II

419

# INTERACTIONS BETWEEN PSEUDOMONADS AND CEREAL LEAVES

by

J JULIAN SMITH, BSc (LEEDS)

A thesis presented for the degree of

Doctor of Philosophy

University of Stirling

September, 1981

Graduation: February 1982

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

Julian Smith ..... Candidate

J. W. Mansfield ..... Supervisor

4th September 1981. ..... Date

ABBREVIATIONS AND CHEMICAL FORMULAE

1. Abbreviations

| | |
|---|---|
| A. | Agrobacterium |
| B. | Bacillus |
| c. | circa |
| cv. | cultivar |
| d | days |
| diam. | diameter |
| E | Erwinia |
| EM | Electron microscope |
| EPS | Extracellular polysaccharide |
| h | hours |
| HR | hypersensitive response |
| lx | lux |
| min | minutes |
| NCPPB | National Collection of Plant Pathogenic Bacteria |
| OD | Optical density |
| P. | Pseudomonas |
| SDW | sterile distilled water |
| SEM | standard error of the mean |
| spp. | species |
| TCH | Thiocarbohydrazide |
| TLC | Thin layer chromatography |
| UV | Ultra violet |
| w/v | weight/volume |
| X. | Xanthomonas |

2. Chemical formulae

| | |
|---|---|
| EtOH | Ethanol |
| $H_2O_2$ | Hydrogen peroxide |
| $MgSO_4$ | Magnesium sulphate |
| $Na_3(C_6H_5O_7).2H_2O$ | Sodium citrate |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| $OsO_4$ | Osmium tetroxide |
| $Pb(NO_3)_2$ | Lead nitrate |

ABSTRACT

Interactions between leaves of oats, wheat and barley and phytopathogenic and saprophytic pseudomonads were investigated.

The mechanisms by which successful pathogenesis or host resistance were achieved were examined. Records of symptom development of oat halo blight incited by P. coronafaciens in a susceptible oat cultivar enabled construction of a symptom key which was subsequently used to clarify the reactions of the cereals to artificial inoculation with pseudomonads. Four types of resistance were elucidated on the basis of symptom appearance. These were: (a) delayed development of symptoms as noted for barley and a reportedly resistant oat line Cc4146 in response to P. coronafaciens; (b) a hypersensitive response (HR) induced by plant pathogens isolated from the Gramineae or with cereal pathotypes; (c) a resistant symptom in response to other plant pathogens and in wheat in response to P. coronafaciens and (d) symptomless resistance following inoculation with the saprophyte P. fluorescens. In contrast to other plants (notably tobacco) the HR was not the general response to non-pathogenic bacteria.

P. coronafaciens multiplied rapidly in susceptible oat leaves, watersoaking and normosensitive collapse of the infiltrated area occurred when a critical level of c. $4 \times 10^6$ bacteria/$cm^2$ leaf had been reached. The HR was marked by a sharp decline in bacterial numbers but in leaves

undergoing the resistant symptom and symptomless resistance bacterial numbers remained constant.

Live bacteria were required during an induction period preceding a latent phase and subsequent hypersensitive or normosensitive (susceptible) collapse. Induction times were generally longer in cereals than in broad bean, French bean or tobacco plants and varied between bacteria when the range of plants were compared. Tissue collapse during hypersensitive or susceptible interactions was prevented by prior infiltration with heat-killed cells of the HR eliciting P. coronafaciens var. atropurpurea and both live and heat-killed cells of P. tabaci (resistant reaction) and P. fluorescens (symptomless). P. coronafaciens (live or heat-killed) was not effective in preventing tissue collapse. Prior infiltration of oat leaves with heat-killed cells of P. coronafaciens var. atropurpurea prevented or delayed electrolyte leakage from tissues undergoing normosensitive or hypersensitive collapse respectively. The protective effect of heat-killed P. coronafaciens var. atropurpurea was neither light dependent nor systemic.

Ultrastructural studies on the interactions revealed that extensive membrane damage and cell collapse coincided with electrolyte leakage during hypersensitive and normosensitive collapse. Little cell collapse was observed during the resistant symptom of P. tabaci or following inoculation with P. fluorescens. In vivo the pathogen P. coronafaciens was associated with an extracellular matrix, polysaccharide in nature, which may play a role in pathogenesis. By contrast, non-pathogenic

bacteria were not associated with extracellular polysaccharide but were often attached to mesophyll cell walls by stalks or enveloping fibrils apparently emanating from host cell walls. The degree of attachment did not increase significantly beyond 12h except for the saprophyte P. fluorescens. Attachment rarely exceeded 30% except in the case of P. fluorescens (51.5%) at 24h after inoculation. The numbers of bacteria attached to host walls were not in amounts likely to contribute to resistance per se. Entrapment in polysaccharide released from host walls during injection of sterile distilled water suspensions (physical entrapment) of pathogenic and non-pathogenic bacteria within intercellular spaces was also recorded.

Extracts from oat leaves previously inoculated with P. coronafaciens, P. coronafaciens var. atropurpurea, P. tabaci or sterile distilled water alone were examined for antimicrobial activity. Antifungal and antibacterial activity was confined largely to ethyl acetate phases of extracts when tested by a variety of bioassay techniques. Antimicrobial activity was found in all extracts and no consistent pattern emerged. The results are discussed on the basis of the presence of both induced and constitutive inhibition in infected leaves.

# C O N T E N T S

Page

EXPERIMENTAL WORK AND RESULTS

CHAPTER 1. THE RESPONSES OF CEREAL LEAVES TO INOCULATION WITH BACTERIA

# INTRODUCTION AND LITERATURE REVIEW

## I GENERAL INTRODUCTION

The hosts of bacterial plant pathogens are many and varied. Indeed, the classic treatise of Smith (1920) lists some 115 genera of flowering plants subject to disease of bacterial origin. Some 30 years later, many more plant hosts were recognised (Elliott, 1951). The bacterial incitants of these diseases fall into five genera; Agrobacterium, Corynebacterium, Erwinia, Pseudomonas and Xanthomonas (Elliott, 1951; Bradbury, 1970). Bacterial pathogens of cereals, an important and widespread group of crops, were some of the earliest to be recognised and Smith (1920) noted the description of 14 reported diseases of Gramineae. This thesis describes studies on interactions between the leaves of the temperate cereals oats, wheat and barley and various pseudomonads including P. coronafaciens, the cause of halo blight of oats initially described by Elliott (1920).

## II REVIEW OF INTERACTIONS BETWEEN PLANTS AND BACTERIA

### 1. Bacterial multiplication

In general, bacteria multiply rapidly within susceptible plants (compatible interactions) but not within resistant tissues (incompatible interactions). These two patterns of change in bacterial numbers were first described by Allington and Chamberlain (1949) who studied the multiplication of X. phaseoli and P. glycinea in French bean (Phaseolus vulgaris) and soybean

(Glycine max). Following an initial period of multiplication in resistant plants, bacterial numbers declined during incompatible interactions. In susceptible plants, multiplication continued until destruction of the tissues occurred. Similarly, P. tabaci multiplied most rapidly in the leaves of a susceptible tobacco cultivar and least in resistant leaves (Diachun and Troutman, 1954). Similar trends have been noted in other plant/bacterium interactions (Chand and Walker, 1964; Ercolani and Crosse, 1966).

In more recent studies symptom development has been closely associated with the multiplication of bacteria to critical numbers within susceptible tissues. Thus Klement et al (1978) found that the time after artificial inoculation at which populations were sufficiently high to cause symptom appearance in tobacco was directly proportional to the initial concentration of inoculum. Thus, higher concentrations of compatible bacteria produced more rapid symptom development but low concentrations exhibiting similar rates of multiplication took longer to attain a leaf population capable of eliciting visible symptoms (Averre and Kelman, 1964; Gross and DeVay, 1977; Hsu and Dickey, 1972; Kawamoto and Lorbeer, 1972; Omer and Wood, 1969; Scharen, 1959; Stall and Cook, 1966; Webster and Sequeira, 1977; Weller and Saettler, 1980).

Saprophytic bacteria are usually limited in growth or even slowly decline in numbers within plant leaves (Klement, 1972; Young, 1974a). Young (1974b) found that a stimulation of the growth of P. fluorescens was achieved if the saprophyte was co-inoculated into bean leaves with

the compatible pathogen P. phaseolicola. He suggested that the pathogen affected host cell membrane permeability, releasing cellular components capable of sustaining bacterial growth, including that of the saprophyte. By contrast, bacteria eliciting the hypersensitive response (HR) characteristic of incompatible interactions also cause increases in host cell permeability but multiply at much lower rates than pathogenic bacteria or decline in numbers during the HR (Cook and Stall, 1968; Klement, 1963; Klement, Farkas and Lovrekovich, 1964).

## 2. Mechanisms of pathogenicity

Bacteria enter plants through natural openings such as stomata and through wounds as described by Goodman (1976). Upon entry into the plant, the extensive multiplication of pathogenic bacteria occurs in the intercellular spaces, or as in some vascular diseases, in the xylem. Successful pathogenicity often depends on the production of toxins and enzymes by the bacteria. Ultrastructural examination of compatible plant/bacteria interactions have proved valuable in elucidating the changes in host cells and tissues leading to the development of macroscopic, visible symptoms. Similarly, the location of bacteria within host tissues has been established (Hearon, Sherald and Kostka, 1980; Wallis et al, 1973).

### A. Toxins

Bacterially produced toxins are an important feature of certain diseases (Patil, 1974). The chlorotic spread characteristic of halo blight of oats caused by

P. coronafaciens is indicative of toxin activity, particularly as Elliott (1920) failed to isolate the pathogen from the distant chlorotic areas of leaf blade. In a comparative study on the pathogenicity of P. coronafaciens and the closely related P. striafaciens (Elliott, 1927), Tessi (1953) concluded that the failure of the latter to produce haloeing of the stripe-blight symptoms of oat leaves was due to its inability to produce a toxin.

Following the observation that a number of phytopathogenic Pseudomonas spp. cause necrotic infection sites surrounded by chlorotic haloes and that these pseudomonads were taxonomically very close (Lelliott, Billing and Hayward, 1966), Sinden and Durbin (1970) postulated that the toxins involved in symptom development were likely to be chemically related. However, only heat labile toxins isolated from cultures of P. coronafaciens and P. tabaci, the incitant of wildfire disease of tobacco, showed any similarity. The toxins produced by both bacteria were tentatively identified as mixtures of two dipeptides containing tabtoxinine-β-lactam residues linked to the amino group of either threonine or serine.

An identical toxin was later described for a Pseudomonas spp. attacking Timothy (Phleum pratense) and the trivial name tabtoxin was proposed (Taylor, Schnoes and Durbin, 1972). The production of the toxin and subsequent chlorosis was light dependent (Durbin and Sinden, 1967). The isolated toxin was capable of inducing chlorosis in leaves in the absence of bacteria. Tabtoxin, involved in both halo blight of oats and wildfire disease

of tobacco, is not host specific and Sinden and co-workers (1969) demonstrated that it was even toxic to mice and rats. Bacterial growth is closely correlated with tabtoxin production (Taylor and Durbin, 1973). Free tabtoxinine-β-lactam produced by P. tabaci was shown by Durbin et al (1978) to induce chlorosis in tobacco leaves and they suggested that it possessed the structural features responsible for the biolgoical activity of the tabtoxins.

Ammonia production has often been implicated in the expression of both incompatible (Goodman, 1972; O'Brien and Wood, 1973) and compatible (Bashan, Okon and Henis, 1980) plant/bacteria interactions but its precise role remains to be defined. Trabulsi (1975) noted an increase in ammonia levels in oat leaves treated with tabtoxin and postulated that the toxin affected ammonia metabolism within the host cell during the course of disease development. Exposure of leaves to ammonia vapour caused ultrastructural changes similar to those caused by isolated toxin and exposure to light exacerbated symptom development. Toxic quantities of ammonia also accumulated in tomato leaves infected with P. tomato prior to symptom formation (Bashan, Okon and Henis, 1980). However, the toxic principle from P. tomato is quite different from tabtoxin (Sinden and Durbin, 1970). An effect of the P. tabaci toxin on the important photosynthetic enzyme Ribulose 1,5-Biphosphate Carboxylase has been demonstrated more recently (Crosthwaite and Sheen, 1979).

A partially purified toxin from P. tagetis caused chlorosis in zinnia (Zinnia elegans) leaves about 3 days

after introduction of the toxin into the stem and Jutte and Durbin (1979) further demonstrated that the toxic effect was confined to the chloroplast. Only 1 day after treatment, chloroplast grana and stromal lamellae showed signs of disorganization, usually at the periphery of the plastid. Changes in chloroplast ultrastructure therefore occurred some time before chlorosis became macroscopically visible.

The chlorosis associated with bean halo blight caused by P. phaseolicola has been attributed to a bacterially produced toxin, phaseolotoxin (Mitchell, 1978). Gnanamanickam and Patil (1976) and Ferguson and Johnson (1980) suggested that phaseolotoxin exerts its effect by interfering with ornithine metabolism within the host cell. Ultrastructural examination of P. phaseolicola cells in a susceptible host revealed that the bacteria were coated in surface vesicles, some of which were ruptured. Sigee and Epton (1975) postulated that the toxin associated with bean halo blight was released by rupture of these vesicles. The proposed relationship between toxin release and the formation of vesicles and blebs by P. phaseolicola was, however, later found to be invalid (Passmoor and Epton, 1980).

B. Enzymes

The enzymic dissolution of plant cell wall components has been implicated in a number of bacteria/plant interactions, notably in soft rots (see Bateman and Millar, 1966). Perombelon and Kelman (1980) point out that although many bacteria possess the ability to produce tissue macerating enzymes, only a few have been associated with decay of

living plant tissue. These include Erwinia spp., Bacillus subtilis, B. megaterium, B. polymyxa, P. marginalis and pectolytic species of Pseudomonas, Clostridium and Flavobacterium. Hildebrand (1972), concluded that with the exception of soft-rotting pseudomonad strains, the role of pectolytic enzymes in plant diseases elicited by Pseudomonas spp. had not been established. Other enzymes involved in pathogenesis include cellulases such as those implicated in geranium stem rot caused by X. pelargonii (Garibaldi, 1972).

Ulstrastructural studies on the spread of bacteria through infected tissues during the development of soft rots indicate that dissolution of cell walls and middle lamellae occurs in advance of the intercellular bacterial front. Thus, Fox (1972) showed that the pectolytic activity of E. carotovora var. atroseptica within potato tubers enabled intercellular spread of the bacterium.

Ultrastructural histopathology of cabbage leaf xylem infected with X. campestris also provided visual evidence of enzymic wall dissolution associated with intense bacterial proliferation (Wallis et al, 1973). There is, however, little evidence for the involvement of cell wall degrading enzymes in diseases caused by leaf spotting phytopathogenic bacteria.

C. Extracellular polysaccharide (EPS)

Johnson (1937) observed that watersoaking of infected areas of leaf tissue was a common feature of compatible plant/bacteria interactions. More recently, Rudolph (1978) demonstrated that a host specific principle from

P. phaseolicola induced watersoaking in bean leaves. The principle involved was later identified as a polysaccharide and further investigation revealed that bacterially produced extracellular polysaccharide (EPS) was responsible (El-Banoby and Rudolph, 1979a; b; 1980). Only susceptible bean leaves were affected and the EPS was considered to be important in maintaining a watersoaked environment within the leaf to allow bacterial proliferation (El-Banoby, Rudolph and Hutterman, 1980).

The slime capsules associated with virulent strains of bacteria are examples of extracellular polysaccharides. It has been postulated that the capsules may prevent the binding of bacteria to host lectins, a process thought to be important as a trigger for the initiation of active resistance mechanisms (Fett and Sequeira, 1980; see Introduction II3B). Ultrastructural evidence for a role of EPS in pathogenicity has been recently demonstrated for E. amylovora by Ayers et al (1979). They showed that the virulence of E. amylovora was correlated with its ability to produce EPS and suggested that EPS may be directly involved in symptom expression and also provide a function essential to the growth of the pathogen in host tissues. More recently, El-Banoby et al (1981) have shown that purified EPS from P. phaseolicola remains in intercellular spaces of susceptible bean leaves following injection but that it is enzymically degraded in resistant leaves.

3. Mechanisms of resistance

Resistance of plants to bacterial pathogens can be

attributed to (a) factors present prior to infection, that is, preformed (constitutive) mechanisms of resistance and (b) processes activated upon infection (induced). The most often quoted mechanism of induced resistance is that of the hypersensitive response (Kelman and Sequeira, 1972). The production of antimicrobial compounds, including phytoalexins, is often associated with the HR (Deverall, 1982).

A. The hypersensitive response (HR)

When injected into leaves incompatible phytopathogenic bacteria typically induce rapid tissue necrosis termed the hypersensitive response. The general occurrence of the HR in tobacco has lead to the use of this response as a diagnostic test for phytopathogenic bacteria (Klement, 1963; Lelliott, Billing and Hayward, 1966). The HR was first reported for plant/bacteria interactions by Klement and co-workers who found that symptom appearance was followed by a rapid decline in bacterial numbers within responding tissues (Klement and Lovrekovich, 1961; Klement, Farkas and Lovrekovich, 1964). The occurrence of the HR has been described in many plants following their challenge with phytopathogenic bacteria. However, the role of the response in the restriction of bacterial multiplication remains unclear.

Tissue collapse during the HR induced by E. amylovora or P. pisi in tobacco is indicative of widespread damage to membranes of cellular organelles in tobacco leaf tissue (Goodman and Plurad, 1971). The plasmalemma, tonoplasts and other cellular membranes were visibly affected by 7h

after inoculation with either bacterium. This damage coincided with and was assumed responsible for the observed tissue collapse and electrolyte leakage associated with the HR. Politis and Goodman (1978) later demonstrated that loose fibrillar material accumulated between tobacco leaf cell plasmalemma and inner cell walls during the HR in response to P. pisi. They suggested that these cell wall appositions consisted of newly synthesised cell wall material that formed as part of the complex reactions leading to host cell death in response to the incompatible bacterium.

Comparative ultrastructural investigations on hypersensitive and susceptible responses indicate that the processes of cell collapse may be similar. Spreading of chloroplast grana, degradation of mitochondria and microbodies and loss of membrane integrity casually related to electrolyte leakage observed in apple leaves inoculated with avirulent or virulent strains of E. amylovora suggested to Goodman and Burkowicz (1970) that the processes in both instances were the same.

Resistant and susceptible cultivars of French bean inoculated with P. phaseolicola led to host cell death at initial infiltration and a second phase which was delayed in the susceptible host. However, chloroplast breakdown in susceptible cells was similar to that in resistant cells indicating that the processes of cell death were similar (Sigee and Epton, 1976). Similar results were noted by Daub and Hagedorn (1980) for bean leaves inoculated with compatible P. syringae or incompatible P. coronafaciens.

Live bacterial cells are a pre-requisite for the

induction of the HR (Klement and Goodman, 1967). By following injections of HR-eliciting bacteria with infiltration of inoculation sites with antibiotics thereby killing bacteria *in vivo* Klement (1971) has identified three stages in the development of the hypersensitive response. These stages are described in Fig. I1. The presence of live bacteria is required only during the short induction time. Killing bacteria with antibiotics during this period results in a failure to elicit the HR. The induction time is followed by a latent period before symptom expression. During the latent period bacteria can be killed but HR expression still occurs. The biochemical processes triggering development of the HR must therefore occur during the induction period.

The numbers of bacteria contained within inocula and thus the number within intercellular spaces is an important factor in induction of the HR. In general, inocula below $5 \times 10^6$ bacteria/ml do not elicit a macroscopic, visible HR collapse (Sequeira, 1976). Turner and Novacky (1974) detected dead host cells selectively stained using Evans blue in symptomless tobacco leaves inoculated with the incompatible pathogen *P. pisi* at concentrations lower than those required to give a confluent HR. After 6h a ratio of 1:1 was observed between the number of dead plant cells and the number of *P. pisi* cells introduced. Thus it appeared that the HR would occur at different levels of inocula, sometimes invisible to the unaided eye. Similar conclusions may be drawn from the work of Essenberg *et al* (1979) who demonstrated that a confluent necrosis could

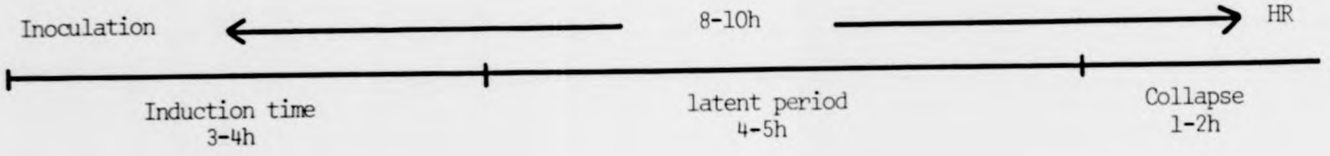



FIG. I1 Development of the hypersensitive reaction (HR) in tobacco leaves infiltrated with the incompatible plant pathogens P. syringae and P. phaseolicola (after Klement, 1971).

be produced by flushing water into sites of immune cotton (Gossypium hirsutum) leaves infiltrated one week previously with low concentrations of X. malvacearum. Essenberg et al suggested that individual cells were affected at low concentrations and that the second infiltration dispersed colonies formed on these cells thus provoking a visible, widespread response.

The expression of the HR is adversely affected by high temperatures (Klement and Goodman, 1967). Klement (1972) found that an incubation temperature of 37°C suppressed the HR development in tobacco leaves and that the thermosensitive part of the HR process was immediately post-induction in the early stages of the latent period. Klement (1972) concluded that the high temperature, although apparently not affecting the factors inducing the HR, probably influenced the biochemical and physiological process of the HR induced in the plant. HR expression in soybean leaves to Pseudomonas spp. is also inhibited by high (31°C) temperature (Holliday et al, 1981).

Humidity may also affect HR development. Saprophytic bacteria do not induce the HR (Klement, Farkas and Lovrekovich, 1964; Lelliott, Billing and Hayward , 1966) but tissue necrosis has been noted in tobacco leaves kept in 100% relative humidity after inoculation with P. fluorescens (Lovrekovich and Lovrekovich, 1970). However, various humidity and light regimes did not affect the HR of Phaseolus vulgaris to Pseudomonas spp. (Lyon and Wood, 1976). The differences recorded between the characteristics of the HR in tobacco and French bean

(Klement, 1972; Lyon and Wood, 1976) emphasise the dangers in generalizing about the HR. The physiological processes of the HR may differ greatly in different species of plants.

