible iron chelating agents alter the recognition of phytoalexin elicitors, either endogenous, or exogenous, thus inhibiting accumulation of phytoalexins to fungitoxic levels, and fungal susceptibility to the phytoalexins may be reduced in the presence of iron chelating agents.

Figures 7.1 and 7.2 show the possible stages at which iron may be involved in determining the outcome of an infection of a plant by a pathogenic fungus.

The results of the work reported in this thesis indicate how iron, and a bacterial siderophore, can affect a few specific fungal diseases of plants. Iron decreases fungal virulence, the siderophore reduces this inhibitory effect thus increasing fungal aggressiveness.

FIGURE 7.1

Schematic diagram indicating the possible stages at which iron and iron chelators are involved in the pre-infection development of a foliar fungal pathogen

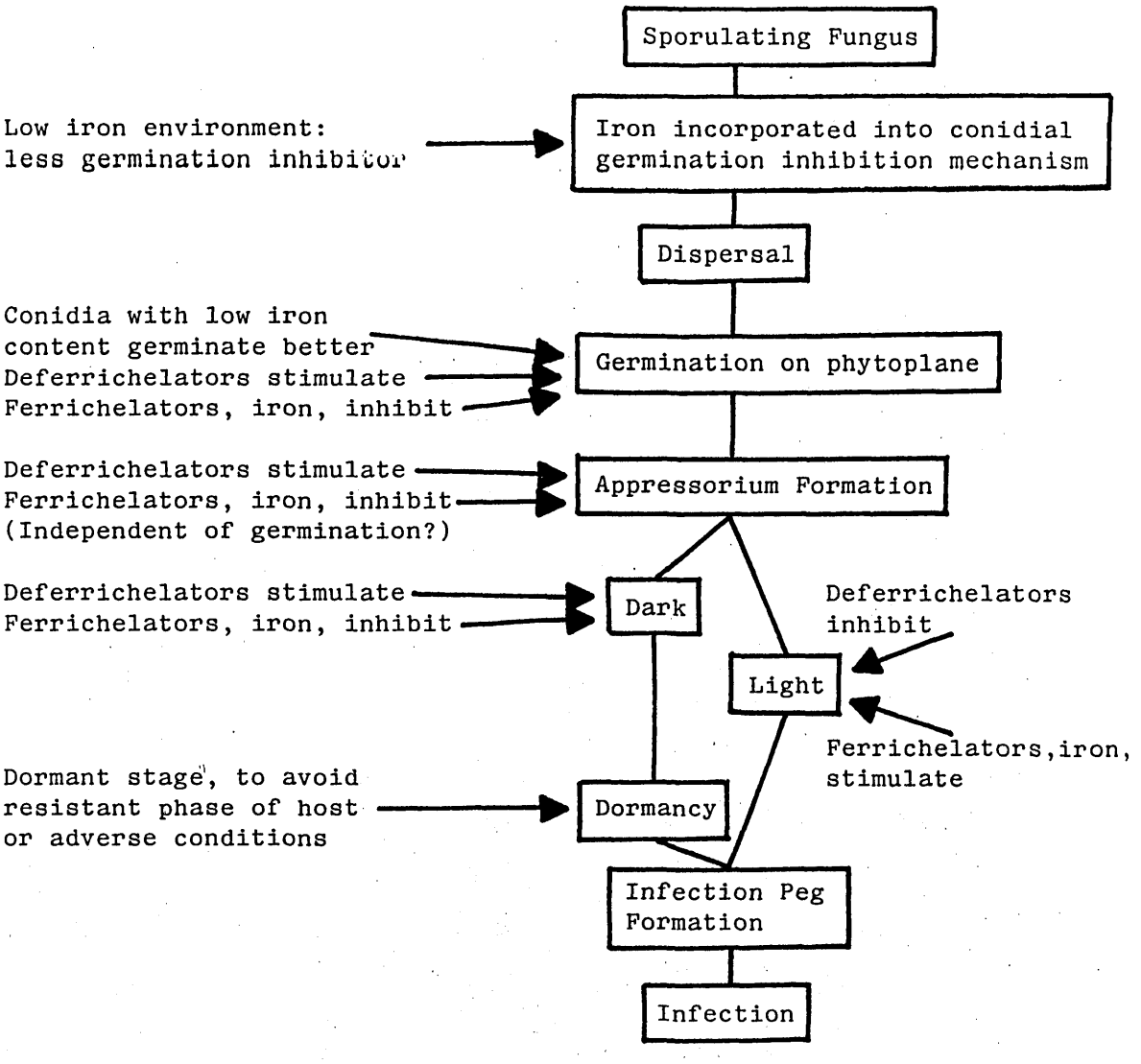
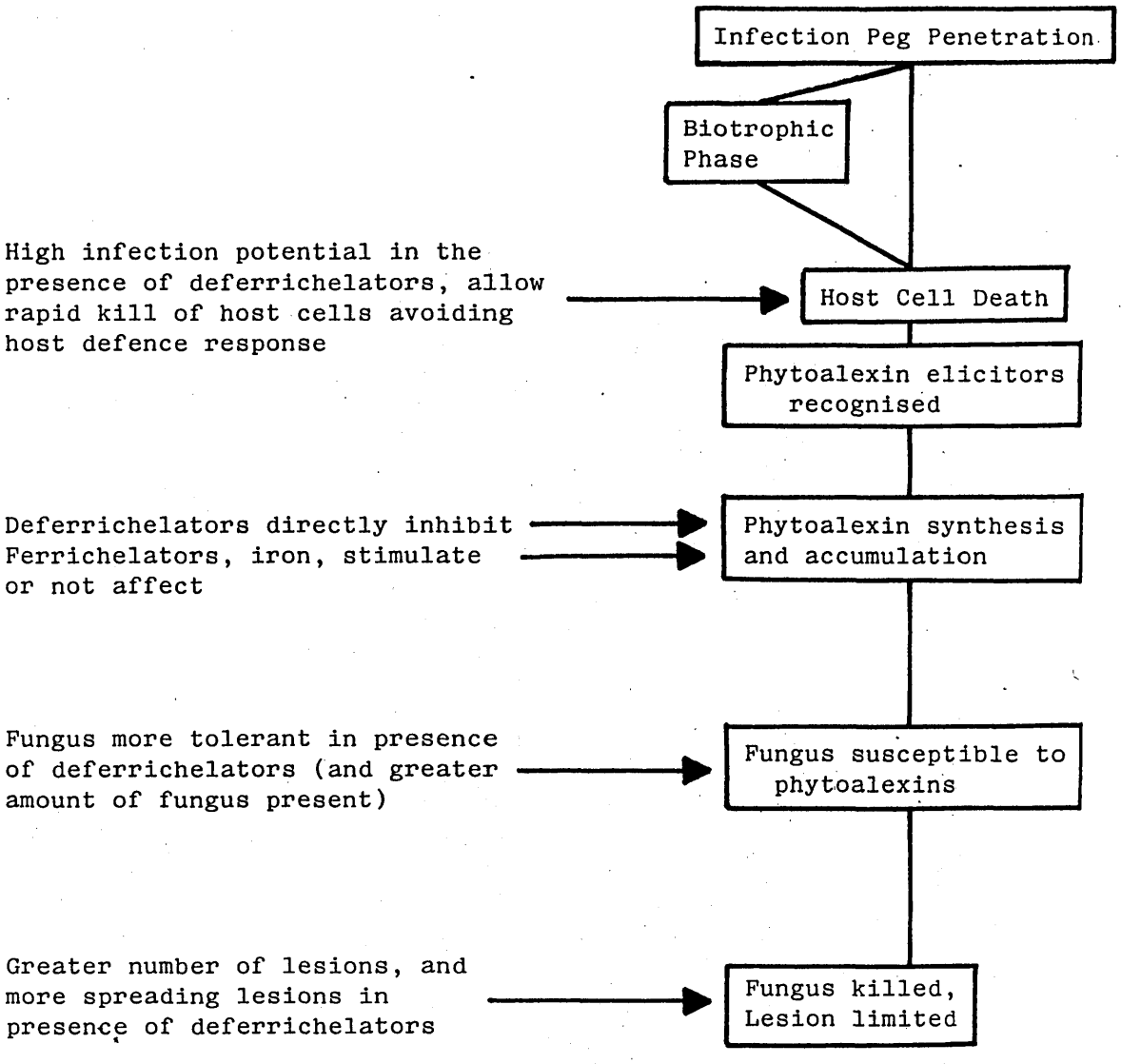


FIGURE 7.2

Schematic diagram indicating the possible stages at which iron and iron chelators are involved in post-infection development of a foliar fungal pathogen



Iron is involved in the host plant's defence processes and strong iron chelators in the infection court probably interfere with the plant's response to attack by a pathogen. It appears that the involvement of iron, and of iron chelating agents, in plant diseases is a widespread phenomenon, and therefore, an important factor in host/pathogen relationships.