The very nature of the symptoms of the HR, loss of turgor of cells followed by collapse and desiccation of the infiltrated leaf area suggests that changes occur in the tissue permeability. Studies on electrolyte leakage from inoculated tissues have confirmed that permeability changes do parallel symptom development (Cook, 1975; Cook and Stall, 1968; Goodman, 1968; 1972; Goto, Takemura and Yamanaka, 1979). Goodman (1968) also demonstrated that SH-containing sulphur compounds mimicked the stimulation of electrolyte leakage adding further weight to his earlier supposition that the HR is mediated by SH-containing compounds produced by the bacteria affecting the S-S bonds in the protein component of membranes (Goodman, 1967). The presence of calcium prevented electrolyte leakage in pepper (Capsicum anuum) inoculated with X. vesicatoria (Cook, 1975; Cook and Stall, 1971). Turgor of host cells does not affect electrolyte loss in hypersensitive leaves (Gulyas et al, 1979). Goodman (1972), as a result of ultrastructural studies, proposed that damage to the plasmalemma was the most likely cause of electrolyte leakage. Furthermore, Cook and Stall (1968) and Goodman (1968, 1972) have suggested that desiccation following tissue collapse was the cause of the drop in numbers of recoverable bacteria during the HR.

Increases in tissue pH, ammonia production and respiration rate have all been recorded in leaves undergoing

the HR (Goodman, 1972; Nemeth and Klement, 1967; O'Brien and Wood, 1973). Ammonia production and pH increases in tobacco inoculated with P. pisi occurred well after HR expression as indicated by electrolyte leakage (Goodman, 1972). This implied that ammonia production and rises in pH were not causally related to the HR as previously suggested (Lovrekovich, Lovrekovich and Goodman, 1970). The same conclusions were drawn by O'Brien and Wood (1973) following studies on the infection of Phaseolus vulgaris by Pseudomonas spp.

Respiration ($O_2$ uptake) in tobacco leaves responding hypersensitively to P. syringae increased to reach maximal rates some 30 min before appearance of the HR (Nemeth and Klement, 1967). The rise was only minimal following inoculation with the pathogen P. tabaci and the saprophyte P. fluorescens. It appeared that increase in tissue respiration was causally related to HR expression. Enzyme changes associated with the HR resulting in cell collapse have also been examined for the same system (Nemeth, Klement and Farkas, 1969). Their results indicated that although enzyme changes during the HR evoked by fungi and viruses were very similar, they were markedly different for bacterially induced hypersensitivity. In reactions evoked by bacteria there was less evidence for an increase in enzyme activities. Nemeth et al suggested that this was because of the comparative rapidity of the HR caused by bacteria.

Lovrekovich and Farkas (1965) found that prior infiltration with heat-killed bacteria would induce protection against wildfire disease in tobacco. The same

treatment was also effective in preventing local lesion formation in tobacco in response to tobacco mosaic virus (Lobenstein and Lovrekovich, 1966). Subsequent experiments by Lozano and Sequeira (1970) demonstrated that heat-killed cells of P. lachrymans and X. axonopodis would prevent the development of the HR in tobacco leaves 18h later with live, HR inducing P. solanacearum. Protection against the HR was reported to be light dependent and systemic. The susceptible reaction of tobacco leaves to P. tabaci and their hypersensitive response to P. glycinea was also prevented by infiltration 18h prior to challenge with heat-killed P. tabaci or P. glycinea (Sleesman, Perley and Hoitink, 1970). The protective effects were not, however, systemic. Heat stable protection fractions were obtained from cells of both bacteria and insoluble cell wall constituents also induced local protection.

Injection of cycloheximide into tobacco leaves at or up to 3h after infiltration with P. pisi delayed the HR for 18h (Pinkas and Novacky, 1971). In vitro and in vivo experiments did not reveal any effects of cycloheximide on bacterial multiplication. The data suggested that cycloheximide exerts its effect on HR expression probably by reducing protein synthesis in the host cell. Furthermore, since cycloheximide did not delay symptom development in tobacco inoculated with compatible P. tabaci it appeared that the HR and pathogenesis were distinctly different phenomena.

The plant hormone, cytokinin, was also shown to prevent the development of the HR in tobacco leaves

infiltrated with $5x10^{-5}$M kinetin 48h prior to injection with P. pisi although bacterial growth in the tissue remained unaffected (Novacky, 1972).

Living bacteria can also prevent the expression of bacterially induced HR. Low concentrations of P. pisi infiltrated into tobacco leaves prevented HR development in response to a subsequent higher concentration of the same bacterium. This protective effect was light dependent and systemic (Novacky, Acedo and Goodman, 1973). Similarly, live cells of an HR inducing bacterium reduced symptom development in French bean leaves following challenge with P. phaseolicola (Omer and Wood, 1969). Electron microscopical studies on the tobacco system showed that apart from minor effects on cell wall density, protected tissue was similar to healthy controls. Interestingly, tobacco leaves injected 4 times with water alone 48h prior to challenge with P.pisi were also protected against the HR (Hanchey, Pastalky and Novacky, 1974). Further investigations revealed that repeated injection of leaves with water or treatment with cotton swabs or carborundum also prevented the HR upon subsequent challenge with the incompatible bacterium. As the pattern of growth of this bacterium was similar to that in leaves protected by low concentrations of the same species the suggestion was made that the protective reactions result from a mild or non-specific injury of the tissue (Novacky and Hanchey, 1976).

Sequeira, Aist and Ainslie (1972) showed that the protective or delaying effect on the HR in tobacco leaves caused by heat-killed cells of P. solanacearum could be

mimicked by both soluble and insoluble cell wall fractions from disrupted bacteria. Partial purification of proteinaceous constituents yielded an active fraction. The peptidoglycan layer of bacterial cell walls was postulated as a likely candidate for a role in the protective response elicited by the crude extracts. However, pure peptidoglycan did not prevent the HR in tobacco leaves in response to P. solanacearum (Wacek and Sequeira, 1973). Results, however, did support the concept that the active component(s) was a glycoprotein associated with the bacterial cell wall but not including the peptidoglycan layer. Independently it was also reported that not only protein fractions from host plant or the incompatible pathogen prevented HR development but also foreign proteins such as albumin would prevent the HR in a non-specific manner in tobacco leaves in response to P. lachrymans, P. morsprunorum and P. syringe (Gaborjanyi, O'Brien and Klement, 1974).

Dispersions of protein-lipopolysaccharides derived from the outer membrane of E. chrysanthemi injected into tobacco prevented confluent HR in response to P. syringae (Mazzuchi and Pupillo, 1976). The protein-lipopolysaccharide complexes of the bacterial outer membrane were thus implicated in the modification of plant responses to phytopathogenic bacteria. Purified lipopolysaccharide from P. solanacearum and non-plant pathogens was effective in preventing the HR elicited in tobacco leaves by live P. solanacearum (Graham, Sequeira and Huang, 1977). When injected into tobacco leaves, purified P. solanacearum

lipopolysaccharide attached to mesophyll cell walls and induced ultrastructural changes in the host cell similar to those induced by attachment of whole heat-killed bacteria. From this series of experiments, bacterial lipopolysaccharide derived from the outer membranes of bacteria emerged as the most likely inducer of the HR and disease resistance in tobacco. More recently, Bonatti and co-workers (Bonatti, Dargeni and Mazzuchi, 1979) have demonstrated that purified protein-lipopolysaccharide complexes of P. aptata protect tobacco against the HR elicited by live cells of the same bacterium. Similarly, protein lipopolysaccharide complexes from a virulent P. tabaci strain prevented the HR and also delayed the susceptible reaction in tobacco leaves (Mazzuchi, Bazzi and Papillo, 1979). They suggested a common mechanism of induced plant tolerance based on host recognition of polysaccharide antigenic determinants.

The nature of the inducer of the HR remains unknown despite several attempts to identify it. It must be produced by live cells in close contact with the plant cell wall and is probably very unstable or readily degraded as discussed by Sequeira (1976). Sequeira et al (1977) showed that attachment and encapsulation of incompatible strains of P. solanacearum to tobacco mesophyll cells appeared to be essential steps in the process that resulted in the HR. Although no quantitative examination of the attachment of bacteria to tobacco cell walls was made by Sequeira et al, they suggested that the close proximity of incompatible bacteria to host cells (attached) was necessary for the transfer of HR-inducing molecules.

The hypersensitive response therefore reflects a specific response of the plant to features of incompatible plant pathogenic bacteria. Convincing evidence that the HR as an induced mechanism of resistance is responsible *per se* for the failure of incompatible plant pathogenic bacteria to multiply within plant leaves is, however, lacking. In this context, the induced *de novo* synthesis of chemical barriers to infection associated with the HR, originally proposed by Muller and Borger (1941), may be important.

B. Attachment of bacteria to cell walls

Ultrastructural studies have revealed that incompatible bacteria can be localized within intercellular spaces by collapsed cells or can be agglutinated *in situ* (Huang, Huang and Goodman, 1975). Incompatible plant pathogenic bacteria can also be localized by attachment to host cells, often enveloped in host wall derived material. This has been described as a reaction strongly associated with resistance (Cason *et al*, 1978; Goodman, Huang and White, 1976; Goodman, Politis and White, 1977; Roebuck, Sexton and Mansfield, 1978). Sequeira *et al* (1977) and Sing and Schroth (1977) further demonstrated that saprophytic bacteria were also attached to host cell walls in a similar manner whereas compatible plant pathogens were not observed to be attached. Huang and Van Dyke (1978) suggested that the attachment of the incompatible *P. pisi* to tobacco callus cells impeded bacterial multiplication whereas the pathogen, *P. tabaci* remained free and was able to multiply. However, it is not clearly understood if

attachment *per se* confers host resistance. It is probably more likely that this intimate contact between host cell and bacterium is required to elicit other processes more strongly implicated in resistance, such as the HR (Atkinson, Huang and Van Dyke, 1981; Sequeira, 1976).

Electron micrographs depicting bacterial attachment could in some instances be differently interpreted following a recent report by Hildebrand *et al* (1980). They report an indiscriminate physical entrapment of incompatible and compatible plant pathogenic and saprophytic bacteria. Water infiltrated into the leaves of bean appears to dissolve certain cell wall materials and subsequent transpiration causes intercellular fluid to recede until films of fibrillar material condensing from dissolved material form at air-water interfaces. Bacteria were commonly observed as trapped behind these films which were more common in younger leaves. The bacteria were not attached to cell walls and multiplication of the pathogen was observed possibly aided by moisture retention caused by the formation of these films.

Attachment involving extensive host cell wall modification does undoubtedly occur, however, and the nature of the enveloping structure is possibly host wall derived (Goodman, Huang and White, 1976) or part plant wall and bacterial in origin (Bonatti, Dargeni and Mazzuchi, 1979). Obukowicz and Kennedy (1981) have suggested that an increase in polyphenol oxidase activity in incompatible combinations may result in tannin formation around bacteria attached to host cell walls.

Callow (1977) has suggested that if the HR involves cell recognition, surface components of host and pathogen are likely to play a major role. Highly specific saccharide binding proteins, lectins, are found on the cell surface of many plants and are capable of binding the glycoprotein portion of cell membranes (Callow, 1975; Sequeira, 1978). The involvement of lectins in plant/bacteria interactions has been clearly demonstrated by Graham and Sequeira (1977) and Sequeira and Graham (1977). Avirulent cells of P. solanacearum were agglutinated by potato (Solanum tuberosum) lectin and the failure of virulent cells to bind was attributed to the presence of a coat of extracellular polysaccharide (see Introduction, II2c) which was not produced by avirulent cells. The binding site of the lectin involved was postulated as the lipid A portion of the lipopolysaccharide layer of the outer bacterial membrane (Sequeira and Graham, 1977). Whatley et al (1980) further demonstrated that the lipopolysaccharide composition of P. solanacearum was closely correlated with its ability to induce the HR in tobacco.

There are a number of recent reports on the agglutination of bacterial cells by plant extracts some of which are not proteins and should not be considered as lectins but agglutinins. These compounds may be involved in recognition but their precise role in the plant/bacteria interaction often remains unclear (Anderson and Jasalavich, 1979; El-Banoby and Rudolph, 1980). Fett and Sequeira (1980, a; b) demonstrated that although an

agglutinin from soybean was most active against more virulent strains of the pathogen X.phaseoli var. sojensis, there was no evidence to suggest that binding of the pathogen to host mesophyll cells might lead to a compatible response. Furthermore, ultrastructural examination of the interaction revealed that only avirulent cells were bound to soybean cell walls. However, Slusarenko and Wood (1981) showed that a non-lectin fraction obtained from cotyledons of Phaseolus vulgaris agglutinated cells of avirulent isolates of P. phaseolicola more actively than it agglutinated cells of virulent isolates.

Sequeira (1976) suggested that the molecule(s) responsible for HR induction may be transferred to host cell from bacteria attached to cell walls and react with a "sensitivity locus" within the host cell. This provides the stimulus for membrane disorganisation (HR collapse) and the release of host cell contents capable of halting bacterial growth. This hypothesis is similar to that proposed by Callow (1977) for recognition and gene control in those instances of varietal specificity governed by gene-for-gene interactions.

C. Antibacterial compounds

(i) Induced chemical resistance

Phytoalexins were originally conceived as antibiotics produced by plants which inhibit the growth of the micro-organisms pathogenic to plants (Muller, 1956). The principle of these "warding-off" compounds has remained but the definition has been modified by Ingham (1973) to include abiotic induction.

In the following discussion of induced chemical resistance only those phytoalexins or antimicrobial compounds implicated in the resistance of plants to bacterial infection will be considered. For a more detailed review of the role of phytoalexins in resistance to fungal pathogens consultation of one of the many review articles is advised (e.g. Deverall, 1977; Mansfield, 1982).

The most detailed studies of the involvement of phytoalexins in bacterial infections concerns the French bean plant and the resistance of leaves of certain cultivars to halo blight caused by P. phaseolicola.

Stholasuta et al (1971) showed that the bean phytoalexin phaseollin accumulated during the HR elicited by an incompatible race of P. phaseolicola but not during the HR caused by P. morsprunorum. Very little phaseollin was detected in bean leaves inoculated with a compatible race of P. phaseolicola. However, phaseollin had no effect on bacterial growth in vitro. O'Brien and Wood (1973b) were the first to postulate a role for phytoalexins in resistance following their detection of the accumulation of antibacterial compounds in bean leaves during HR expression. Subsequent work by Lyon and Wood (1975) and Gnanamanickam and Patil (1977a) showed that high concentrations of phenolics accumulating during the HR in bean leaves were the flavonoid phytoalexins phaseollin, coumestrol, phaseollin isoflavan and kievitone. Much lower levels were detected in susceptible reactions and in both resistant and susceptible reactions phytoalexin accumulation closely followed necrosis

of plant cells. The stone-fruit pathogen P. mors-prunorum also induced a rapid HR and phytoalexin accumulation but the saprophyte P. fluorescens did not elicit symptoms or changes in isoflavonoid concentrations (O'Brien and Wood, 1973 a; b; Lyon and Wood, 1975). Gnanamanickam and Patil (1977b) and Patil and Gnanamanickam (1976) found that phaseotoxin, produced by P. phaseolicola, suppressed hypersensitive cell death and also phytoalexin accumulation.

Lyon and Wood (1975) and Gnanamanickam and Patil (1977a; b) used experimental systems in which attached leaves were inoculated and incubated under low relative humidities, under which conditions the collapse and desiccation of tissue during the HR, even in the absence of accumulation of inhibitory compounds would be expected to impede bacterial proliferation (Rudolph, 1980). Webster and Sequeira (1977), however, incubated detached bean pods in a saturated atmosphere thereby reducing the effect of desiccation on bacterial multiplication. Thus, they demonstrated that virulent and avirulent isolates of P. syringae multiplied equally rapidly in bean pods during the first 9 days after inoculation. However, only virulent isolates proceeded to produce spreading, watersoaked lesions; lesions caused by avirulent isolates remaining small and localized. Changes in lesion appearance were followed by changes in bacterial populations, multiplication of the avirulent isolate being restricted. Ethyl acetate extracts from tissues undergoing the resistant reaction became highly inhibitory to both

strains of P. syringae at the time of restriction of bacterial multiplication within infected pods, but extracts from tissue developing spreading lesions, or healthy pods, lacked inhibitory activity. Results obtained with these crude extracts provide convincing evidence for the involvement of phytoalexins in resistance. The concentration of phaseollin within these extracts paralleled their antibacterial activity but Webster and Sequeira did not detect any significant effect of purified phaseollin on the multiplication of P. syringae. The inhibitory activity of the crude extracts was attributed to an unidentified phenolic compound.

The results of bioassays carried out by Webster and Sequeira (1977) raise the problem of conflicting evidence on the antibacterial activity of compounds initially recognised as phytoalexins because of their fungitoxic properties. Thus, although Lyon and Wood (1975) also found phaseollin to be inactive, coumestrol was antibacterial in their assays and must therefore be considered as a phytoalexin despite its lack of fungitoxicity (Sherwood et al, 1970). Coumestrol, kievitone, phaseollin and phaseollin isoflavan were all found to be inhibitory to P. phaseolicola in the bioassays employed by Gnanamanickam and Patil (1977). Although there appears to be evidence for the accumulation of antibacterial phytoalexins during the resistance of French bean to plant pathogenic bacteria, opinions differ on the relative importance of individual compounds.

The in vitro sensitivity of bacteria to phytoalexins

(particularly isoflavonoids) often differs according to the bioassay technique employed. Thus, Wyman and Van Etten (1978) showed that of 6 isoflavonoid phytoalexins assayed against a range of plant pathogenic bacteria, only kievitone exhibited strong antibacterial activity as indicated by direct addition to a bacterial lawn or liquid culture. Further work has demonstrated that isoflavonoid phytoalexins and the broad bean (Vicia faba) phytoalexin wyerone were selectively toxic to Gram-positive bacteria (Gnanamanickam and Mansfield, 1981; Gnanamanickam and Smith, 1980). This selectivity was also noted by O'Neill (1981) for the activity of hydroxyflavan phytoalexins derived from narcissus. In general, this work supports the observation that phytopathogenic bacteria are not very sensitive to well known phytoalexins.

The response of soybean leaves to compatible and incompatible races of P. glycinea is closely comparable to that of French bean leaves to P. phaseolicola. Keen and Kennedy (1974) found that the HR in soybean caused by incompatible races of P. glycinea, or the non-pathogen P. lachrymans was associated with the rapid accumulation of glyceollin and coumestrol which were demonstrated to be antibacterial in vitro. Bruegger and Keen (1978) and Keen et al (1981) have further demonstrated a relationship between the soybean HR/glyceollin accumulation mechanism and the restriction of the populations of incompatible plant pathogenic pseudomonads. Little accumulation of phytoalexin was detected following inoculation with a compatible race of P. glycinea or the saprophyte P. fluorescens.

Among the widely studied phytoalexins of the Solanaceae, several have been implicated in resistance to bacterial infection. Thus, Lyon and Bayliss (1975) demonstrated that the sesquiterpenoid rishitin isolated from potato tuber rots caused by E. carotovora var. atroseptica was inhibitory to this and 5 other bacteria in vitro. Lyon (1978) later suggested that rishitin exerted its effect by acting directly on the bacterial cell membrane in a manner similar to that of a cationic surfactant or membrane bound antibiotic. Rishitin may be an important factor contributing to the resistance of potato tubers to E. carotovora var. atroseptica. Hammerschmidt and Kuc (1979) have recently demonstrated that the phytoalexin phytuberin occurs in tobacco leaves undergoing the HR in response to P. lachrymans and may be involved in resistance expression.

The accumulation of unidentified inhibitors of bacterial growth has been reported for a number of plant/bacteria interactions. Stall and Cook (1968) examined the response of pepper leaves to X. vesicatoria and succeeded in extracting inhibitors from the intercellular spaces of leaves, the sites of bacterial multiplication, using the technique devised by Klement (1965). Unidentified phytoalexins accumulated more rapidly and reached higher concentrations in extracts from leaves undergoing the hypersensitive reaction than the susceptble response.

Mansfield (1982) describes the value of extracting intercellular fluids in allowing the recovery of compounds

to which bacteria will almost certainly be exposed within the infected plant. The application of this technique may help to clarify the role of phytoalexins in French bean and soybean and would also be suitable in examining phytoalexin involvement in those interactions apparently involving localized bacteriostasis such as that of cotton leaves resistant to X. malvacearum, described by Essenberg et al (1979).

(ii) Pre-formed (constitutive) resistance

Pre-existing chemical barriers to infection have been demonstrated for a number of plant species as discussed by Ingham (1973). In some instances, antimicrobial compounds may be involved in resistance by their release from inactive or mildly active precursors under the influence of enzymes normally separated from their precursors in different parts of healthy tissues. An example of this type of pre-formed inhibitor is an antifungal compound isolated from wheat (Triticum aestivum) identified as 6-methoxy-2(3)-benzoxazolinone (Hietala and Wahlroos, 1956; Wahlroos and Virtanen, 1959). Induced phytoalexin synthesis, unlike pre-formed inhibitors, is thought to occur de novo from remote precursors (Deverall, 1977).

The role in resistance of plants to fungal pathogens has been inferred for a number of pre-formed inhibitors but a similar role in resistance to plant pathogenic bacteria is not so well documented. In the following discussion only those pre-formed inhibitors identified from cereals and those exhibiting antibacterial activity will be considered.

The resistance of oat (Avena sativa) to the take-all fungus Gaeumanomyces graminis var. tritici has been attributed to a pre-formed glycosidic inhibitor avenacin, found in roots and shoots (Turner, 1953; 1956). The structure of this saponin is known as is the structure of similar saponins found in oat leaves, 2,6-desgluco-avenacosid A and B (Luning and Schlosser, 1976). Luning and Schlosser (1975) had previously shown that the aglycone forms of the avenacosid saponins produced by the activity of a β-glucosidase were extremely fungitoxic.

Defago (1977) states that the toxic action of the saponin is an interaction with sterols of the fungal membrane resulting in cell death. Bacteria can only be affected when sterols are incorporated into the growth medium (Deacon, personal communication). Avenacin in oats is well established as playing a major part in the resistance of oats to fungal attack (Holden, 1980) and Turner (1961) has shown that the success of a pathogen lies in its ability to degrade enzymically avenacin upon infection.

A similar system of pre-informed inhibition appears to operate in maize (Zea mays) and wheat. An antifungal compound identified as 6-methoxy-2(3)-benzoxazoline was shown to be derived from a glucoside precursor of limited antifungal activity. The conversion to the aglycone form resulted in strong antifungal activity (Hietala and Wahlroos, 1956; Wahlroos and Virtanen, 1959). This same activity, however, was not implicated in more recent work on the resistance of wheat to fungal attack (Baker and Smith, 1977; Deverall, Wong and McLeod, 1979).

Whitney and Mortimore (1961) showed that the resistance of sweet corn (Zea mays var. saccharata) to the bacterial pathogen X. stewartii was possibly associated with the levels of a pre-formed inhibitor identified as 6-methoxy-benzoxalinone. Hartman et al (1975) also demonstrated that soft-rotting Erwinia spp. were inhibited by a differentially inhibitory fraction from maize, subsequently identified as 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) by Corcuera et al (1978). A role for DIMBOA or its degradation product, 6-methoxybenzoxazolinone, in resistance of maize to bacterial pathogens was thus inferred. However, the recent experiments of Lacy et al (1979) have shown that although several soft-rotting Erwinia spp. pathogenic to maize were susceptible, DIMBOA was not the primary means of plant resistance but possibly exerted selection pressure for strains pathogenic to maize at some level other than that of primary host resistance.

A steroid glycoalkaloid found in expressed tomato (Lycopersicon esculentum) sap, tomatine, was found to be fungitoxic to tomato pathogens (Arneson and Durbin, 1967). Mohanakumaran et al (1969) showed that bacterial wilt resistant tomato plants have higher concentrations of tomatine than susceptible ones. Concentrations in resistant plants were found to be bacteriostatic in vitro and Mohanakumaran et al suggested that tomatine was involved in the resistance of tomato to the wilt bacterium, P. solanacearum.

Antibacterial activity has been detected in crude or partially purified healthy plant extracts of garlic (Lund

and Lyon, 1975) and poplar (Van Hoof *et al*, 1980) but a possible role in the resistance of these plants to bacterial pathogens remains undefined. Kelman and Sequeira (1972) have discussed the apparent importance of pre-formed chemical resistance in certain species of plant, notably maize. As noted previously (Introduction II3Bi) certain plant pathogenic bacteria are seemingly insensitive to recognised plant phytoalexins, investigation into the role of pre-formed inhibitors may therefore prove to be more fruitful in establishing a chemical component of resistance to bacterial pathogens.

## III HALO BLIGHT OF OATS

With the current interest in physiological approaches to plant pathogenesis, plant/bacteria interactions have been widely studied with members of the Solanaceae receiving perhaps disproportionate attention. With the exception of rice (*Oryza sativa*) pathogens e.g. *X. translucens* f. sp. *oryzicola* causing bacterial leaf streak of rice (Sridhar *et al*, 1979), little work has been directed towards bacterial diseases of cereals. The majority of cereal diseases caused by bacteria are reported from North America (Hagborg, 1974). Of these diseases, several are of economic importance notably basal glume rot of wheat incited by *P. atrofaciens*. *X. translucens* f. sp. *cerealis* is an important bacterial blight of wheat, barley and to a lesser extent oats and rye (*Secale cereale*) (Jones, Johnson and Reddy, 1917).

Maize is particularly susceptible to bacterial attack

in particular by E. stewartii, C. nebraskense and P. andropogonis eliciting Stewarts' wilt, Goss's wilt and bacterial stripe respectively (Carlson et al, 1979). Chocolate spot of corn is caused by P. syringae (Ribeiro et al, 1977) which is similar to holcus spot elicited by P. holci (Kendrick, 1926). These few examples serve well to illustrate the range and variability of bacterial pathogens of cereals.

One of the few bacterial diseases of temperate cereals to reach economic importance is the widely reported halo blight of oats caused by P. coronafaciens (Elliott) Stevens. This is a relatively well studied pathogen in as much as the disease syndrome has been well described by several authors. Elliott (1920) was the first worker to identify P. coronafaciens as the cause of a halo blight occurring throughout the oat growing sections of the central and Eastern States of North America. Different isolates recovered by Elliott were all pathogenic to oats and some were found to be pathogenic to wheat, barley and rye. A similar disease described for bromegrasses, ryegrasses and fescues was attributed to a closely related bacterium, P. coronafaciens var. atropurpurea (Reddy and Godkin, 1923; Tominaga, 1968).

Halo blight of oats was first identified in Scotland by Noble (1949) who attributed the source to be infested seed. P. coronafaciens was later reported in England and Wales (Davies, Noble and Norman, 1954) and a subsequent report of the disease in Wales by Griffiths and Peregrine (1956) confirmed the source as infested North American seed. The disease has since been confirmed in Africa (Harder and

Harris, 1973) and West Germany (Muller, 1964). Oat is usually described as the principal host but isolates of P. coronafaciens have been identified from barley, rye, Triticale and a few pasture grasses.

Symptoms

Lesions are first visible on leaf blades as light green, oval spots 4-5mm in diameter which progress to necrotic lesions bordered by a chlorotic margin (Elliott, 1920). The chlorotic margins spread rapidly from the point of infection, the lesions often coalesce and the whole leaf blade may become affected until death of the blade (drying and browning) occurs. Kingsolver (1964) further demonstrated that the pathogen can attack oats from the time the seed ruptures to when the plant is mature. P. coronafaciens has also been associated with crown rot, rosette blight, tiller blight as well as blade blight of winter oats. Roane and Kuriger (1976) have shown that in cool weather bacteria from infected blades may infect the crown via the midrib. The crown rot occurs in late winter and often causes the whole plant to turn orange/brown with death of the plant resulting if the cool weather persists. In warmer temperatures the plant, albeit stunted, often recovers but may fail to head. This has lead to the confusion of attributing some of these symptoms to Barley Yellow Dwarf Virus. Cunfer and Schaad (1976) and Cunfer et al (1978) have recorded a similar multiplicity of symptoms for halo blight of rye. They reported that the range of symptoms seen to occur under field conditions were dependent upon the weather and that P. coronafaciens could

be isolated from haloed, scorched and mildly chlorotic leaves, glumes and florets. Cunfer and co-workers concluded that a failure to recognise all these symptoms may lead to an underestimation of yield losses due to the disease or to an inaccurate diagnosis of the causal agent.

Resistance and control

Chemical control of bacterial diseases is mainly limited to seed treatments. Elliott (1920) achieved partial control of halo blight by treating oat seeds with formaldehyde but a considerable improvement was made by using antibiotics (Griffiths and Peregrine, 1960).

A more promising approach to control is that of breeding for plant resistance. Surveys of North American oat cultivars revealed only a few resistant to halo blight (Caldwell, Mulvey and Compton, 1946; Kingsolver, 1944). Griffiths and Peregrine (1960) confirmed these results and also described a hexaploid oat line Cc4146 (*Avena sativa* x *A. ludoviciana*) as "possessing a type of resistance which approached immunity". The inheritance of resistance to halo-blight in oats was later shown to be governed by a single pair of genes with resistance dominant over susceptibility (Cheng and Roane, 1968; Griffiths and Peregrine, 1964).

## IV OBJECTIVES

The principal aim of this study was to determine the physiological processes underlying the success or failure of pseudomonads to colonize and cause disease symptoms in leaves of oats and other cereals. Interactions between cereals and

pseudomonads were chosen for this work because they had not been studied in any detail at the start of my research programme. The absence of information concerning the interactions meant that it was possible to approach the topic in an entirely objective manner, generalizations which have been made on the resistance of plants to bacteria, based on work on other host/bacterium interactions, were not assumed to apply to the cereals.

Experiments reported in Chapter I were therefore designed to provide novel and essential data on the biology of interactions, including studies on symptom development, bacterial multiplication and the occurrence of protection phenomena in cereals. As a result of the findings of these experiments two aspects of resistance processes were selected for further examination; the attachment of bacteria to oat cell walls, and the occurrence of antibacterial compounds in infected oat leaves. The aims of the ultrastructural study on the binding of bacteria to walls were to examine the value of histochemical staining in studying bacteria *in vivo* and to use selected techniques for a quantitative analysis of the significance of attachment to plant cell walls. Studies on antimicrobial compounds were designed to examine the presence of preformed and induced inhibitors of bacterial growth in oats, and to test the toxicity of the compounds to virulent and avirulent bacteria.

# MATERIALS AND METHODS

## I GENERAL

### 1. Plant Material

#### A. Source of seeds

Seeds of oat cultivars used in experiments were kindly provided by Dr B Clifford, Welsh Plant Breeding Institute, Aberystwyth, and were multiplied at the University of Stirling. Wheat cv. Maris Huntsman and Barley cv. Golden Promise were obtained from the Biology Department, University of Stirling.

Tobacco (Nicotiana tabacum) cv. Burley was kindly provided by Professor H Meidner, Biology Department, University of Stirling. Seeds of French bean (Phaseolus vulgaris) cv. Canadian Wonder and broad bean (Vicia faba) cv. Aquadulce, were obtained from stock at the University Gardens, Stirling.

#### B. Growth of plants

Oat, wheat and barley seeds were sown in 15cm pots of John Innes No 2 compost and raised in greenhouses supplemented with fluorescent lights to maintain a 16h photoperiod. When oat and barley plants were at the Feeke's 10.0 to 10.1 ("in boot") growth stage, and wheat at growth stage 5 (Large, 1954) they were transferred to a growth room ($20^{o}C$, 16h photoperiod, $1.05 \times 10^{4}$lx at soil level) for experiments.

French bean, broad bean and tobacco plants were grown under the same conditions. Tobacco and broad bean plants

were used when between 4 to 6 leaves were fully expanded and French beans when the first trifoliate was just beginning to expand. All plants were watered daily from below.

2. Bacteria and Fungi

A. Origin and maintenance of cultures

Twenty-six isolates of Pseudomonas coronafaciens (Elliott) Stevens and 5 isolates of P. coronafaciens var. atropurpurea were obtained with other plant pathogenic and saprophytic pseudomonads from the National Collection of Plant Pathogenic Bacteria (NCPPB), Plant Pathology Laboratory, Harpenden. Pseudomonas coronafaciens (NCPPB 1348) and P. coronafaciens var. atropurpurea (NCPPB 2397) were used in all experiments unless otherwise stated. Other bacteria used were P. phaseolicola (Burke) Dows. Race 1 (NCPPB 1321), P. fluorescens (Trevison) Mig. (NCPPB 1964), P. syringe van Hall (NCPPB 281), P. morsprunorum Wormold (NCPPB 560) and P. tabaci (Wolf and Foster) Stevens (NCPPB 2706). As a non-indigenous plant pathogen, P. tabaci was used in strict accordance with the conditions stated on the licence (PH 1/1979) issued under the Destructive Pests and Diseases of Plants (Scotland) Order 1966 by the Department of Agriculture and Fisheries for Scotland, Edinburgh.

The bacteria were received in glass ampoules which were scored with a glass cutter before breaking open. Nutrient broth (Oxoid) was added to the ampoule and agitated. The contents were then plated onto nutrient

agar (Oxoid) and incubated at 25°C.

Bacillus megaterium was obtained as a nutrient agar culture from Dr M Horne (University of Stirling).

All bacteria were cultured on nutrient agar plates at 25°C for 24h for experimental purposes. Cultures were maintained on nutrient agar plates and storage media slopes at 4°C.

Cultures of the fungus Cladosporium herbarum were obtained from the Stirling University Culture Collection and grown on Czapek Dox agar at 25°C. Conical flasks (250ml) containing c. 40 ml of the medium were inoculated with sterile distilled water washings from sporulating cultures, the excess water being poured off prior to incubation. A suitably sporulating culture was obtained within 6-12 days.

B. Preparation of bacterial suspensions

Bacterial suspensions were obtained by washing a 24h nutrient agar plate culture with sterile distilled water. The resultant suspension was then washed twice by centrifugation ($5.6 \times 10^3$g for 10 mins). The final pellet was resuspended in sterile distilled water.

The suspensions were then adjusted spectrophotometrically to the desired concentration (Unicam SP1800 Spectrophotometer). The concentration used in all experiments unless otherwise stated was $10^8$ viable cells/ml. Optical densities (OD) were measured at 620nm, 1cm path length and those corresponding to $10^8$ cells/ml are shown in Table M.1. Counts of viable bacteria were determined by plating dilutions of suspensions of known OD onto nutrient agar plus sucrose (5% w/v) and recording the number of resultant colonies after 3d incubation at 25°C.

TABLE M.1. Optical densities of bacterial suspensions in sterile distilled water to yield a concentration of $10^8$ viable cells $ml^{-1}$

| Bacterium | OD (620nm, 1cm path length) |
|---|---|
| *P. coronafaciens* | 0.12 |
| *P. coronafaciens* var. *atropurpurea* | 0.12 |
| *P. phaseolicola* | 0.12 |
| *P. syringae* | 0.12 |
| *P. fluorescens* | 0.10 |
| *P. tabaci* | 0.09 |
| *P. morsprunorum* | 0.13 |

3. Inoculation of plant material

The bacterial suspension was drawn up into a 5ml plastic syringe (sterile, pre-packed) fitted with a fine gauge hypodermic needle (B-D26G ⅜). The adaxial epidermis of the first and second leaves of the cereals was gently pierced and infiltrated until a 2 to 3cm length of leaf appeared watersoaked. Several injections were necessary in order to infiltrate entire leaf sections (Plate A). Fully expanded leaves of tobacco, broad bean and the primary leaves of French bean were infiltrated in the same manner but required only one injection to allow infiltration of approximately 1 $cm^2$ of interveinal tissue (Klement, 1963). Following inoculation leaves were gently washed in a stream of distilled water and appeared normal after 10 to 30 min.

4. Culture Media

A. Nutrient agar

| Contents: | | |
|---|---|---|
| | Oxoid nutrient agar | 28g |
| | Distilled water | 1000ml |

The agar and distilled water were mixed and placed in a steamer until the agar had dissolved. Aliquots were dispensed into glass bottles and autoclaved for 15 min at 1 $kg/cm^2$. Plates were poured when the molten agar had cooled.

B. Nutrient agar plus sucrose (5% w/v)

| Contents: | | |
|---|---|---|
| | Oxoid nutrient agar | 28g |
| | Sucrose (Fisons analytical grade) | 50g |
| | Distilled water | 1000ml |

The constituents were mixed and placed in a steamer until

PLATE A Injection of cereal leaf with bacterial suspension using hypodermic syringe and fine needle.

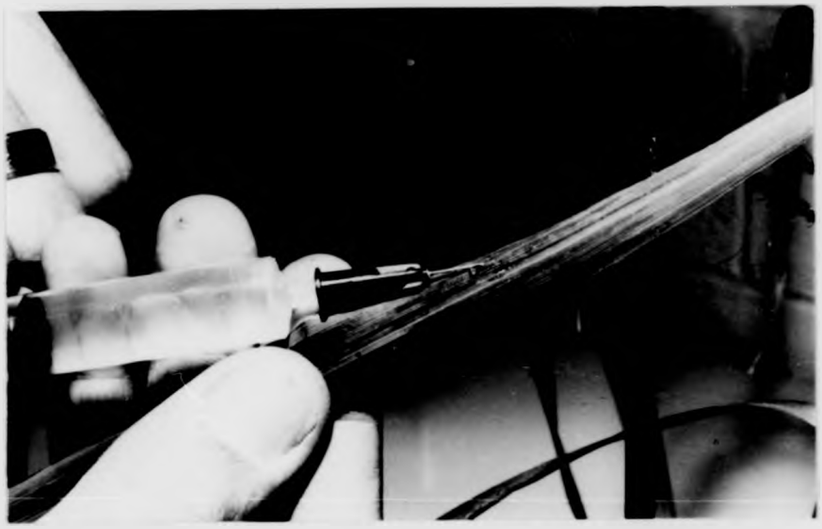


PLATE A Injection of cereal leaf with bacterial suspension using hypodermic syringe and fine needle.

the agar had dissolved. Plates were poured after autoclaving as described above.

C. Nutrient broth

| Contents: | | |
|---|---|---|
| | Oxoid nutrient broth | 13g |
| | Distilled water | 1000ml |

The nutrient broth powder and distilled water were thoroughly mixed, dispensed into glass bottles and autoclaved as above.

D. Storage medium

| Contents: | | |
|---|---|---|
| | Difco yeast extract | 3g |
| | Oxoid Bacteriological Peptone | 5g |
| | Glucose (Fisons, AR Grade) | 5g |
| | $CaCO_3$, powdered | 20g |
| | Oxoid Agar No 3 | 15g |
| | Distilled water | 1000ml |

The constituents were mixed and placed in a steamer until the agar had completely dissolved. Aliquots were dispensed into McCartney screw-top glass bottles and autoclaved as above. The bottles were inclined at a slight angle in order to produce slopes following autoclaving (Anon, 1968).

E. Czapek Dox agar (modified)

| Contents: | | |
|---|---|---|
| | Oxoid Czapek Dox agar (modified) | 45.4g |
| | Distilled water | 1000ml |

The agar and distilled water were placed in a steamer until the former had completely dissolved. Aliquots were dispensed into 250ml conical flasks, stoppered with cotton wool and autoclaved as above.

## II RESPONSES OF CEREAL LEAVES TO BACTERIA

### 1. Assessment of response

Following infiltration of plant leaves with bacterial suspensions, symptoms were recorded daily for 14 days noting the time of appearance, severity or absence of macroscopic symptoms. A simple disease rating index was constructed with which bacteria and cereal cultivars could be later assessed. The disease index is described in detail in Table 1.1 in Chapter 1 of the results.

### 2. Estimation of Bacterial populations in leaves

The numbers of bacteria in leaves were estimated by sampling leaf discs cut from infiltrated areas using a cork borer (4mm diameter). Three discs were cut from each leaf and ground by hand with a ground glass homogenizer (Quickfit) in 3ml sterile distilled water. A 10-fold dilution series of the homogenate was prepared and 0.2ml volumes spread using a bent glass rod on plates of nutrient agar plus sucrose (5% w/v) (Lyon and Wood, 1976). Colonies were counted from 3 replicate plates prepared from each dilution after 3 days incubation at 25°C. These counts were converted to log number bacteria/$cm^2$ leaf.

### 3. Characteristics of the hypersensitive response (HR) and normosensitive collapse

#### A. Estimation of induction periods

Solutions of the antibiotic streptomycin (Streptomycin sulphate, Sigma Chemical Company) was injected at hourly intervals into sites previously infiltrated with bacterial

suspensions and the appearance or absence of response recorded (Klement, 1971). A concentration series of streptomycin was prepared and assayed against suspensions of bacteria and the antibiotic was found to be most effective at 500µg/ml. This concentration was selected for use in all experiments.

B. Electrolyte leakage

Four 1cm diameter leaf discs were cut from infiltrated areas and placed in 5ml de-ionized water in glass vials kept in a water bath at 20°C. Conductivity of the bathing solution was measured immediately and again after 6h using a conductivity electrode (K=1.0) and meter (Portland Electronics Model P310). The increase in conductivity (µmhos) was calculated as an indication of electrolyte leakage.

4. Prevention of hypersensitive and normosensitive collapse

A. Preparation of heat-killed cells

Dense suspensions of c. $5 \times 10^9$ cells/ml were obtained by the method previously described and heat-killed by placing them in a water bath at 100°C for 15 min. Direct plating of aliquots onto nutrient agar plus sucrose (5% w/v) confirmed the efficacy of the treatment.

B. Infiltration of plant leaves

Heat-killed bacterial suspensions were injected into plant leaves by the technique described previously. Viable suspensions of $10^8$ bacteria/ml were then injected into these sites at subsequent intervals under a range of experimental conditions.

C. Assessment of response

Treated leaves were examined daily and the degree of symptom expression recorded in terms of the percentage of leaf area affected. It was thus possible to describe and assess the responses as percentage protection against hypersensitive or normosensitive collapse in comparison with untreated control sites.

III ULTRASTRUCTURAL STUDIES

1. Preparation of material for electron microscopy

A. Chemicals

Chemicals were either of electron microscopy or analytical grade. Correct procedures were adopted in the handling and storage of the highly toxic chemicals used.

B. Fixation, dehydration and embedding

Infiltrated areas were cut from leaves and small areas of tissue c. $2mm^2$ were sampled using a sharp razor blade. The fixature used was 2% glutaraldehyde in 50mM sodium cacodylate buffer, pH 7.2 (Sabatini et al, 1963). The tissue was infiltrated with fixative, i) by vacuum infiltration then left at c. 20°C for 2-3h, ii) by rotation (Taab rotator) for 3-4h at c. 20°C using muslin plugs to ensure submersion of tissue in fixative. Following fixation the tissue was rinsed in 50mM sodium cacodylate buffer, pH 7.2 for 1h. The tissue was then fixed in 2% $OsO_4$ in 50mM sodium cacodylate buffer, pH 7.2 overnight (c. 16h).

Following fixation the tissue was rinsed in 50mM sodium cacodylate buffer, pH 7.2 for 1h prior to dehydration

in a series of increasing EtOH (BDH Chemicals) concentrations. Propylene oxide was used as the link agent between the dehydration and embedding of the tissue.