7.2 Conclusions

1. Siderophores produced by saprophytic microorganisms stimulate germination of several plant pathogens.
2. Ferrated siderophores inhibit germination, but it is not known if the active iron is ferric or ferrous.
3. Siderophores stimulate appressorium formation (and reduce germ tube length), and in Colletotrichum spp. increase the proportion of appressoria which are heavily melanised.
4. Ferrated siderophores inhibit appressorium formation and increase the proportion which are hyaline. (Hyaline appressoria have no dormant phase.)
5. The presence of siderophores in the infection court reduces phytoalexin accumulation, and by inference, the ability of the host to respond to infection.
6. Siderophores accelerate colonisation and lesion formation, presumably through an increase in infection potential (1 & 3) and the direct effect on the host (5).

7. Fully ferrated siderophores reduce lesion development by decreasing infection potential (2 & 4) and by provoking infection at a resistant stage of the host (4).

BIBLIOGRAPHY

ADIKARAM, N.K.B., BROWN, A.E. & SWINBURNE, T.R. (1982). Rotting of immature Capsicum frutescens L. fruit by iron-depleted Glomerella cingulata (Stonem.). Physiological Plant Pathology, 21, 171-177.

ALBERT, A. (1960). Metal-binding agents. IN Selective Toxicity, pp. 145-168. London: Methuen & Co. Ltd.

ANDERSON, A. (1978). Initiation of resistant responses in bean by mycelial wall fractions from three races of the bean pathogens Colletotrichum lindemuthianum. Canadian Journal of Botany 56, 2247-2251.

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

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

BAILEY, J.A. & MANSFIELD, J.W. (1982). Phytoalexins. Glasgow: Blackie.

BÉKÉSI, P. (1979). The effect of pollen of some weed species on germination of conidia of Botrytis cinerea. Acta Phytopathologica Academiae Scientiarum Hungaricae 14, 379-382.

BIRAGHI, A. (1934). On the biological significance of alleged appressoria of the genus Gloeosporium. Bollitino Stazione Patologica Vegetale Rome 14, 202-210.

BLAKEMAN, J.P. (1971). The chemical environment of the leaf surface in relation to growth of pathogenic fungi. IN Ecology of leaf Surface Microorganisms, pp.255-268. London: Academic Press.

BLAKEMAN, J.P. (1972). Effect of plant age on inhibition of Botrytis cinerea spores by bacteria on beetroot leaves. Physiological Plant Pathology 2, 143-152.

BLAKEMAN, J.P. (1973). The chemical environment of leaf surfaces with special reference to spore germination of pathogenic fungi. Pesticide Science 4, 575-588.

BLAKEMAN, J.P. & BRODIE, I.D.S. (1977). Competition for nutrients between epiphytic microorganisms and germination of spores of plant pathogens on beetroot leaves. Physiological Plant Pathology 10, 29-42.

BLAKEMAN, J.P. & PARBERY, D.G. (1977). Stimulation of appressorium formation in Colletotrichum acutatum by phylloplane bacteria. Physiological Plant Pathology 11, 313-325.

BRODIE, I.D.S. & BLAKEMAN, J.P. (1976). Competition for exogenous substrates in vitro by leaf surface microorganisms and germination of conidia of Botrytis cinerea. Physiological Plant Pathology 9, 227-239.

BROWN, A.E. & SHARMA, H.S.A. (1985). A possible role for ferrous complexes in fungal disease suppression: glume blotch and net blotch of cereals. Phytopathologische Zeitschrift 113, in press.

BROWN A.E. & SWINBURNE, T.R. (1971). Benzoic acid: an antifungal compound formed in Bramley's Seedling apple fruits following infection by Nectria galligena Bres. Physiological Plant Pathology 1, 469-475.

BROWN, A.E. & SWINBURNE, T.R. (1980). The resistance of immature Banana fruits to anthracnose (C. musae (Berk. & Curt.)Arx.). Phytopathologische Zeitschrift 99, 70-80.

BROWN, A.E. & SWINBURNE, T.R. (1981). Influence of iron and iron chelators on formation of progressive lesions by C. musae on banana fruits. Transactions of the British Mycological Society 77, 119-124.

BROWN, A.E. & SWINBURNE, T.R. (1982). Iron-chelating agents and lesion development by Botrytis cinerea on leaves of Vicia faba. Physiological Plant Pathology 21, 13-21.

COMMONWEALTH MYCOLOGICAL INSTITUTE (1968). Plant Pathologist's Handbook. Reading: Lampert Gilbert Ltd.

CSÁKY, T.Z. (1948). On the estimation of bound hydroxamine in biological materials. Acta Chemica Scandinavica 2, 450-454.

DAVID, C.N. (1974). Ferritin and iron metabolism in Phycomyces. IN Microbial Iron Metabolism, pp.149-158. (Edited by J.B. Neilands). New York: Academic Press.

DEN DOOREN DE JONG, L.E. (1926). Bijdrage tot de kennis van het mineralisatieprocess. Thesis. Technische Hoogeschool, Delft, pp.1-199.

DUKE, M.M. (1928). The genera Vermiculuria and Colletotrichum. Transactions of the British Mycological Society 13, 156-184.

EDNEY, K.L. (1983). Top fruit. IN Post Harvest Pathology of Fruit and Vegetables, pp.44-71. London: Academic Press.

EMMETT, R.W. & PARBERY, D.G. (1975). Appressoria. Annual Review of Phytopathology 13, 147-167.

FINKELSTEIN, R.A., SCIORTINO, C.V. & McINTOSH, M.A. (1983). Role of iron in microbe-host interactions. Reviews of infectious diseases 5, 5759-5777.

GNANAMANICKHAM, S. & PATIL, S. (1977). Phaseotoxin suppresses bacterially induced hypersensitive reaction and phytoalexin synthesis in bean cultivars. Physiological Plant Pathology 10, 169-177.

GRAHAM, A.H. & HARPER, D.B. (1983). Distribution and transport of iron in conidia of Colletotrichum musae in relation to the mode of action of germination stimulants. Journal of General Microbiology 129, 1025-1034.

GROSS, D.C. (1982). Evidence that syringomycin, produced by Pseudomonas syringae pv. syringae, is a ferric siderophore. Phytopathology 72, 941.

GROVER, R. (1971). Participation of host exudate chemicals in appressorium formation by Colletotrichum piperatum. IN Ecology of Leaf Surface Microorganims. London: Academic Press, pp. 509-528.

HARPER, A.M. & STRANGE, R.N. (1981). Characterisation of the nutrients required by Botrytis cinerea to infect broad bean leaves. Physiological Plant Pathology 19, 153-167.

HARPER, D.B. & SWINBURNE, T.R. (1979). 2,3-Dihydroxy-benzoic acid and related compounds as stimulants of germination of conidia of Colletotrichum musae (Berk. & Curt.) Arx. Physiological Plant Pathology 14, 363-370.

HARPER, D.B., SWINBURNE, T.R., MOORE, S.K., BROWN, A.E. & GRAHAM, A.H. (1980). A role for iron in the germination of conidia of Colletotrichum musae. Journal of General Microbiology 121, 169-174.

HARGREAVES, J., MANSFIELD, J., COXON, D. & PRICE, K. (1976). Wyerone epoxide as a phytoalexin in Vicia faba and its metabolism by Botrytis cinerea and B. fabae in vitro. Phytochemistry 15, 1119-1121.

HARGREAVES, J., MANSFIELD, J. & ROSSALL, S. (1977). Changes in phytoalexin concentrations in tissues of the broad bean plant (Vicia faba, L.) following inoculation with species of Botrytis. Physiological Plant Pathology 11, 227-242.

HAYASHI, T., TAKATSUKI, A. & TAMURA, G. (1982). Effect of Brefeldin A on biosynthesis of cellular components of Candida albicans. Agricultural Biological Chemistry 46, 2241-2248.

HIDER, R.C. (1985). The facilitation of iron uptake in bacteria and plants by substituted catechols. Proceedings of the NATO Advanced Research Workshop on Iron, Siderophores and Plant Disease. In press.

HILL, H.A.O. (1981). Iron: an element well fitted for its task? IN The Biological Chemistry of Iron Dordrecht, Reidel. pp 3-13.

HOLT, J.G. & KRIEG, N.R. (1984). Bergey's Manual of Systematic Bacteriology. Baltimore: Williams & Wilkins.