Tissue was finally embedded in epoxy (low viscosity) resin (EMscope Laboratories Limited, EMIX resin kit). The procedures described are outlined in Fig. M.1

C. Sectioning

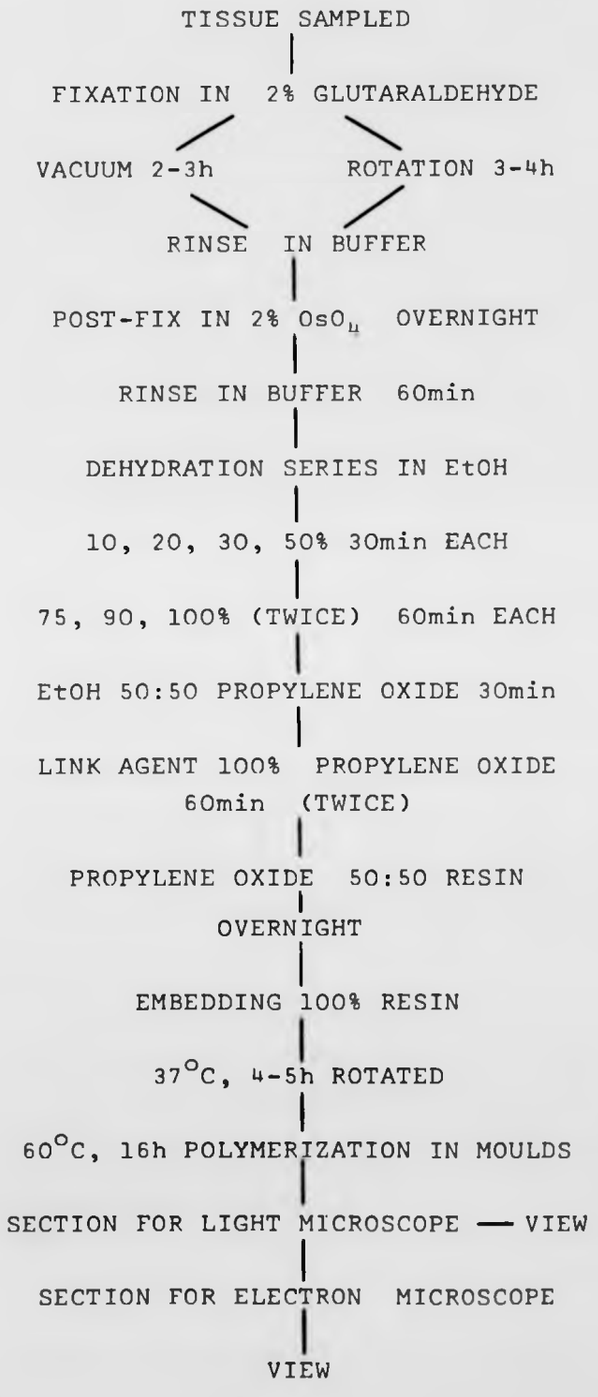
(i) For light microscope

Resin blocks containing tissue were trimmed to an appropriate size using a small hacksaw blade and razor blade prior to sectioning. Thin sections c. 2µm thick were then cut using an LKB Pyramitome 11800 fitted with a glass knife. Resultant sections were carefully removed from the blade and placed in a drop of distilled water on a clean, grease-free glass slide. After drying on a hot plate, sections were stained with toluidine blue (see Materials and Methods III 2A) and viewed under the light microscope (Nikon x 40 objective, x10 eyepieces). Areas for viewing in the electron microscope were then selected and the block face trimmed with a sharp razor blade to leave the desired area on a raised "mesa" to facilitate the cutting of ultrathin sections.

(ii) For electron microscope

Ultrathin sections c. 90mµ thick were cut with glass knives using an LKB Ultratome III. The sections collected as ribbons in a plastic trough filled with clean distilled water fitted to the knife using dental wax. The ribbons of ultrathin sections were collected on uncoated 400 mesh copper grids (unless stated otherwise) and allowed to dry.

FIGURE M.1 Preparation of plant material for the electron microscope.



After staining the sections were viewed under transmission electron microscope (Jeol Jem 100C transmission electron microscope, accelerating voltage 60-80 kv).

2. Staining and cytochemistry

A. Toluidine blue

Content: 1% w/v dissolved in 1% aqueous borax solution. Sections on glass slides were covered with the stain for 2 min, rinsed with distilled water and dried. Bacteria were stained dark blue within the intercellular spaces (see Appendix 1).

B. Uranyl acetate/lead citrate

(i) Uranyl acetate. Content: 2% w/v dissolved in clean distilled water and stored at $4^{o}C$. Used by itself, uranyl acetate gives a general enhancement of contrast in tissues fixed with $OsO_4$ (Glauert, 1965).

(ii) Lead citrate. This was prepared by the method of Reynolds (1963) as follows:

a) a solution of $Pb(NO_3)_2$ (1.33g), $Na_3(C_6H_5O_7).2H_2O$ (1.76g) in 30ml distilled water was mixed in a 50ml volumetric flask.

b) The solution was shaken vigorously for 1 min and then allowed to stand for 30 min to ensure the complete conversion of lead nitrate to lead citrate.

c) Carbonate-free N-NaOH (8ml) was then added and the solution made up to 50ml with distilled water and mixed by inversion. The lead citrate dissolved and the solution was ready for use (pH 12.0).

Lead citrate singularly or in conjunction with uranyl acetate

provides a general increase in contrast.

(iii) Staining of sections on copper grids was achieved by placing the grids (sections on stain) on a drop of uranyl acetate maintained on a grease-free Parafilm strip in a Petri dish for 30 min. The grids were then removed, flushed with distilled water and dried before being placed on a drop of lead citrate stain maintained in the same manner as above. Pellets of NaOH were included in the Petri dish with lead citrate in an attempt to absorb any free carbonate which would otherwise be readily absorbed by the lead citrate and thus impair staining. After 30 min on lead citrate the grids were removed and flushed sequentially with distilled water, 0.02M NaOH and finally distilled water before drying.

C. Silver proteinate

Ultrathin sections mounted on uncoated 400 mesh gold grids were immersed in 1% periodic acid for 30 min, washed with distilled water and placed in 0.2% (w/v) thiocarbohydrazide (TCH, Agar Aids) in 20% acetic acid for 2, 12, 24, 48 and 72h. After washing in series of 15, 10 and 5% acetic acid solutions (10 min each) the grids were then washed in distilled water for 30 min before placing in an aqueous 1% solution of silver proteinate (Biological stain, Agar Aids) in the dark for 30 min. The sections were finally washed twice for 10 min in distilled water before drying and viewing. Controls included were the ommission of (a) periodic acid oxidation, (b) thiocarbohydrazide, (c) silver proteinate (Hickey and Coffey), 1978) and (d) the oxidation of sections with 5-10% $H_2O_2$ instead of

periodic acid (Scannerini and Bonfante-Fasolo, 1979).

Silver proteinate staining is derived from the periodic acid-Schiff (PAS) stain and enables demonstration of polysaccharides with free glycol groups (Roland, 1978). The reactions involved are summarized in Fig M.2.

D. Ruthenium red

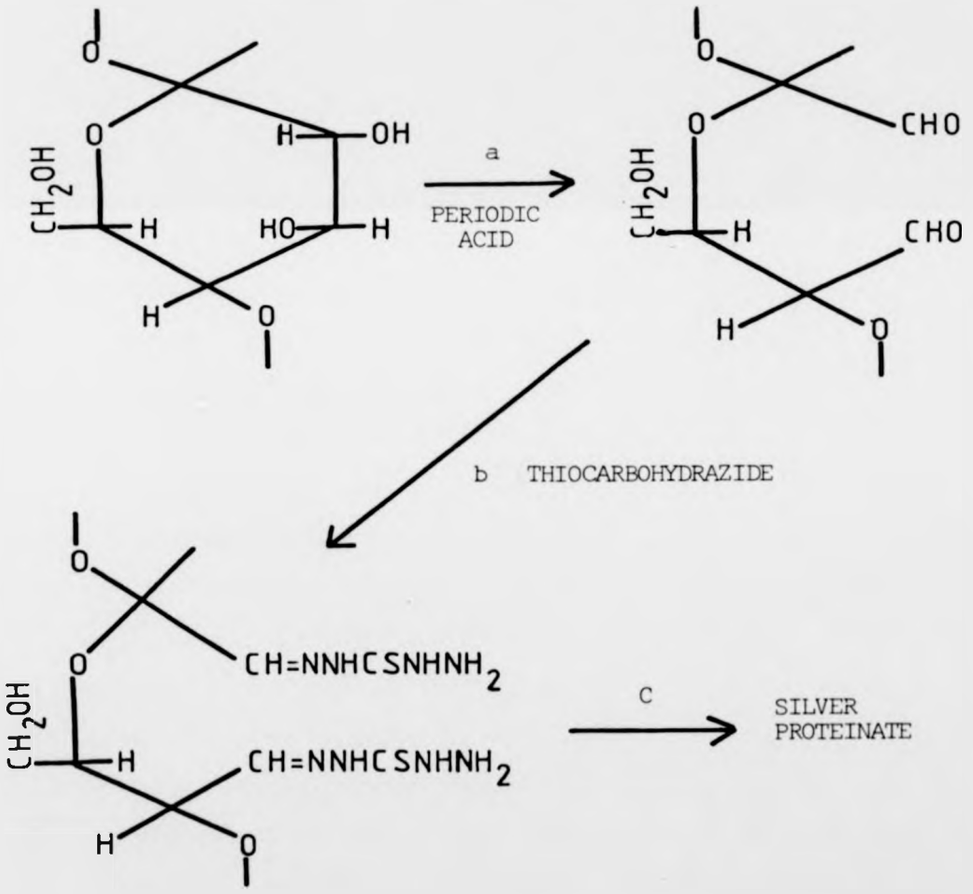
The staining procedure adopted was a modified version of that described by Luft (1971), ruthenium red stain (BDH Chemicals) applied during fixation. Tissue was fixed as previously described in 2% glutaraldehyde in buffer containing 0.05-0.1% ruthenium red (5-10 mg/10ml) and then rinsed in buffer containing 0.05-0.1% ruthenium red. Finally, the tissue was post-fixed in $OsO_4$ containing 0.05-0.1% ruthenium red before dehydration and embedding in the way previously described. Sections required no further staining but uranyl acetate / lead citrate was found to enhance contrast considerably.

Ruthenium red is an inorganic, intensely coloured compound used traditionally as a stain for pectins in plant anatomy. The presence of $OsO_4$ is essential to enhance its poor electron density by producing dense deposits but maintaining the affinity of the dye for acidic polysaccharides (Luft, 1971; Blanquet, 1976 a:b).

3. Assessment of bacterial attachment to plant cell walls

Bacteria were counted from single sections viewed in the electron microscope and assigned to different categories on the basis of the interaction observed as described in the results. At least 100 bacteria were counted from

FIGURE M.2 Visualization of oxidised polysaccharides by silver proteinate staining (after Roland, 1978). (a) Oxidation of glycols (b) Condensation of aldehydes (c) Final visualization



repeat tissues viz. independant innoculation and fixation. The data were analysed by a $X^2$ test.

## IV THE PRODUCTION OF ANTIMICROBIAL COMPOUNDS BY OAT LEAVES

### 1. Chemicals

All solvents used were of analytical grade except for acetone and hexane which were of laboratory reagent grade.

### 2. Preparation of oat leaf extracts

Care was taken to allow minimal exposure of extracts to light and high temperatures (>35$^{o}$C).

Oat leaf extracts were prepared by a modified facilitated diffusion procedure (Keen, 1978). Infiltrated areas were cut from leaves previously inoculated with the test bacteria, quickly weighed and placed in a 250ml conical flask containing 40% aqueous ethanol (15ml solvent/g fresh weight tissue). The tissue was infiltrated under vacuum and the flasks stoppered with cotton wool plugs before being placed on a rotary shaker (110r.p.m., 25$^{o}$C) for 24h. The extract was then filtered and extracted twice with ethyl acetate. The pooled ethyl acetate fractions were dehydrated with $MgSO_4$ and taken to dryness under vacuum. Residues were resuspended in 100% ethanol (1ml ≡ 4g fresh weight of tissue), transferred to vials and stored at -20$^{o}$C. The aqueous phases remaining after partition with ethyl acetate were freeze-dried and stored at -20$^{o}$C until required when the residues were resuspended in sterile distilled water (1ml water was added to the extract from 4g fresh weight of tissue).

3. Thin layer chromatography (TLC)

All TLC was conducted using precoated plates (Merck, silica gel 60 $F_{254}$, 0.25mm thick). Volumes of samples representing the extract from 0.4g fresh tissue were applied to plates using drawn out Pasteur pipettes. Chromatograms were developed in hexane/acetone (2:1) in chromatography tanks lined with tissue paper soaked in the solvent. Plates were then air dried and bands located by examination under UV radiation at 254 or 366nm (Camag Universal Lamp). Any bands detected were marked with pencil prior to bioassay.

4. Bioassay Media

A. Sensitest agar

| Contents: | Oxoid Sensitest agar | 32g |
|---|---|---|
| | Distilled water | 1000ml |

The agar and distilled water were mixed and placed in a steamer until the agar had dissolved prior to dispensing into glass bottles. The agar was autoclaved for 15 min at $1kg/cm^2$ ($121^oC$) and allowed to cool before pouring plates.

B. Water agar (0.7% w/v)

| Contents: | Oxoid Agar Bacteriological (Agar No 1) | 7g |
|---|---|---|
| | Distilled water | 1000ml |

The agar and distilled water were mixed and placed in a steamer until the agar had dissolved. Approximately 90ml was decanted into 100ml conical flasks and autoclaved. Cooled agar was kept molten in a water bath at $45^oC$ until required.

C. Peptone water (0.1% w/v)

| Contents: | | |
|---|---|---|
| | Bacteriological peptone | 1g |
| | NaCl | 5g |
| | Distilled water | 1000ml |

The constituents were thoroughly mixed and dispensed into glass bottles before autoclaving.

D. Czapek Dox liquid medium

| Contents: | | |
|---|---|---|
| | Oxoid Czapek Dox liquid medium | 33.4g |
| | Distilled water | 1000ml |

The constituents were thoroughly mixed and dispensed into glass bottles before autoclaving.

5. Bioassay techniques

A. Antibacterial TLC plate bioassay

Thin layer chromatograms of extracts, dried after developing, were sprayed with a dense suspension (c. $5 \times 10^9$ bacteria/ml) of the test bacterium in nutrient broth (Shandon Laboratory Spray Gun). The sprayed plates were dried in a stream of cold air just sufficient to remove any film of water and give a translucent appearance before being incubated at 25°C overnight in plastic boxes lined with filter paper to maintain a high humidity. After incubation the plates were removed and dried until opaque before being sprayed immediately with aesculin spray (2g aesculin [BDH], 1g ammonium ferric citrate, 5g Difco yeast extract in 1000ml distilled water). The plates were then re-incubated as before to allow hydrolysis of the aesculin to occur (Lund and Lyon, 1975).

The hydrolysis of aesculin resulted in the development

of a yellow/brown colour, the zones of inhibition of bacterial growth remaining colourless.

B. Antifungal TLC bioassay

Antifungal compounds were detected on thin layer chromatograms of extracts by the technique described by Klarman and Sanford (1968). A dense suspension of Cladosporium herbarum spores was prepared by adding c. 20ml sterile Czapek Dox liquid medium with a drop of Triton X-100 as a wetting agent to sporulating cultures and the surface disturbed using a glass rod. The resultant suspension was filtered through muslin before spraying onto a developed TLC plate. The sprayed plate was then incubated in a humid box at 25°C for 4 days. The presence of antifungal compounds was revealed as areas of white silica gel where the dark green fungus had failed to grow.

C. Seeded agar plate bioassay

Ten ml of a dense suspension of test bacteria in sterile distilled water were added to 90ml molten (45°C) 0.7% (w/v) water agar in a 100ml conical flask and mixed. The seeded soft agar was then poured over a layer of previously set Sensitest agar and allowed to set. Antibiotic assay discs (Millipore 10mm diam.) loaded with 50µl of the test extract (representing the extract from 0.2g fresh tissue) and allowed to dry were then gently placed on the seeded agar surface. This procedure was a modification of that used by Gnanamanickam and Smith (1980). Alternatively, 10µl drops (representing the extract from 0.04g fresh tissue) of extracts were applied directly to the seeded agar surface (Wyman and Van Etten, 1978). Plates were then incubated at 30°C and

the occurence and size of inhibition zones in the bacterial lawn were recorded.

D. Liquid culture bioassay

The antibacterial effect of oat leaf extracts was assayed by a liquid culture assay based on growth of the test bacterium in 0.1% peptone water (Lyon and Bayliss, 1975). Two hundred and fifty µl aliquots representing the extract from 1g fresh tissue were pipetted into small sterile test-tubes and ethyl acetate fractions were taken to dryness and resuspended in 20µl EtOH. Aqueous extracts were not taken to dryness. Peptone water (0.1%) suspensions of test bacteria were adjusted to $10^8$ to $10^9$ bacteria/ml and added to the test solutions to give a final volume of 1ml and the liquid cultures were then incubated for 24h at 30°C with periodic agitation. Ten-fold dilution series in 0.1% peptone water were prepared from the liquid cultures at 0h and 24h and 0.1ml volumes of the appropriate dilutions plated onto nutrient agar plus sucrose (5% w/v). Colonies were counted after 3d incubation at 30°C and the number of viable bacteria/ml calculated to assess any effects of the extracts on bacterial viability and growth over the incubation period of 24h.

# EXPERIMENTAL WORK AND RESULTS

## CHAPTER 1

## THE RESPONSES OF CEREAL LEAVES TO INOCULATION WITH BACTERIA

In order to classify the types of interactions between cereal leaves and bacteria several phytopathogens and a saprophyte were inoculated into leaves of oats, wheat and barley and symptom development recorded. Representative interactions were subsequently selected for detailed examination.

### 1. Symptom development

#### A. P. coronafaciens and P. coronafaciens var. atropurpurea isolates.

Twenty-six isolates of P. coronafaciens and 5 isolates of P. coronafaciens var. atropurpurea were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB) and suspensions of $10^8$ bacteria/ml were injected into leaves of oat cv. Milford, barley cv. Golden Promise and wheat cv. Maris Huntsman. An additional oat plant, line Cc4146, reported as possessing a high degree of resistance to P. coronafaciens (Griffiths and Peregrine, 1960) was also tested. Symptoms were recorded from at least 2 sites on the first and second leaves of two plants.

#### (i) Observations

Oat cv. Milford was susceptible to 25 isolates of P. coronafaciens tested, watersoaking and collapse of the infiltrated area (normosensitive collapse) occurring by 3 days after inoculation. This was followed by confluent necrosis of the infiltrated area by 4 to 5 days when

chlorosis began to spread towards the tip of the leaf blade (Plate 1.1a). Leaves often became totally chlorotic and desiccated above the infiltration site by 10 days after inoculation.

A localized HR developed in response to P. coronafaciens NCPPB isolate 1327 which originated from rye (Secale cereale). This HR was expressed by the collapse of inoculation sites between 24 and 36h followed by desiccation 3 to 4 days after inoculation. The rye isolate of P. coronafaciens also caused a similar response in other cereals tested. Oat line Cc4146 and to a lesser degree barley, proved moderately susceptible to the other isolates of P. coronafaciens, necrosis and yellowing progressing beyond infiltrated areas by 7 days after inoculation. In wheat leaves, the area infiltrated with P. coronafaciens isolates, other than NCPPB 1327, became very pale yellow by 4 days after inoculation. The yellowing sympton became more pronounced with time but remained distinctly localized to the infiltrated area (Plate 1.1b). There was no indication of tissue collapse in the manner of the HR observed with isolate 1327.

The five isolates of P. coronafaciens var. atropurpurea tested caused a typical HR in all of the cereal leaves. Infiltrated tissue became glazed by 18h after inoculation and collapsed forming a distinctly localized, pale brown lesion between 1 and 3 days (Plate 1.1c).

(ii) Disease symptom key

A ten-point disease sympton key was constructed from the symptom records based on the reaction of the susceptible oat cv. Milford to the P. coronafaciens isolates. The key

PLATE 1.1 Symptoms produced in response to c. $10^8$ bacteria/ml sterile distilled water in (a) oats cv. Milford inoculated 5 days previously with P. coronafaciens (NCPPB 1348), note collapsed infiltrated area and spreading chlorosis at blade margins; (b) resistant reaction of wheat cv. Maris Huntsman, yellowing localised to infiltrated area 7 days after inoculation with P. coronafaciens (NCPPB 1348) and (c) the hypersensitive collapse of the infiltrated area of oat cv. Milford 5 days after inoculation with P. coronafaciens var. atropurpurea (NCPPB 2397).

c. $10^8$
ter in
5 days
(NCPPB
d area

of wheat
calised
inoculation
48) and
of the
ford 5 days
afaciens



(c)

(b)

(a)

PLATE 1.1

c. $10^8$
ter in
5 days
(NCPPB
d area
e
of wheat
calised
inoculation
48) and
of the
ford 5 days
afaciens



(c)

(b)

(a)

PLATE 1.1

includes symptoms both in and beyond the infiltrated area (Table 1.1, Plate 1.2). The key was used for a quantitative assessment of the development of the susceptible symptoms where These occurred in cereals 3 and 7 days after inoculation with P. coronafaciens and P. coronafaciens var. atropurpurea (Table 1.2). Application of symptom key rating clearly demonstrated the ability of all but one P. coronafaciens isolate to induce disease in susceptible oat leaves. Pathogenic isolates varied in virulence. Oat line Cc4146 and barley were clearly moderately susceptible to artificial inoculation with P. coronafaciens, whereas wheat was resistant but expressed the HR in only one instance. P. coronafaciens var. atropurpurea, as previously noted, induced an HR in each of the cereals.

B. Differential response of certain oat cultivars to P. coronafaciens.

The response of 10 oat cultivars to inoculation with P. coronafaciens (NCPPB 1348) was classified by means of the sympton key. Milford was the most susceptible cultivar tested, line Cc4146 was moderately susceptible but only Clinton showed a marked resistance to P. coronafaciens. The other cultivars were all susceptible (Table 1.3).

It was as a result of these experiments that the combination of oat cv. Milford and P. coronafaciens isolate 1348 was chosen for further studies on the susceptible response. Similarly, P. coronafaciens var. atropurpurea was chosen for its ability to induce the HR in cereal leaves.

TABLE 1.1 Disease symptom key expressing response of oat cv. Milford leaves to inoculation with P. coronafaciens

| Disease Rating [a] | | Description of symptoms [b] |
|---|---|---|
| 1 | ia | No symptoms |
| | sa | No symptoms |
| 2 | ia | Patchy watersoaking |
| | sa | No symptoms |
| 3 | ia | Patchy chlorosis with watersoaking |
| | sa | No symptoms |
| 4 | ia | Coalescing chlorosis and brown flecks |
| | sa | No symptoms |
| 5 | ia | Pale brown lesions with slight chlorosis |
| | sa | No symptoms |
| 6 | ia | Pale brown lesions with chlorotic margins, discrete haloes |
| | sa | No symptoms |
| 7 | ia | Coalescing haloes (chlorotic with brown patches) |
| | sa | Marginal chlorosis to i.a. |
| 8 | ia | Collapse and desiccation |
| | sa | Spreading chlorosis, at blade margins especially |
| 9 | ia | collapse and desiccation |
| | sa | Extreme spreading chlorosis with brown patches |
| 10 | ia | Collapse and desiccation |
| | sa | Collapse and desiccation |

[a] On scale 1-10, each stage need not necessarily precede the next.

[b] Based largely on the reaction of oat cv. Milford to a suspension of $10^8$ bacteria/ml of P.coronafaciens (NCPPB 1348). Symptom development in the infiltrated area (ia) and area surrounding the infiltrated area (sa) were noted.

PLATE 1.2 The response of oat cv. Milford to inoculation with a suspension c. $10^8$ bacteria/ml sterile distilled water of *P. coronafaciens* (NCPPB 1348) (a) 2; (b) 4; (c) 7; (d) 9 and (e) 10 days after inoculation. Note the progression from collapsed and watersoaked infiltrated area (a) through desiccation (b) to spreading chlorosis (c) and (d). By 10d (e) chlorosis and necrosis are widespread, the former symptom affecting the whole blade above the infiltrated area.

to
$10^8$
ter of
a) 2;
days
ogression
nfiltrated
) to
. By 10d
widespread,
whole
a.