ISHIDA, N. & AKAI, S. (1969). Relation of temperature to germination of conidia and appressorium formation in Colletotrichum lagenarium. Mycologia 61, 382-386.

JACOBS, A. & WORWOOD, M. (1974). Iron in biochemistry and medicine. London: Academic Press.

KING, E.O., WARD, M.K. & RANEY, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluoroscin. Journal of Laboratory and Clinical Medicine 44, 301-307.

KLOEPPER, J., LEONG, J., TEINTZE, M. & SCHROTH, M. (1980). Pseudomonas siderophores: a mechanism explaining disease-suppressive soils. Current Microbiology 4, 317-320.

KUBO, Y., SUZUKI, K., FURUSAWA, I. & YAMOMOTO, M. (1985). Melanin biosynthesis as a prerequisite for penetration by appressoria of Colletotrichum lagenarium: site of inhibition by melanin-inhibiting fungicides and their action on appressoria. Pesticide Biochemistry and Physiology 23, 47-55.

LEBEN, C. & DAFT, G. (1967). Epiphytic bacteria and the germination of Colletotrichum lagenarium conidia on cucumber leaves. Phytopathology 57, 527-529.

LEHNINGER, A.L. (1970). Biochemistry. New York: Worth.

LENNE, J. & PARBERY, D.G. (1976). Phyllosphere antagonists and appressorium formation in Colletotrichum gloeosporioides. Transactions of the British Mycological Society 66, 334-336.

LEONG, S. & NEILANDS, J.B. (1982). Siderophore production by phytopathogenic microbial species. Archives of Biochemistry and Biophysics 218, 351-359.

LIGHT, P.A. & CLEGG, R.A. (1974). Metabolism in iron-limited growth. IN Microbial Iron Metabolism. New York: Academic Press. pp. 35-61.

MANSFIELD, J. & DEVERALL, B. (1974). The rates of fungal development and lesion formation in leaves of Vicia faba during infection by B. cinerea and B. fabae. Annals of Applied Biology 76, 77-89.

McCRACKEN, A.R. & SWINBURNE, T.R. (1979). Siderophores produced by saprophytic bacteria as stimulants of germination of conidia of Colletotrichum musae. Physiological Plant Pathology 15, 331-340.

MESSENGER, A. & BARCLAY, R, (1983). Bacteria, iron and pathogenicity. Biochemical Education 11, 54-63.

MEYER, J-M. & ABDALLAH, M. (1978). The fluorescent pigment of Pseudomonas fluorescens, biosynthesis, purification, and physico-chemical properties. Journal of General Microbiology 107, 319-328.

MEYER, J-M. & HORNSPERGER, J. (1978). Role of pyoverdine, the iron-binding fluorescent pigment of Pseudomonas fluorescens in iron transport. Journal of General Microbiology 107, 329-331.

MIEHLE, B.R. & LUKEJIC, F.L. (1972). Studies on conidial germination and appressorium formation by Colletotrichum trifolii Bain & Essary. Canadian Journal of Microbiology 18, 1263-1269.

MILGROM, L. (1985). Ligninase; biotechnology's new money-spinner? New Scientist 1456, 16-17.

MUSSELL, H. & STAPLES, R. (1971). Phytoalexin-like compounds apparently involved in strawberry resistance to Phytophthora fragariae. Phytopathology 61, 515-517.

MUZZARELLI, R. (1973). Natural chelating polymers. London: Pergamon.

NEILANDS, J.B. (1973). Chemistry of iron in biological systems. IN Metal ions in biological systems. New York: Plenum Press. pp. 13-42.

NEILANDS, J.B. (1974). Microbial Iron Metabolism, A comprehensive treatise. New York: Academic Press.

NEILANDS, J.B. (1981a). Iron transport and adsorbtion in microorganisms. Annual Review of Nutrition 1, 27-46.

NEILANDS, J.B. (1981b). Microbial iron compounds. Annual Review of Biochemistry 50, 715-731.

NEILANDS, J.B. (1983). Siderophores. IN Iron binding proteins without cofactors or sulpher clusters. New York: Elsevier Science.

OKU, H., SHIRAISHI, T. & OUCHI, S. (1977). Suppression of induction of phytoalexin, pisatin. Naturwissenschaften 64, 643-644.

PARBERY, D. & BLAKEMAN, J.P. (1978). Effect of substances associated with leaf surfaces on appressorium formation by Colletotrichum acutatum. Transactions of the British Mycological Society 70, 7-19.