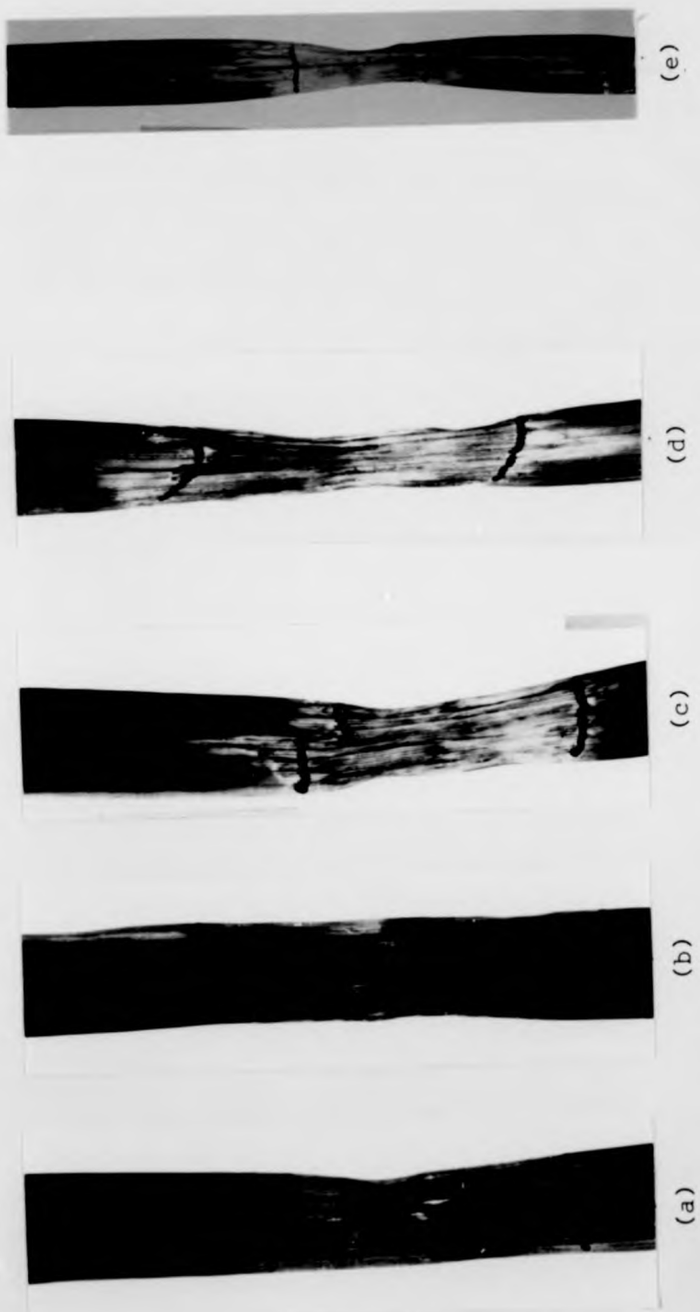



PLATE 1.2

to
$10^8$
ter of
a) 2;
days
ogression
nfiltrated
) to
). By 10d
widespread,
e whole
a.




PLATE 1.2

TABLE 1.2 Disease symptom rating for isolates of P. coronafaciens and P. coronafaciens var. altropurpurea inoculated into leaves of oats, barley and wheat

| Isolate (NCPPB) | Host origin [a] | Disease symptom rating [b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Oat | | | | Barley | | Wheat | |
| | | cv. Milford | | line Cc4146 | | cv. Golden Promise | | cv. Maris Huntsman | |
| | | 3d | 7d | 3d | 7d | 3d | 7d | 3d | 7d |
| P. coronafaciens | | | | | | | | | |
| 197 | | 4 | 8 | 3 | 7 | 3 | 6 | [c] | - |
| 600 | | 3 | 8 | 2 | 6 | 2 | 6 | - | - |
| 874 | | 4 | 8 | 1 | 5 | 1 | 1 | - | - |
| 876 | | 1 | 5 | 1 | 1 | 1 | 1 | - | - |
| 1253 | | 5 | 7 | 4 | 6 | 1 | 4 | - | - |
| 1327 | Secale cereale | HR [d] | HR | HR | HR | HR | HR | HR | HR |
| 1348 | | 3 | 10 | 2 | 6 | 2 | 7 | - | - |
| 1349 | | 2 | 3 | 2 | 3 | 1 | 3 | - | - |
| 1350 | | 3 | 10 | 3 | 9 | 3 | 6 | - | - |
| 1351 | | 3 | 8 | 3 | 7 | 1 | 6 | - | - |
| 1352 | | 3 | 9 | 3 | 7 | 3 | 6 | - | - |
| 1353 | | 3 | 10 | 3 | 9 | 1 | 6 | - | - |
| 1354 | | 2 | 9 | 2 | 8 | 2 | 7 | - | - |
| 1355 | | 3 | 10 | 3 | 9 | 3 | 9 | - | - |
| 1356 | | 3 | 7 | 3 | 6 | 3 | 6 | - | - |
| 1357 | | 3 | 10 | 2 | 7 | 2 | 7 | - | - |
| 2395 | | 3 | 10 | 2 | 7 | 3 | 7 | - | - |
| 2419 | | 3 | 8 | 2 | 7 | 2 | 6 | - | - |
| 2420 | | 3 | 7 | 3 | 6 | 3 | 6 | - | - |
| 2481 | | 3 | 7 | 3 | 6 | 3 | 6 | - | - |
| 2680 | | 3 | 7 | 3 | 7 | 1 | 4 | - | - |
| 2681 | | 3 | 10 | 3 | 7 | 2 | 5 | - | - |
| 2707 | S. cereale | 2 | 6 | 1 | 3 | 2 | 4 | - | - |
| 2714 | Triticale | 1 | 3 | 1 | 3 | 1 | 3 | - | - |
| 2816 | | 3 | 7 | 3 | 5 | 1 | 4 | - | - |
| 2823 | | 3 | 10 | 2 | 5 | 1 | 1 | - | - |
| P. coronafaciens var. atropurpurea | | | | | | | | | |
| 1328 | Bromus sp. | HR | HR | HR | HR | HR | HR | HR | HR |
| 1768 | Agrotis sp. | HR | HR | HR | HR | HR | HR | HR | HR |
| 1769 | Arrhenatherum elatius | HR | HR | HR | HR | HR | HR | HR | HR |
| 2396 | Westerwold rye grass | HR | HR | HR | HR | HR | HR | HR | HR |
| 2397 | Lolium multiflorum | HR | HR | HR | HR | HR | HR | HR | HR |

[a] Avena sativa unless stated; [b] Mean recorded from at least 2 sites, 3d and 7d after inoculation with $10^8$ bacteria/ml; [c] Resistant symptom (See Text sections 1.1A(i) and 1.1C for description); [d] Hypersensitive response.

TABLE 1.3 The response of different oat cultivars to inoculation with P. coronafaciens (NCPPB 1348)

| Cultivar | Disease symptom rating [a] 3d | 7d |
|---|---|---|
| Milford | 3 | 10 |
| Cc4146 | 2 | 6 |
| Cc4346 | 3 | 8 |
| Siluria | 2 | 8 |
| Orlando | 3 | 8 |
| Mandarin | 3 | 8 |
| Nelson | 3 | 7 |
| Maris Oberon | 3 | 8 |
| Maris Tabard | 3 | 8 |
| Clinton | 1 | 3 |

[a] Mean rating recorded from 5 sites, 3 and 7d after inoculation with a suspension of $10^8$ bacteria/ml.

C. Responses of cereal leaves to a range of phytopathogenic and saprophytic bacteria

The plant pathogens P. morsprunorum, P. phaseolicola and P. tabaci did not induce the HR in cereals, causing only a pale yellowing of the infiltrated area which appeared 3 to 4 days after inoculation. This response, like that of wheat to P. coronafaciens was categorized as a resistant symptom to distinguish it from the typical HR elicited by P. coronafaciens var. atropurpurea (Plate 1.3a, b). By contrast, the P. syringae isolate tested (ex. Syringa vulgaris) caused an HR very similar to that elicited by P. coronafaciens var. atropurpurea in each cereal (Plate 1.3c). No symptoms developed following inoculation with the saprophyte P. fluorescens or sterile distilled water alone (Plate 1.3d).

The responses of the cereal leaves to the pseudomonads tested are summarized in Table 1.4.

D. The effect of inoculum concentration on symptom development

(i) Susceptible (normosensitive) response

The resistance of oat line Cc4146 and also barley to P. coronafaciens was more clearly expressed when concentrations were reduced to $10^7$ or $10^6$ bacteria/ml. At an inoculum concentration of $10^7$ bacteria/ml many lesions produced in Cc4146 and barley were localized as necrotic patches surrounded by pale yellow haloes within infiltrated areas which did not develop confluent watersoaking or necrosis. No symptoms developed in Cc4146 or barley following inoculation with $10^6$ bacteria/ml.

PLATE 1.3 Symptoms produced 5 days after inoculation with c. $10^8$ bacteria/ml sterile distilled water in oat cv. Milford by (a) P. morsprunorum, (b) P. tabaci, (c) P. syringae and (d) P. fluorescens. Note the paling of the infiltrated area in the resistant symptoms (a) and (b) strictly localized to the infiltrated area as is the hypersensitive collapse seen in (c). The saprophyte induced no symptoms (d).

inoculation
e distilled

tabaci,
orescens.
rated area
) and (b)
iltrated area
apse seen in
no symptoms




PLATE 1.3

r inoculation
le distilled

tabaci,
uorescens.
rated area
) and (b)
iltrated area
lapse seen in
d no symptoms

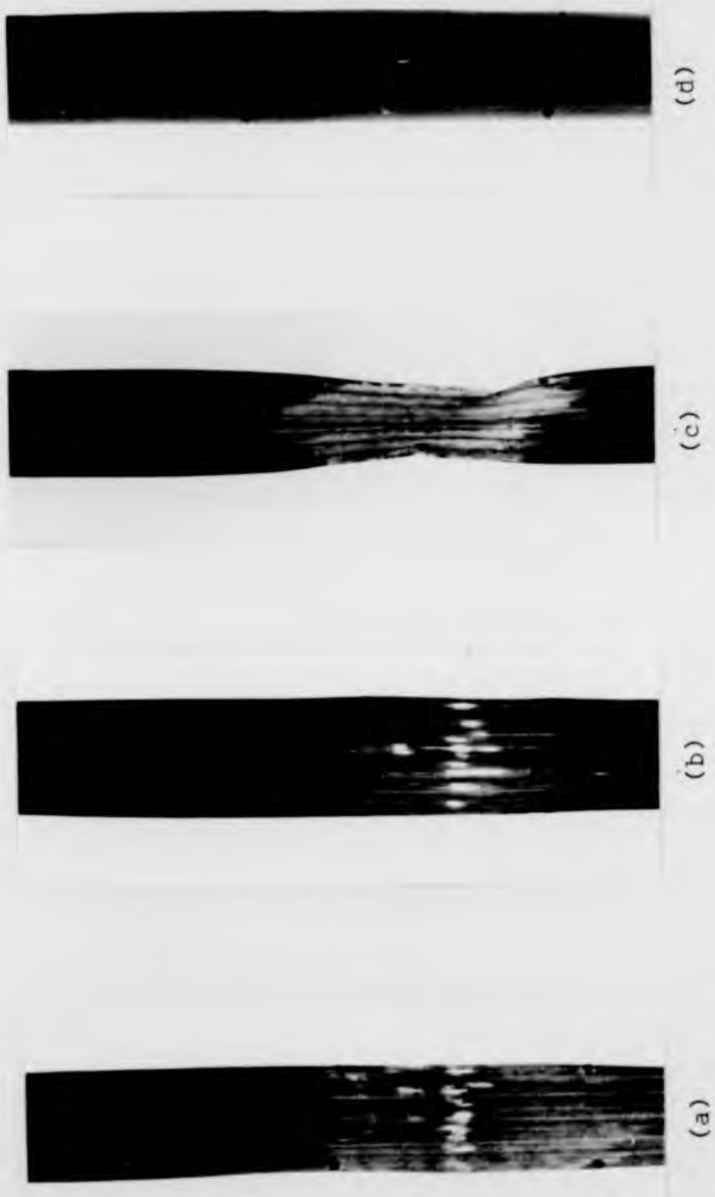



PLATE 1.3

TABLE 1.4 Reactions of oats, barley and wheat to pseudomonads following artificial inoculation with a suspension of c. $10^8$ bacteria/ml in sterile distilled water.

| Bacterium | Reaction in cereal leaves | | | |
|---|---|---|---|---|
| | Oats | | Barley | Wheat |
| | cv. Milford | line Cc4146 | | |
| *P. coronafaciens* | S [a] | MS [a] | MS | RR |
| *P. coronafaciens* var. *atropurpurea* | HR [a] | HR | HR | HR |
| *P. fluorescens* | NS [a] | NS | NS | NS |
| *P. morsprunorum* | RS [a] | - [b] | RS | RS |
| *P. phaseolicola* | RS | RS | RS | RS |
| *P. syringae* | HR | - | HR | HR |
| *P. tabaci* | RS | - | RS | RS |

[a] S = susceptible, MS = moderately susceptible, RS = resistant symptom, HR = hypersensitive response, NS = No symptoms.

[b] Not tested.

Symptom appearance and the development of spreading lesions in oat cv. Milford were merely delayed at the lower inoculum concentrations.

(ii) Hypersensitive response (HR)

A concentration of $5 \times 10^6$ bacteria/ml was required for the induction of a confluent HR in oat leaves by P. coronafaciens var. atropurpurea (Plate 1.4). At $10^6$ bacteria/ml this bacterium produced either localized patches of necrosis within infiltrated areas or no macroscopically visible symptoms. No symptoms were produced by P. morsprunorum, P. phaseolicola or P. tabaci at $10^7$ or fewer bacteria/ml. Increasing bacterial numbers to $10^9$/ml sometimes enhanced the yellowing of areas infiltrated with the phytopathogens causing a resistant symptom but always failed to cause tissue collapse or desiccation even by 10 days after inoculation.

2. Bacterial populations in leaves following inoculation

The changes in bacterial numbers in leaves following inoculation were determined from at least 2 repeated experiments and are expressed as log number bacteria/$cm^2$ leaf in Figs. 1.1-1.7. The most detailed studies were conducted on oats with pertinent comparisons made in wheat and barley.

A. Oats

The development of disease symptoms in oat cv. Milford by P. coronafaciens was closely correlated with bacterial multiplication (Fig. 1.1). Watersoaking and normosensitive collapse of the infiltrated area occurred when the pathogen


PLATE 1.4 The effect of inoculum concentration on expression of the hypersensitive response (HR) elicited by P. coronafaciens var. atropurpurea (2397) in oat cv. Milford. A confluent browning of the infiltrated area was noted only for suspensions of c.$5 \times 10^6$ bacteria/ml or greater; severity of the HR was proportional to inoculum concentration.

PLATE 1.4 The effect of inoculum concentration on expression of the hypersensitive response (HR) elicited by P. coronafaciens var. atropurpurea (2397) in oat cv. Milford. A confluent browning of the infiltrated area was noted only for suspensions of c.$5 \times 10^6$ bacteria/ml or greater; severity of the HR was proportional to inoculum concentration.

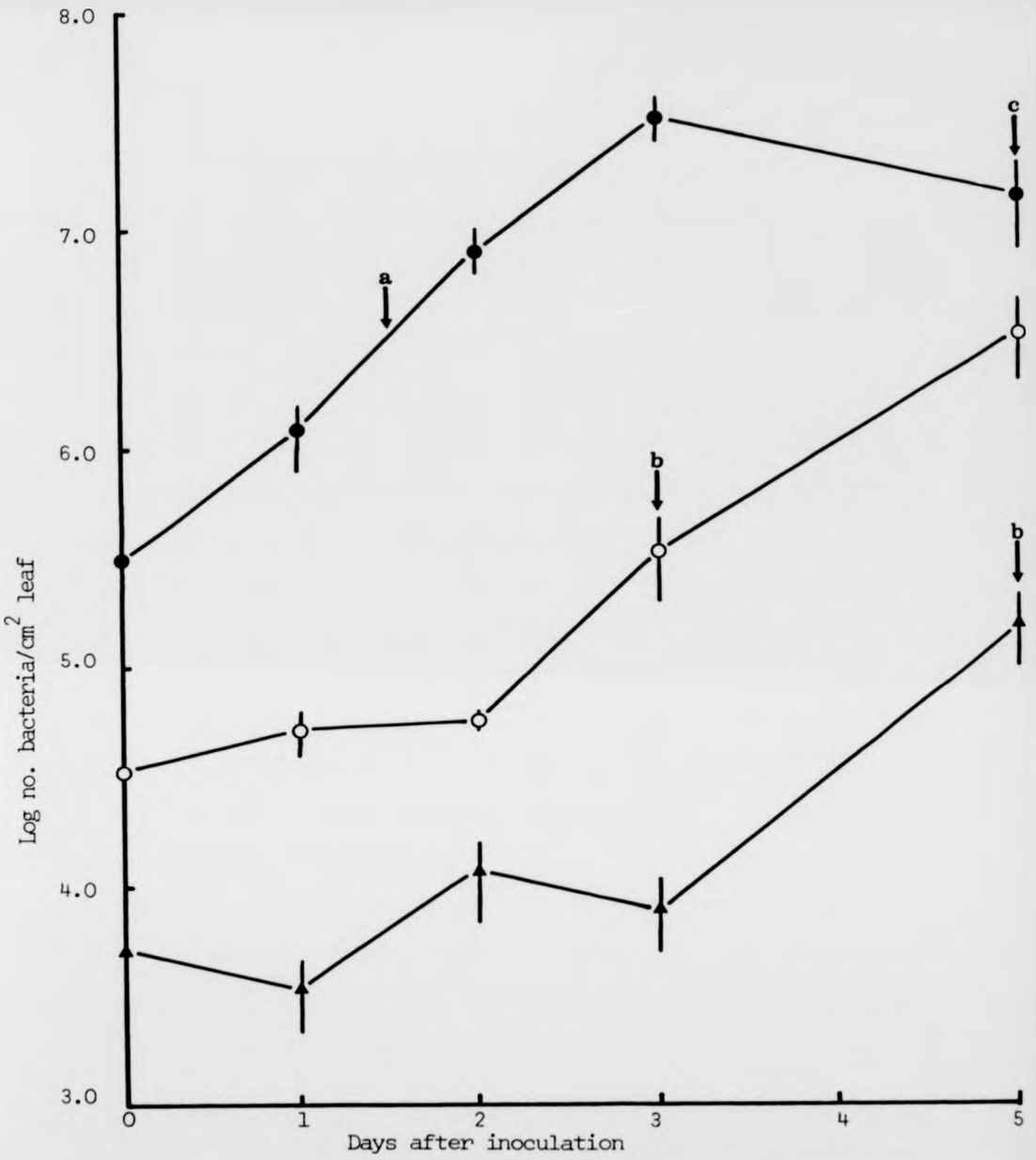



FIG. 1.1 Populations of P. coronafaciens (NCPPB 1348) in oat cv. Milford following inoculation with $10^8$(●), $10^7$(○) and $10^6$(▲) bacteria/ml. Arrows mark symptom appearance: a, collapsed and watersoaked infiltrated area; b, haloes in infiltrated area; c, spreading chlorosis. Data were combined from 2 repeated experiments, bars indicate ±SEM.

had multiplied to a critical level of c. $4x10^6$ bacteria/$cm^2$ leaf. The time at which the population was reached depended upon the initial inoculum concentration. Multiplication was much less rapid in line Cc4146 at $10^8$ bacteria/ml and was not detected at the lower inoculum concentrations (Fig. 1.2).

Little or no multiplication of P. fluorescens was recorded in either cv. Milford or line Cc4146. The numbers of P. phaseolicola recovered from oat leaves also remained relatively constant or decreased slightly with time after inoculation (Fig. 1.3). The same was true of P. tabaci in cv. Milford. P. morsprunorum, however, declined in numbers following inoculation into this cultivar. The HR caused by P. coronafaciens var. atropurpurea was associated with a rapid decrease in bacterial numbers apparent by 1 day after inoculation before the response of infiltrated tissue was fully expressed.

Although P. tabaci failed to multiply in oat leaves, the same isolate did multiply in its host plant, tobacco, in a manner similar to that recorded for P. coronafaciens in oat cv. Milford (Fig. 1.4).

B. Barley

Patterns of bacterial multiplication in barley leaves were related to symptom development as observed for oats. The production of halo blight symptoms by P. coronafaciens was associated with rapid bacterial multiplication (Fig. 1.5). P. phaseolicola and P. fluorescens maintained constant populations or gradually declined following inoculation. Levels of P. coronafaciens var. atropurpurea dropped during

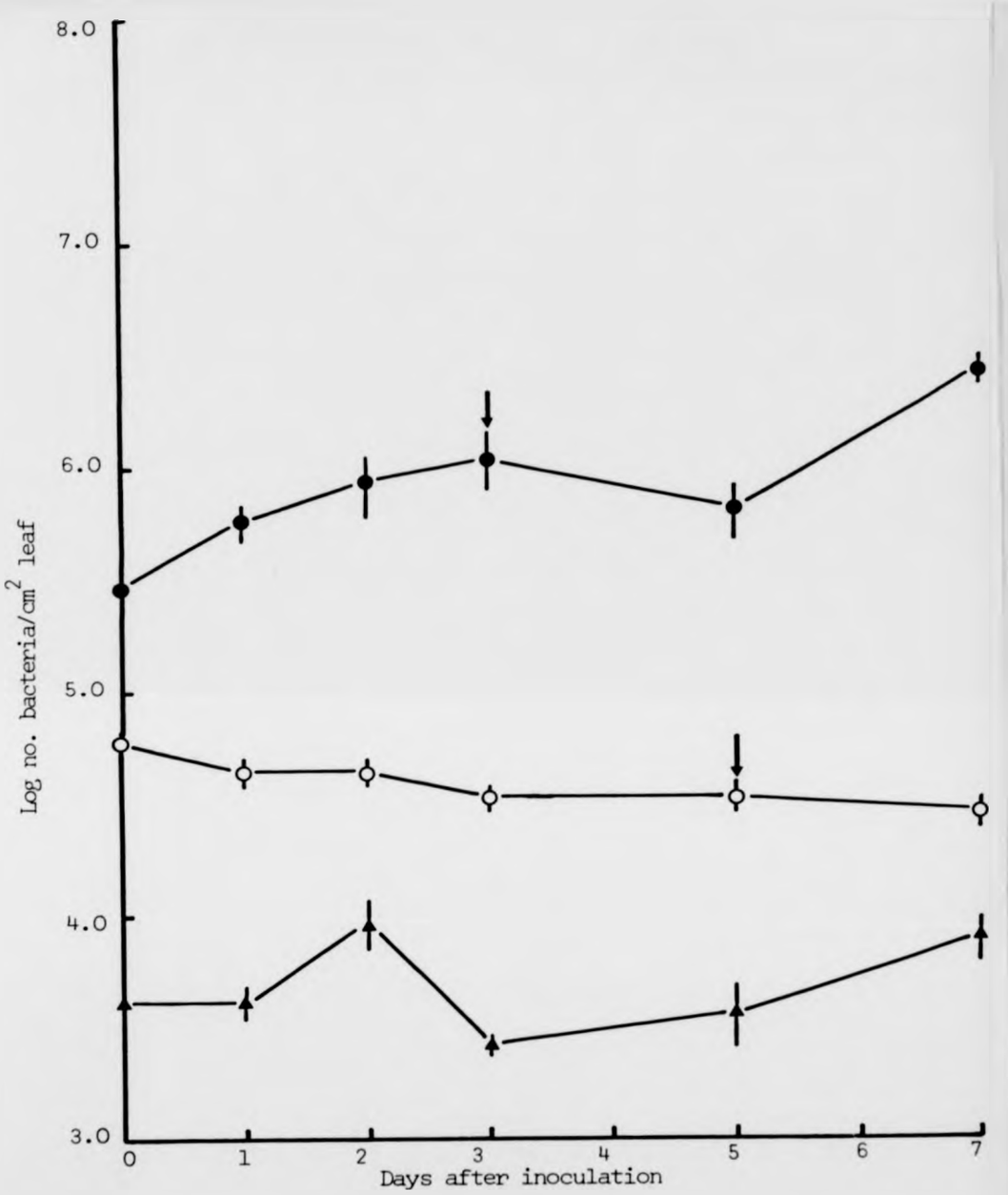



FIG. 1.2 Populations P. coronafaciens (NCPPB 1348) in oat line Cc4146 following inoculation with $10^8$(●), $10^7$(○) and $10^6$(▲) bacteria/ml. Arrows mark appearance of haloes in infiltrated areas. Data were combined from 2 repeated experiments. Bars indicate ±SEM.

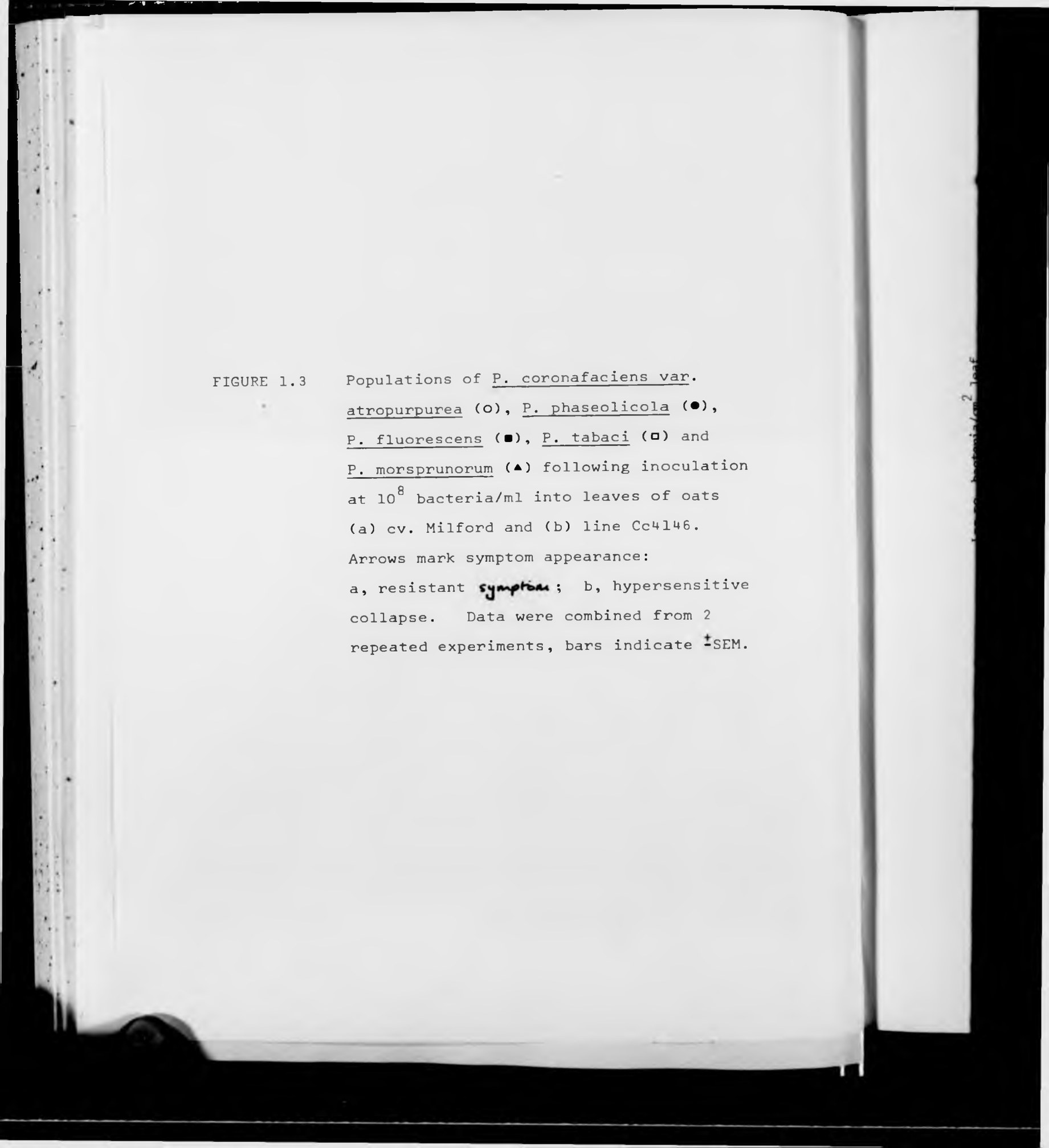


FIGURE 1.3 Populations of P. coronafaciens var. atropurpurea (○), P. phaseolicola (●), P. fluorescens (■), P. tabaci (□) and P. morsprunorum (▲) following inoculation at $10^8$ bacteria/ml into leaves of oats (a) cv. Milford and (b) line Cc4146. Arrows mark symptom appearance: a, resistant symptom; b, hypersensitive collapse. Data were combined from 2 repeated experiments, bars indicate ±SEM.

ens var.
icola (●),
i (□) and
g inoculation
es of oats
Cc4146.
ce:
ypersensitive
ed from 2
ndicate ±SEM.

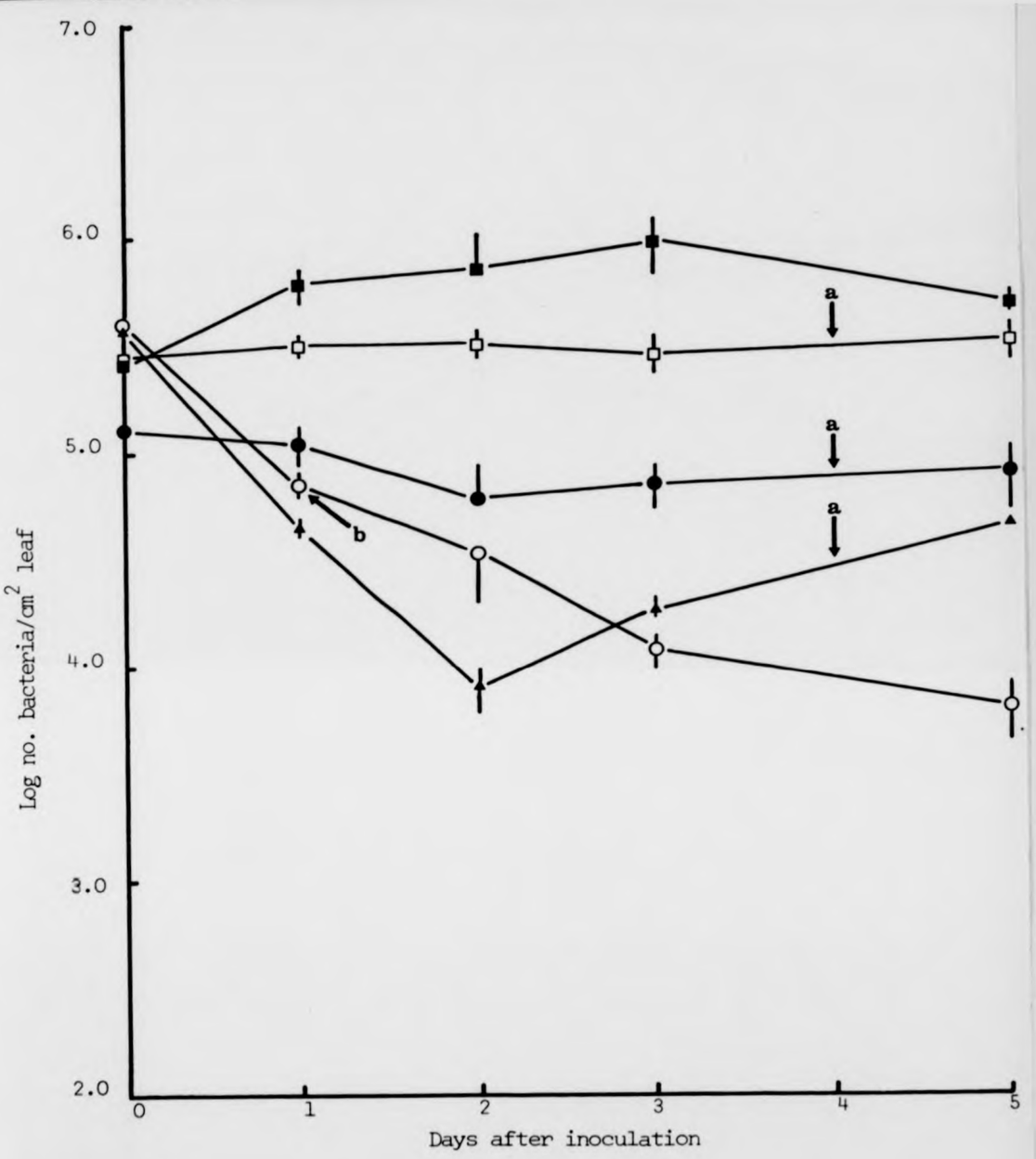



FIG. 1.3a

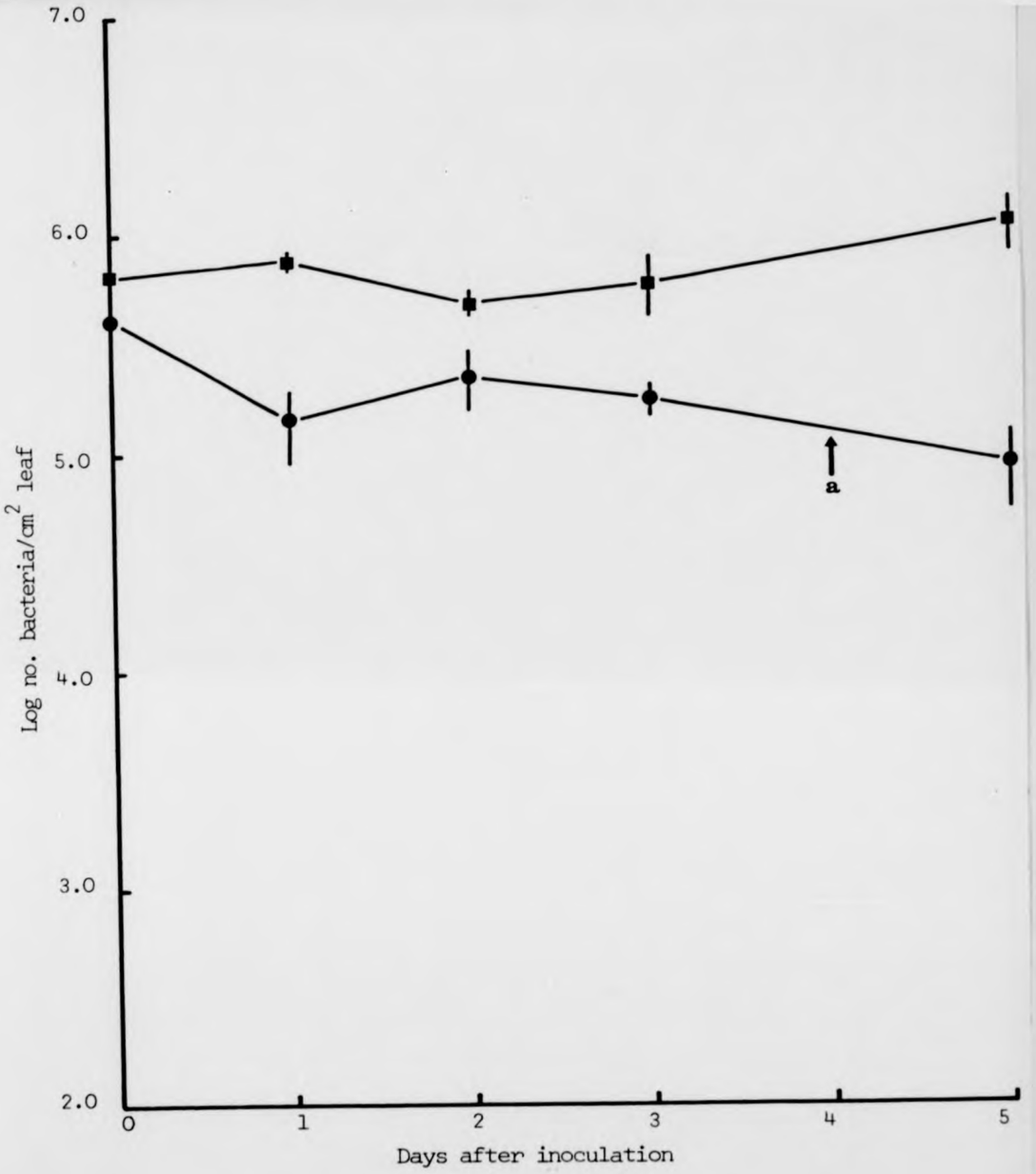

7.0
6.0
5.0
4.0
3.0
2.0
Log no. bacteria/cm$^2$ leaf
a
0
1
2
3
4
5
Days after inoculation

FIG. 1.3b

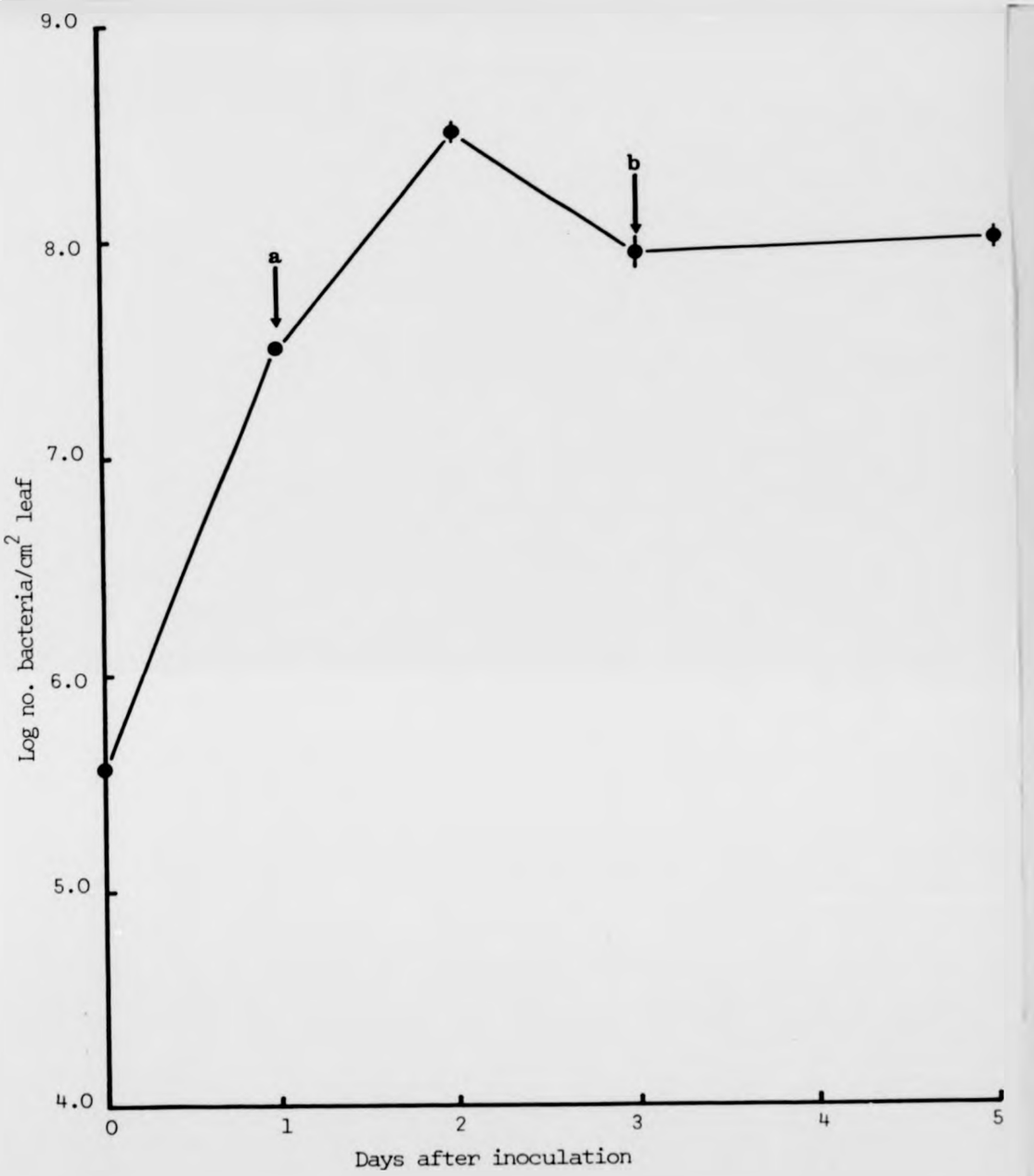



FIG. 1.4 Population of P. tabaci following inoculation at $10^8$ bacteria/ml into leaves of tobacco cv. Burley. Arrows mark symptom appearance: a, collapsed and watersoaked infiltrated area; b, spreading chlorosis. Data were combined from 2 repeated experiments, bars indicate ±SEM.

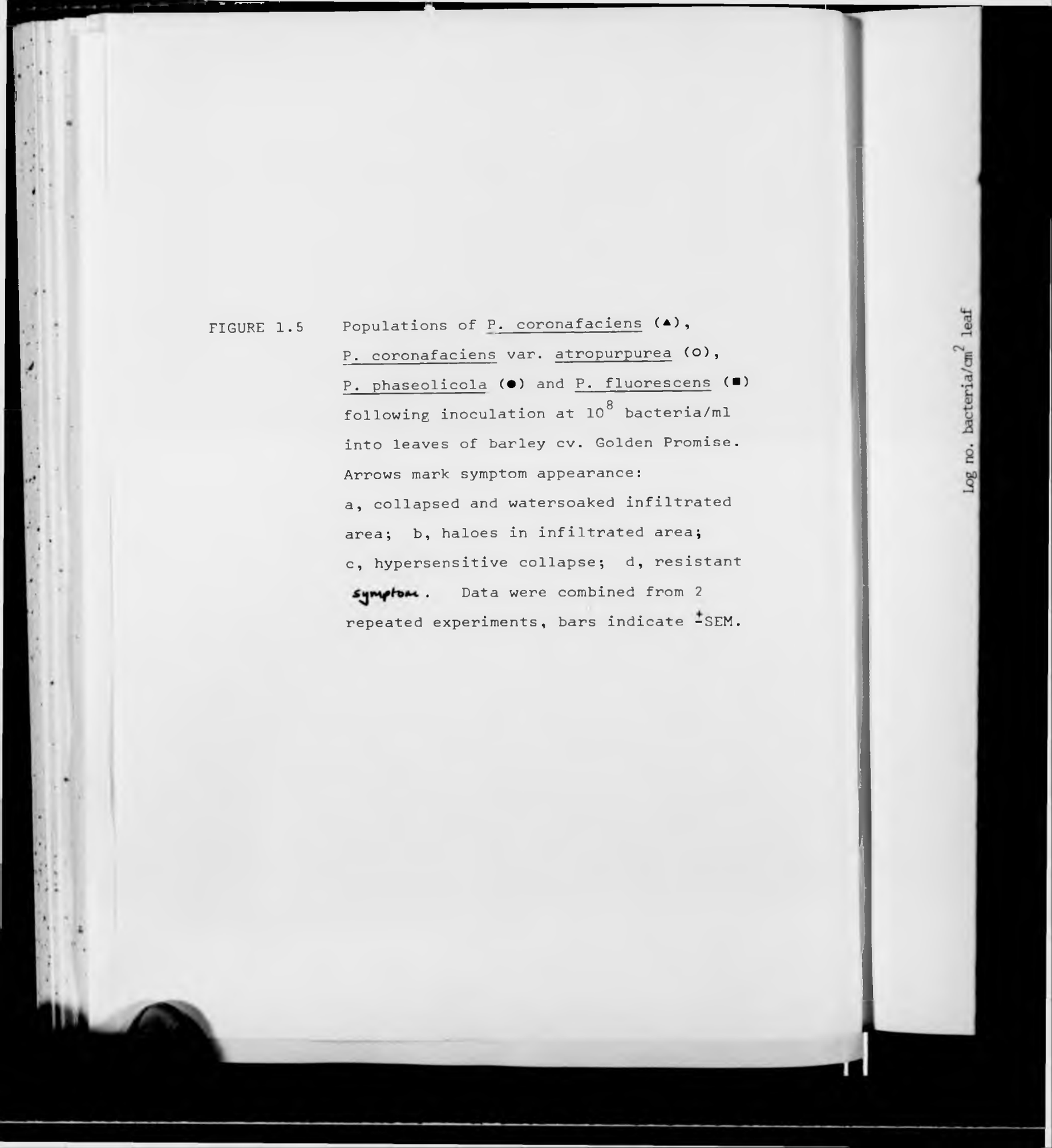



FIGURE 1.5 Populations of P. coronafaciens (▲), P. coronafaciens var. atropurpurea (○), P. phaseolicola (●) and P. fluorescens (■) following inoculation at $10^8$ bacteria/ml into leaves of barley cv. Golden Promise. Arrows mark symptom appearance: a, collapsed and watersoaked infiltrated area; b, haloes in infiltrated area; c, hypersensitive collapse; d, resistant symptom. Data were combined from 2 repeated experiments, bars indicate ±SEM.

ns (▲),
purea (○),
uorescens (■)
bacteria/ml
den Promise.
e:
infiltrated
ed area;
d, resistant
d from 2
dicate ±SEM.