POWELL, P.E., CLINE, G.R., REID, C.P.P. & SZANISZLO, P.J. (1980). Occurrence of hydroxamate siderophore iron chelators in soils. Nature 287, 833-834.

PREECE, T.F., BARNES, G. & BAYLEY, J.M. (1967). Junctions between epidermal cells as sites of appressorium formation by plant pathogenic fungi. Plant Pathology 16, 117-118.

PREECE, T.F. & DICKINSON, C. (1971). Ecology of leaf surface microorganisms. London: Academic Press.

RAHE, J.E. & KUC, J. (1970). Metabolic nature of the infection limiting effect of heat on bean anthracnose. Phytopathology 60, 1005-1009.

REED, C.A. (1981). Oxidation states, redox potentials and spin states. IN The Biological Chemistry of Iron. Dordrecht: Reidel. pp. 13-25.

ROSSALL, S. & MANSFIELD, J.W. (1980). Investigation of the causes of poor germination of Botrytis species on broad bean leaves (Vicia faba L.). Physiological Plant Pathology 16, 369-382.

ROSSALL, S., MANSFIELD, J.W. & HUTSON, R. (1980). Death of Botrytis cinerea and B. fabae following exposure to wyerone derivatives, in vitro, and during infection development in broad bean leaves. Physiological Plant Pathology 16, 135-146.

ROSSALL, S. & MANSFIELD, J.W. (1981). Nutrients and lesion formation by Botrytis cinerea on leaves of Vicia faba. Transactions of the British Mycological Society 76, 172.

SKOROPAD, W.P. (1967). Effect of temperature on ability of Colletotrichum graminicola to form appressoria and penetrate barley leaves. Canadian Journal of Plant Science 47, 431-434.

SKOROPAD, W.P. (1972). The effect of some herbicides and fungicides on formation of appressoria in Colletotrichum graminicola. Proceedings of the Canadian Phytopathology Society 39, 41.

SWINBURNE, T.R. (1976). Stimulants of germination and appressoria formation by Colletotrichum musae (Berk. & Curt.) Arx. in banana leachate. Phytopathologische Zeitschrift 87, 74-90.

SWINBURNE, T.R. (1981). Iron and iron chelating agents as factors in germination, infection and aggression of fungal pathogens. IN Microbial ecology of the phylloplane. London: Academic Press. pp.227-243.

SWINBURNE, T.R. (1983). Quiescent infections in post-harvest diseases. IN Post-harvest pathology of fruit and vegetables. London: Academic Press. pp.1-21.

SWINBURNE, T.R. & BROWN, A.E. (1983). Appressoria development and quiescent infections of banana fruit by Colletotrichum musae. Transactions of the British Mycological Society 1, 176-178.

TEINTZE, M., HOSSAIN, M.B., BARNES, C.L., LEONG, J. & van der HELM, D. (1981). Structure of Ferric Pseudobactin, a siderophore from a plant growth promoting Pseudomonas. Biochemistry 20, 6446-6457.

TEINTZE, M. & LEONG, J. (1981). Structure of Pseudobactin A, a second siderophore from plant growth promoting Pseudomonas B10. Biochemistry 20, 6457-6462.

TIETJEN, K.G. & MATERN, U. (1984). Induction and suppression of phytoalexin biosynthesis in cultured cells of Safflower, Carthamus tinctorius L. by metabolites of Alternaria carthami Chowdhury. Archives of Biochemistry and Biophysics 229, 136-144.

TUKEY, H.B. (1971). Leaching of substances from plants. IN Ecology of leaf surface microorganisms. London: Academic Press. pp.67-89.

VAN BURGH, P. (1950). Some factors affecting formation and penetrability of Colletotrichum phomoides. Phytopathology 40, 29.

VAUGHN, D. (1969). The stimulation of invertase development in aseptic storage tissue slices by humic acids. Soil Biology and Biochemistry 1, 15-28.

VAUGHN, D. (1974). A possible mechanism for humic acid action on cell elongation in root segments of Pisum sativum under aseptic conditions. Soil Biology and Biochemistry 6, 241-247.

VAUGHN, D. & ORD, B. (1983). Influence of humic acid on iron required for the formation of hydroxy-proline in discs of Beta vulgaris L. storage tissue. Plant and Soil 73, 27-34.

WEINBERG, E.D. (1978). Iron and infection. Microbial Reviews 42, 45-66.