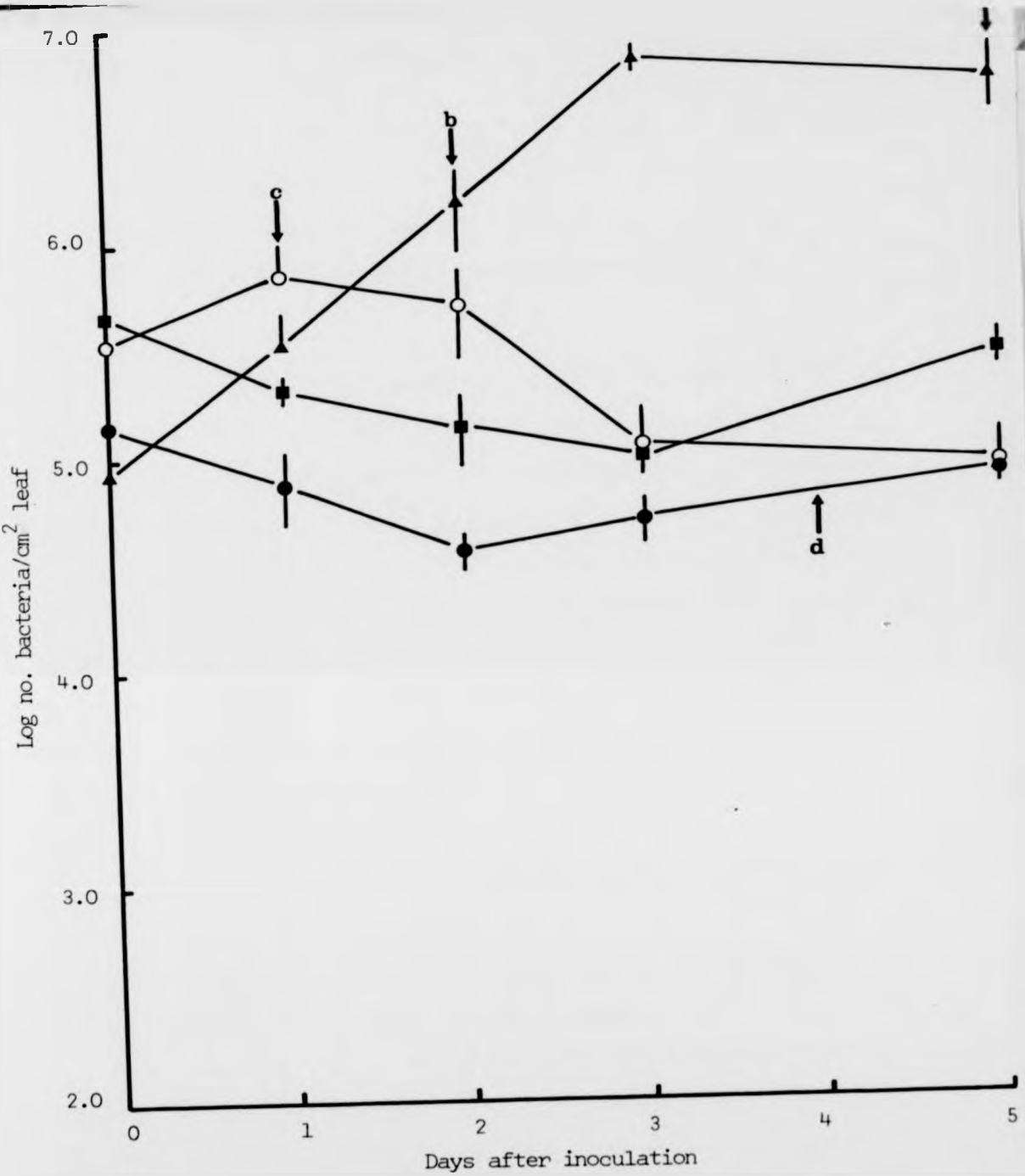



FIG. 1.5

the expression of the HR but this decrease was less marked in barley than in oats. A long term experiment with P. coronafaciens in barley showed that viable bacteria could be recovered 3 weeks after inoculation (Fig. 1.7).

C. Wheat

Similar trends in bacterial numbers described for oats and barley were apparent in wheat leaves undergoing similar symptom development. In the resistant symptoms (P. coronafaciens, P. phaseolicola) and with P. fluorescens, constant or slightly declining populations were recorded. By contrast, the numbers of P. coronafaciens var. atropurpurea fell rapidly during HR expression (Fig. 1.6). Viable bacteria could be recovered from wheat leaves undergoing a resistant symptom in response to P. coronafaciens 4 weeks after inoculation (Fig. 1.7).

3. Characteristics of the hypersensitive response (HR) and normosensitive collapse

A. Estimation of induction times in oat leaves and comparison with other plant species

The hypersensitive and normosensitive collapse of tobacco leaves inoculated with phytopathogenic bacteria are characterized by an induction time during which viable bacteria must be present to elicit an irreversible chain of events leading to symptom expression following a post-induction latent period (Klement and Goodman, 1967; Klement, Hevesi and Sasser, 1978). By following inocula of bacteria with an antibiotic injection (streptomycin 500ppm) at hourly intervals, the periods during which viable

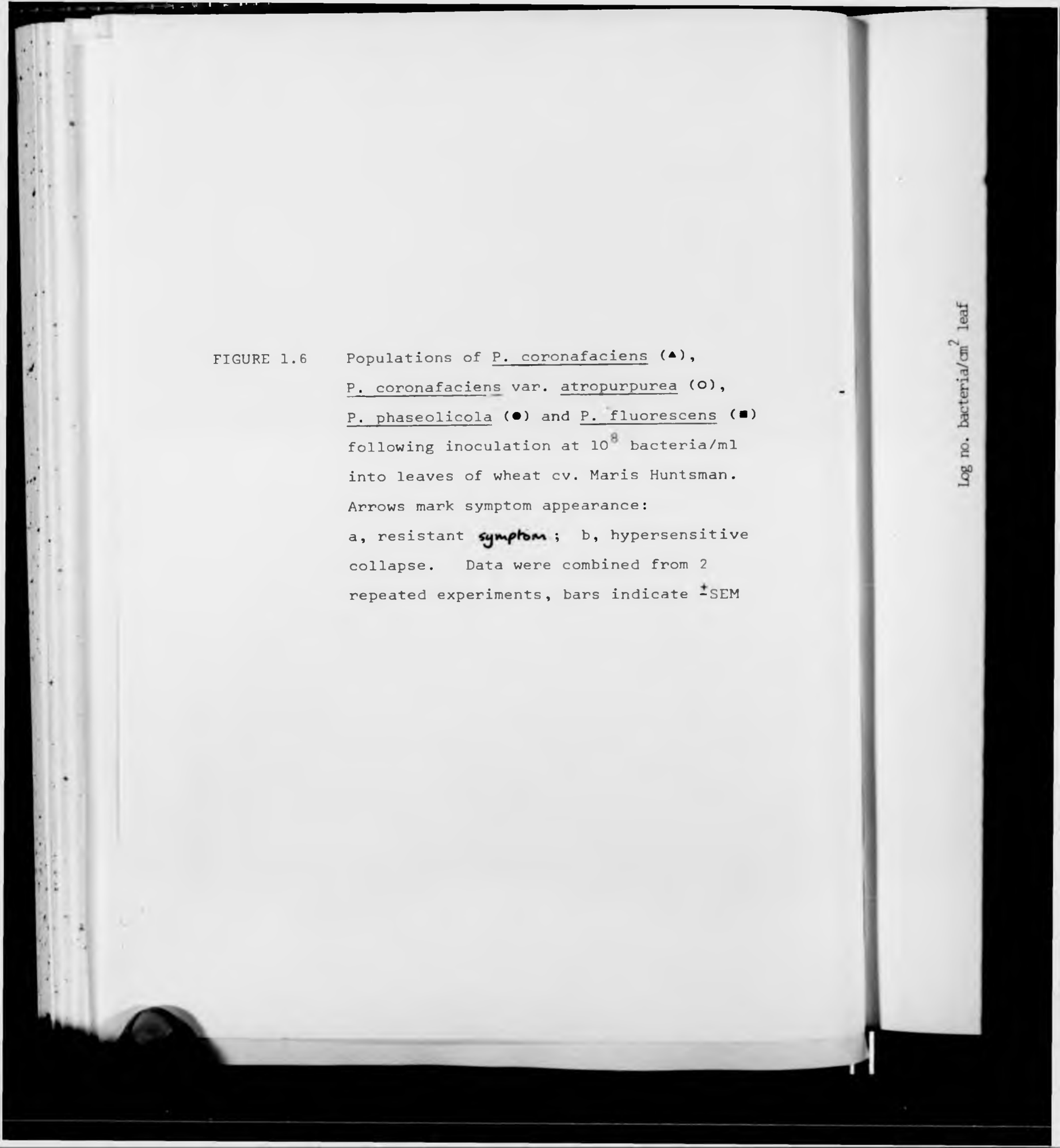



FIGURE 1.6 Populations of *P. coronafaciens* (▲), *P. coronafaciens* var. *atropurpurea* (○), *P. phaseolicola* (●) and *P. fluorescens* (■) following inoculation at $10^8$ bacteria/ml into leaves of wheat cv. Maris Huntsman. Arrows mark symptom appearance: a, resistant symptom; b, hypersensitive collapse. Data were combined from 2 repeated experiments, bars indicate ±SEM

ns (▲),
purea (○),
uorescens (■)
bacteria/ml
s Huntsman.
e:
persensitive
d from 2
ndicate ±SEM

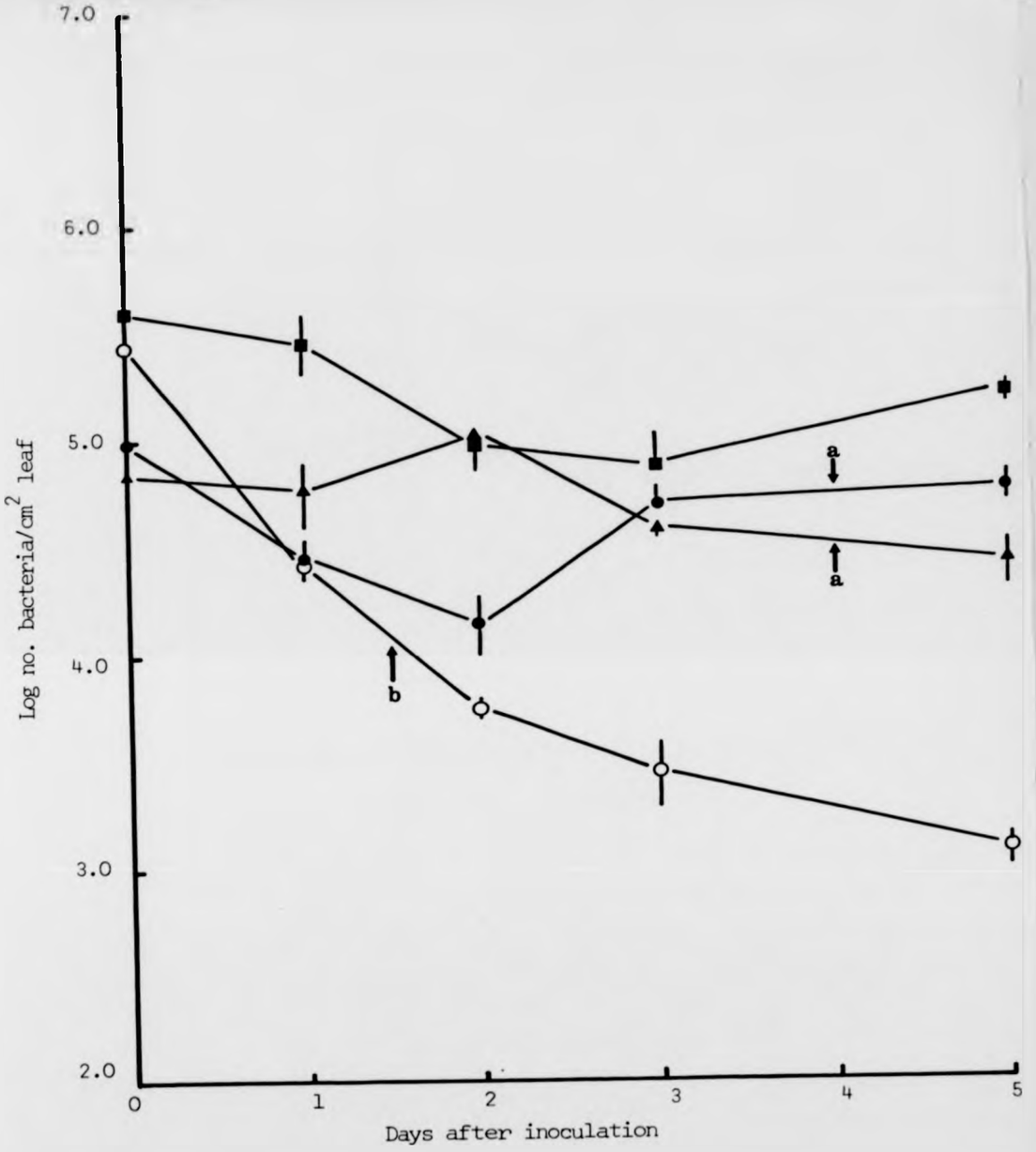



FIG. 1.6

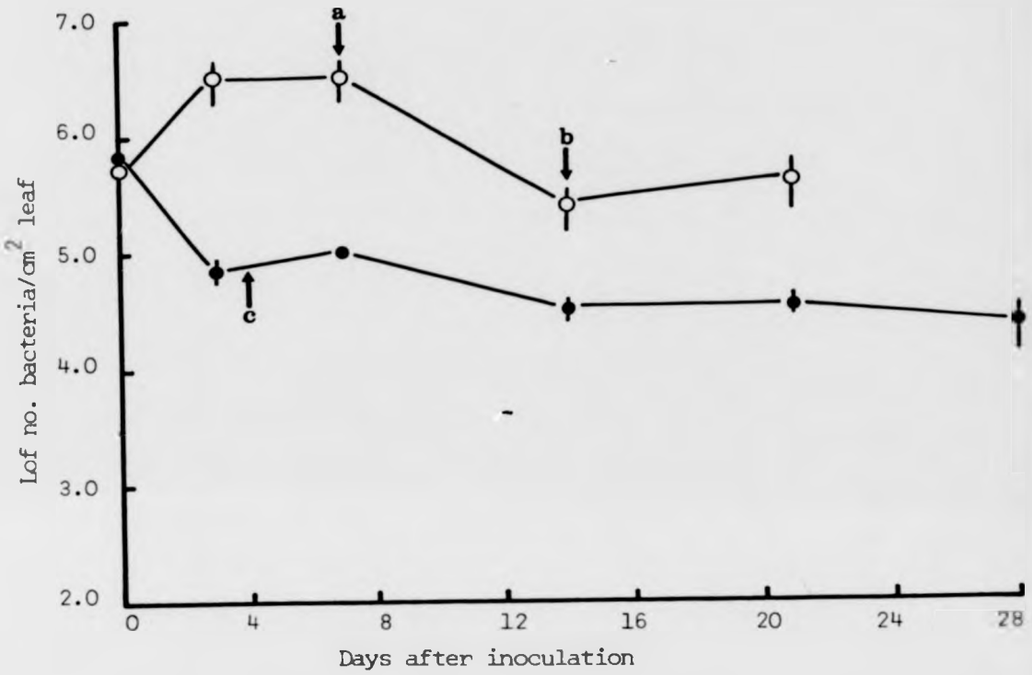



FIG. 1.7 Long time course population study of P. coronafaciens in wheat cv. Maris Huntsman (●) and barley (○). Arrows denote symptom appearance: a, collapsed and watersoaked infiltrated area; b, spreading chlorosis; c, resistant symptom. Data were combined from 2 repeated experiments, bars indicate ±SEM.

bacteria are required to elicit the final responses in oats were determined.

(i) Hypersensitive response (HR)

Suspensions of a range of HR-eliciting phytopathogens were inoculated into leaves and the infiltrated areas subsequently injected with streptomycin solution. Induction times determined are given in table 1.5 (see also Plate 1.5 and Fig. 1.8).

In general, induction times were longest in oats, French bean and broad bean and shortest in tobacco. Symptom appearance followed a similar pattern (Table 1.6). Thus P. coronafaciens var. atropurpurea required an induction time of 3-4h in oat leaves compared with 1-2h in tobacco. It is noteworthy that P. tabaci, P. phaseolicola and P. morsprunorum elicited a clearly defined HR in the plants other than their hosts despite producing only a resistant symptom in oat leaves. P. Syringae, the only other phytopathogen to elicit an HR in oat leaves, produced a faster reaction than P. coronafaciens var. atropurpurea.

(ii) Normosensitive collapse

Pseudomonas coronafaciens, P. phaseolicola and P. tabaci required long induction times to produce normosensitive collapse in their respective hosts, oats, French bean and tobacco. The time between inoculation and the appearance of symptoms was considerably longer in susceptible than hypersensitive reactions (Table 1.6, see also Plate 1.6 and Fig. 1.9).

TABLE 1.5 Induction times for hypersensitive collapse in oat and tobacco in response to P. coronafaciens var. atropurpurea determined by streptomycin injection

| Time of second infiltration[b] (h) | Appearance of Hypersensitive response[a] Oat[e] Streptomycin[c] | SDW[c] | Tobacco[e] Streptomycin[c] | SDW[c] |
|---|---|---|---|---|
| 0 | - | +++ | - | +++ |
| 1 | - | +++ | - | +++ |
| 2 | - | +++ | ++ | +++ |
| 3 | - | +++ | +++ | +++ |
| 4 | ++ | +++ | +++ | +++ |
| 5 | +++ | +++ | +++ | +++ |
| 6 | +++ | +++ | +++ | +++ |
| NI[d] | +++ | +++ | +++ | +++ |

[a] Appearance of HR after 3d recorded on visual scale. - no HR, +++ complete HR of infiltrated area.

[b] Following first infiltration of $10^8$ bacteria/ml of P. coronafaciens var. atropurpurea (NCPPB 2397).

[c] SDW = sterile distilled water. Streptomycin and SDW were the secondary inocula.

[d] NI = no secondary infiltration.

[e] Induction times were 3-4h in oat and 1-2h in tobacco.
The time of earliest symptom appearance was 18h in oat and 8h in tobacco.

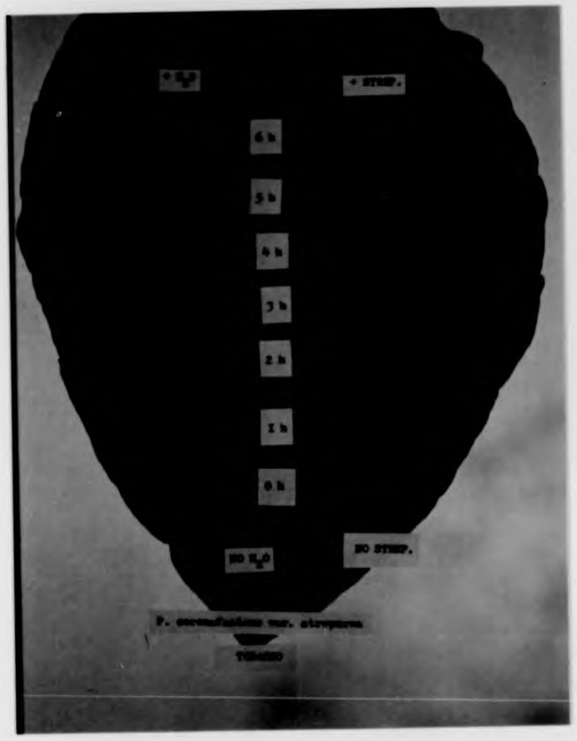


PLATE 1.5 Determination of induction time of the HR elicited by P. coronafaciens var. atropurpurea in tobacco using the streptomycin injection technique. Note confluent HR of infiltrated areas treated with streptomycin 2h or more after inoculation.

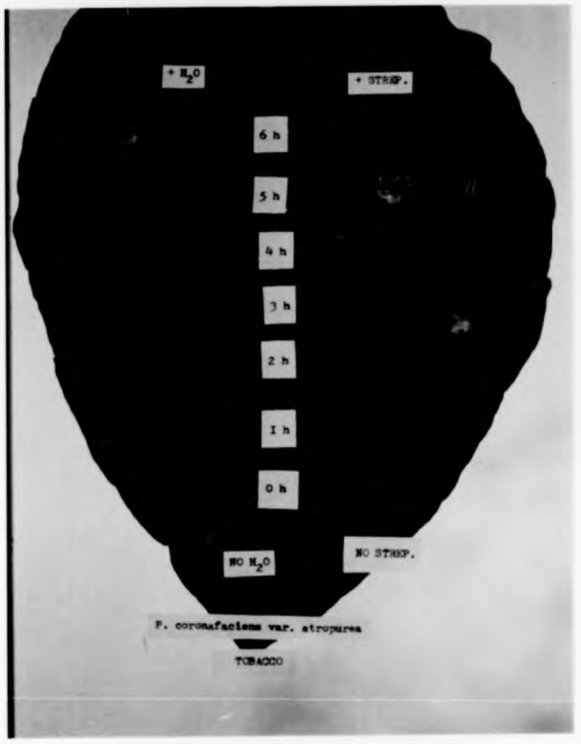



PLATE 1.5 Determination of induction time of the HR elicited by P. coronafaciens var. atropurpurea in tobacco using the streptomycin injection technique. Note confluent HR of infiltrated areas treated with streptomycin 2h or more after inoculation.

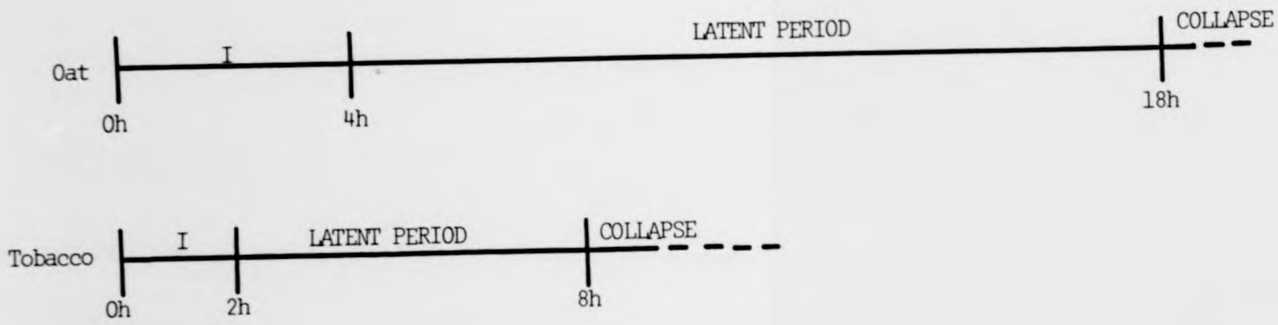



FIG. 1.8 Diagramatic representation of induction times and latent periods prior to appearance of the hypersensitive response (HR) in leaves of oat and tobacco elicited by a suspension of P. coronafaciens var. atropurpurea ($10^8$ bacteria/ml).

TABLE 1.6 Induction times determined by streptomycin injection and the times of earliest symptom appearance during hypersensitive and normosensitive collapse in response to pseudomonads [a]

| Bacterium | | Inoculated Plant | | | |
|---|---|---|---|---|---|
| | | Oats | French bean | Broad bean | Tobacco |
| *P. coronafaciens* | I [b] | 18-24[d] | 3-4 | 4-5 | 1-2 |
| | SA[c] | 48 | 12 | 12 | 8 |
| *P. coronafaciens* var. *atropurpurea* | I | 3-4 | 3-4 | 3-4 | 1-2 |
| | SA | 18 | 12 | 12 | 8 |
| *P. morsprunorum* | I | -[e] | 2-3 | 4-5 | 2-3 |
| | SA | - | 8 | 18 | 8 |
| *P. phaseolicola* | I | - | 24-36[d] | 2-3 | 2-3 |
| | SA | - | 48 | 8 | 8 |
| *P. syringae* | I | 2-3 | 2-3 | 3-4 | 1-2 |
| | SA | 12 | 8 | 12 | 8 |
| *P. tabaci* | I | - | 3-4 | 2-3 | 12-18[d] |
| | SA | - | 12 | 12 | 24 |

[a] Leaves were injected with suspensions of c.$10^8$ bacteria/ml sterile distilled water.

[b] I = induction time (h).

[c] SA = symptom appearance (h) after inoculation. The earliest symptom observed was glazing of the abaxial epidermis.

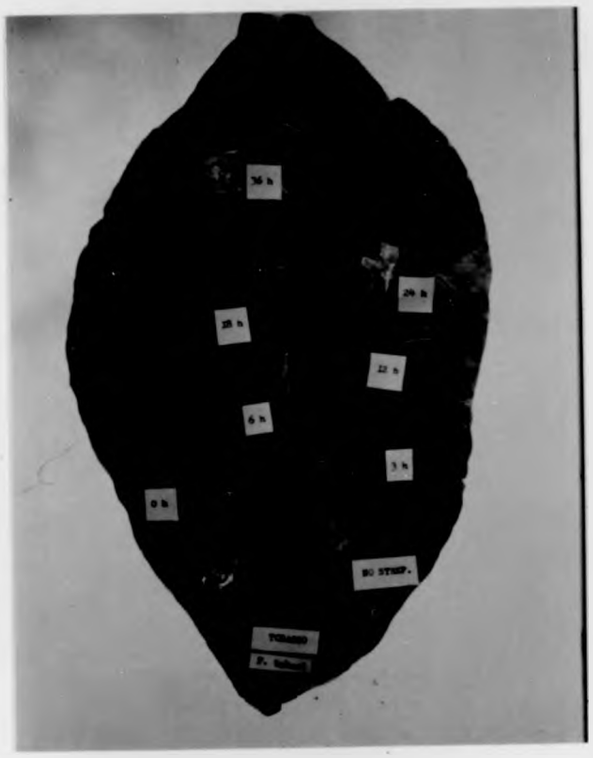
[d] Normosensitive collapse in response to compatible bacterium.

[e] Resistant symptom.

PLATE 1.6 Determination of induction times for normosensitive collapse in leaves in response to e. $10^8$ bacteria/ml of (a) P. tabaci in tobacco, (b) P. phaseolicola in French bean and (c) P. coronafaciens in oat cv. Milford using the streptomycin injection technique. Note confluent normosensitive collapse of infiltrated areas treated with streptomycin 18h in (a) 36h in (b) and 24h in (c) or more after inoculation.

times for
leaves in
/ml of
b) P.
and (c)
Milford
ction
normo-
trated areas
8h in
c) or more

(a)




(b)

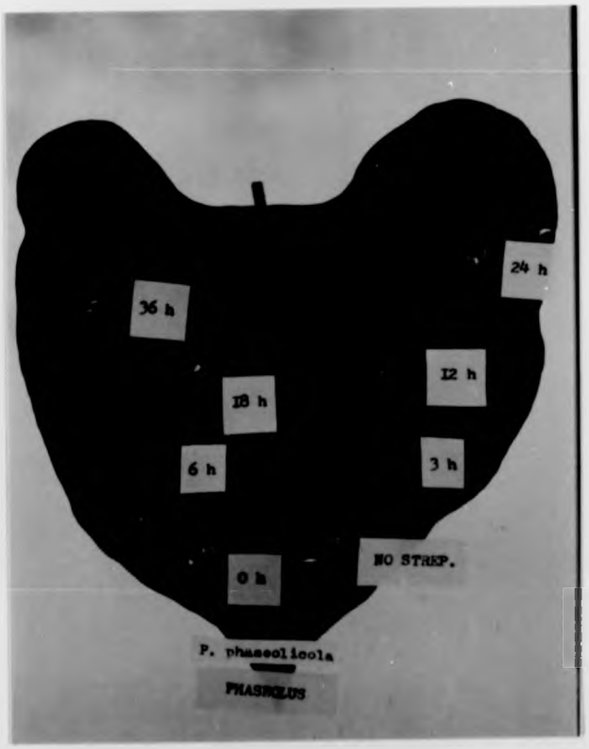
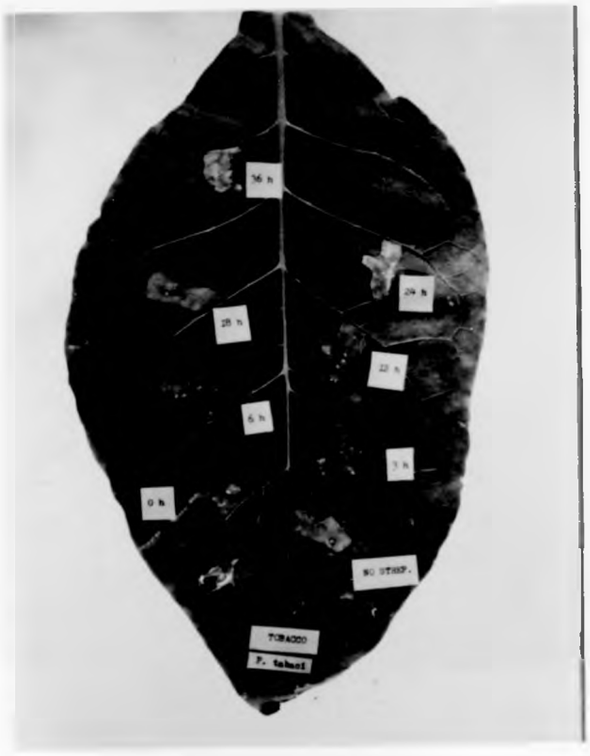



PLATE 1.6

times for
leaves in
/ml of
) P.
and (c)
Milford
ction
normo-
trated areas
8h in
c) or more

(a)




(b)

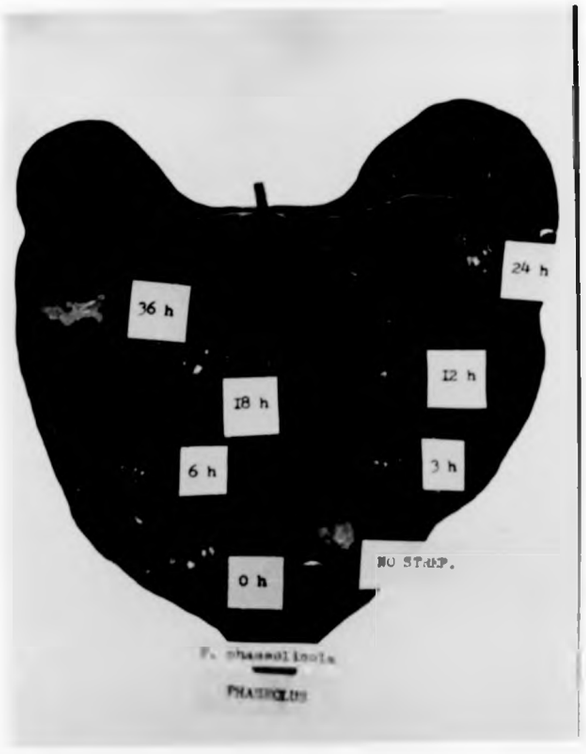



PLATE 1.6

(c)

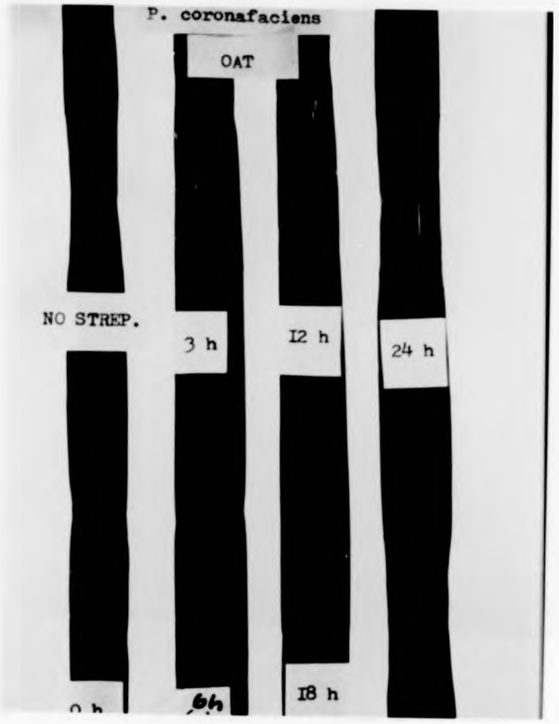



PLATE 1.6

(c)

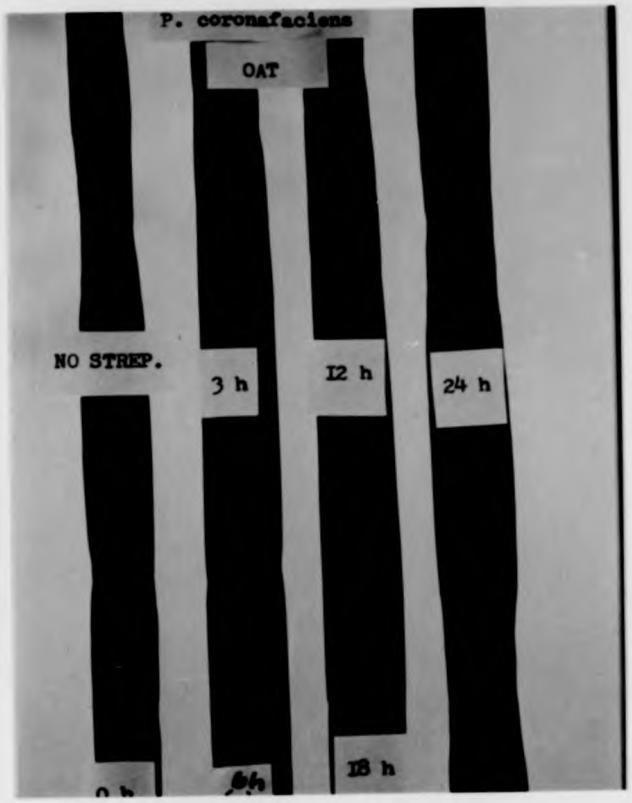



PLATE 1.6

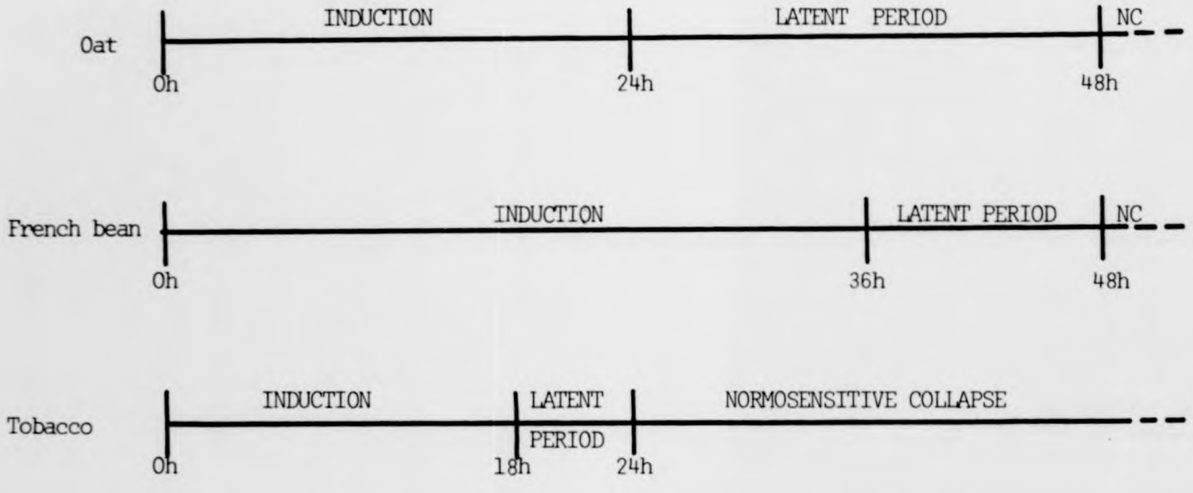



FIG. 1.9 Diagramatic representation of induction times and latent periods prior to normosensitive collapse (NC) in leaves of oat, French bean and tobacco elicited by P. coronafaciens, P. phaseolicola and P. tabaci respectively.

B. Prevention of the hypersensitive response (HR) and normosensitive collapse

Pre-treatment of tobacco leaves with heat-killed bacteria can prevent the HR and also protect against the multiplication of pathogenic bacteria (Kelman and Sequeira, 1972; Lovrekovich and Farkas, 1965; Novacky, Acedo and Goodman, 1973; Sequeira, 1976; Sequeira and Hill, 1974). Prior inoculation with live cells of an HR inducing bacterium also reduced symptom development in French bean following challenge with P. phaseolicola (Omer and Wood, 1969). The occurrence of protection phenomena in oat leaves was therefore examined and the results obtained are summarized in Table 1.7. The percentage of the infiltrated area protected from collapse was recorded 3 and 6 days after secondary infiltration.

Heat-killed cells of P. coronafaciens var. atropurpurea prevented HR expression and also protected oat leaves against normosensitive collapse. By contrast, dead cells of the pathogen, P. coronafaciens were only effective in slightly delaying normosensitive and HR collapse (Plate 1.7, Table 1.7). Pre-treatment with live cells of either bacterial species did not appear to influence symptom development following secondary infiltration.

Living and dead cells of P. fluorescens protected against P. coronafaciens, but only delayed HR expression as indicated by decreasing percentage protection between 3 and 6 days after secondary infiltration with P. coronafaciens var. atropurpurea. Cells of P. tabaci were particularly effective (either live or dead) against both the HR and normosensitive collapse (Table 1.7).

TABLE 1.7 Prevention of the hypersensitive response (HR) and normosensitive collapse in oat leaves

| Primary Infiltration [a] | | % Protection against hypersensitive collapse (*P. coronafaciens* var. *atropurpurea*) [b] | | % Protection against normosensitive collapse (*P. coronafaciens*) [b] | |
|---|---|---|---|---|---|
| | | 3 days | 6 days | 3 days | 6 days |
| *P. coronafaciens* | LIVE | 0(-) | 0(-) | 0(-) | 0(-) |
| | DEAD | 12.5(0-25) | 0(-) | 12.5(0-50) | 0(-) |
| *P. coronafaciens* var. *atropurpurea* | LIVE | 0(-) | 0(-) | 0(-) | 0(-) |
| | DEAD | 95(-) | 90(-) | 100(-) | 100(-) |
| *P. fluorescens* | LIVE | 72.5(50-80) | 45(25-80) | 100(-) | 100(-) |
| | DEAD | 72.5(50-95) | 25(-) | 90(80-100) | 50(-) |
| *P. tabaci* | LIVE | 100(-) | 100(-) | 100(-) | 100(-) |
| | DEAD | 100(-) | 75(-) | 100(-) | 100(-) |
| Water | | 0(-) | 0(-) | 0(-) | 0(-) |

[a] Leaves were infiltrated with suspensions of live or dead bacteria ($10^8$ or $5x10^9$ bacteria/ml respectively), 24h before challenge with $10^8$ bacterial/ml of *P. coronafaciens* var. *atropurpurea* to induce the HR or *P. coronafaciens* to induce normosensitive collapse.

[b] The percentage of infiltrated area protected from collapse was recorded. Results are the means from at least 2 repeated experiments each with at least 2 inoculation sites recorded 3 and 6 days after secondary infiltration. The maximum range between replicate sites is given in parentheses.

PLATE 1.7 Prevention of the HR and normosensitive collapse in oat cv. Milford. Sites previously infiltrated with suspensions of $5 \times 10^9$ heat-killed bacteria/ml of (a) P. coronafaciens, (b) P. coronafaciens var. atropurpurea or (c) sterile distilled water were challenged with (from left to right) sterile distilled water, suspensions of $10^8$ live bacteria/ml of P. coronafaciens and P. coronafaciens var. atropurpurea. Photographs were taken 4 days after the challenge inoculation, note localized prevention of symptom development at some inoculation sites delineated by pen marks on leaf surfaces.

PLATE 1.7

...sensitive
... Sites
...spensions
.../ml of (a)
...afaciens
...ile distilled
...om left to
...,
...ria/ml of
...afaciens var.
...ere taken
...oculation,
...symptom
... sites
...f surfaces.

Prevention of the HR by prior inoculation with heat-killed P. coronafaciens var. atropurpurea was examined in more detail. Protection was achieved between 12 and 18h after inoculation with heat-killed bacteria and was effective within the infiltrated area for at least 6 days. Protection was consistently reduced by about 20% when leaves were kept in the dark before being challenged with P. coronafaciens var. atropurpurea but was clearly not light dependent (Table 1.8). The protection achieved was highly localized to the initially infiltrated area of leaf tissue (see Plate 1.7). The HR developed in the usual way at other sites in the same leaf and in other leaves on the same plant; there was no indication of a systemic protective effect (Table 1.9).

C. Electrolyte leakage from oat leaves following inoculation with bacterial suspensions

The very nature of hypersensitive and normosensitive collapses is indicative of cell death and concomitant permeability changes related to loss of membrane integrity of responding mesophyll cells. Changes in the permeability of cells within oat tissues undergoing various types of response were therefore examined by recording electrolyte leakage from discs cut from infiltrated leaves.

Increases in the conductivity of bathing solutions were closely correlated with the appearance and extent of macroscopically visible symptoms in the excised discs. Considerable electrolyte leakage was recorded from tissue undergoing the HR or normosensitive collapse in response to P. coronafaciens var. atropurpurea and P. coronafaciens

TABLE 1.8 Effect of timing and light on prevention of the hypersensitive response (HR) in oat leaves

| Time of Challenge Infiltration (h) [a] | % Protection against HR [b] | |
|---|---|---|
| | Light | Dark [c] |
| 0 | 0 | 0 |
| 6 | 0 | 0 |
| 12 | 0 | 0 |
| 18 | 90 | 85 |
| 24 | 85 | 25 |
| 36 | 57.5 | 37.5 |

[a] Leaves were infiltrated with $5 \times 10^9$ bacteria/ml heat-killed P. coronafaciens var. atropurpurea and challenged at intervals with $10^8$ live bacteria/ml of the same species to induce hypersensitive collapse.

[b] The percentage of infiltrated area protected against collapse was estimated to the nearest 10% after 3 days. Results are the means of at least two inoculation sites.

[c] Silver foil was wrapped over sites of primary infiltration and removed at the time of challenge.

TABLE 1.9 Non-systemic nature of the prevention of hypersensitive response (HR) in oats

| Interval between protection treatment and challenge infiltration (d) [a] | % Protection against hypersensitive collapse (*P. coronafaciens* var. *atropurpurea*)[b] | | | | |
|---|---|---|---|---|---|
| | Leaf 1 [a] | Leaf 2 [a] | | | Leaf 3 [a] |
| | | a | b | c | |
| 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 0 | 50 | 0 | 0 |
| 3 | 0 | 0 | 75 | 0 | 0 |
| 6 | 0 | 0 | 60 | 0 | 0 |

[a] Site b on leaf 2 was infiltrated with $5 \times 10^9$ heat-killed bacteria/ml *P. coronafaciens* var. *atropurpurea*. Following this $10^8$ live bacteria/ml of the same bacterium were infiltrated at intervals at the same site, above and below this site on leaf 2 (a and c respectively) and in leaves above (leaf 1) and below (leaf 3) leaf 2.

[b] The percentage of the infiltrated area protected from collapse was estimated to the nearest 10%. Results are the means of 2 repeated experiments each with 2 inoculation sites.

respectively (Fig. 1.10). By contrast, no electrolyte leakage was recorded from discs taken from leaves undergoing the resistant symptom in response to P. tabaci or following inoculation with P. fluorescens (Fig. 1.10). Prevention of the normosensitive collapse by prior inoculation with heat-killed cells of P. coronafaciens var. atropurpurea also prevented electrolyte leakage (Fig. 1.11).


FIGURE 1.10 Electrolyte leakage from leaf discs of oats cv. Milford previously infiltrated with a suspension containing $10^8$ bacteria/ml P. coronafaciens (●), P. coronafaciens var. atropurpurea (○), P. tabaci (□) and P. fluorescens (■). Infiltration with sterile distilled water (▲) was used as a control. Some points are offset to accommodate bars giving range of means from 2 repeated experiments. Arrows denote symptom appearance; a, hypersensitive response; b, normosensitive collapse.

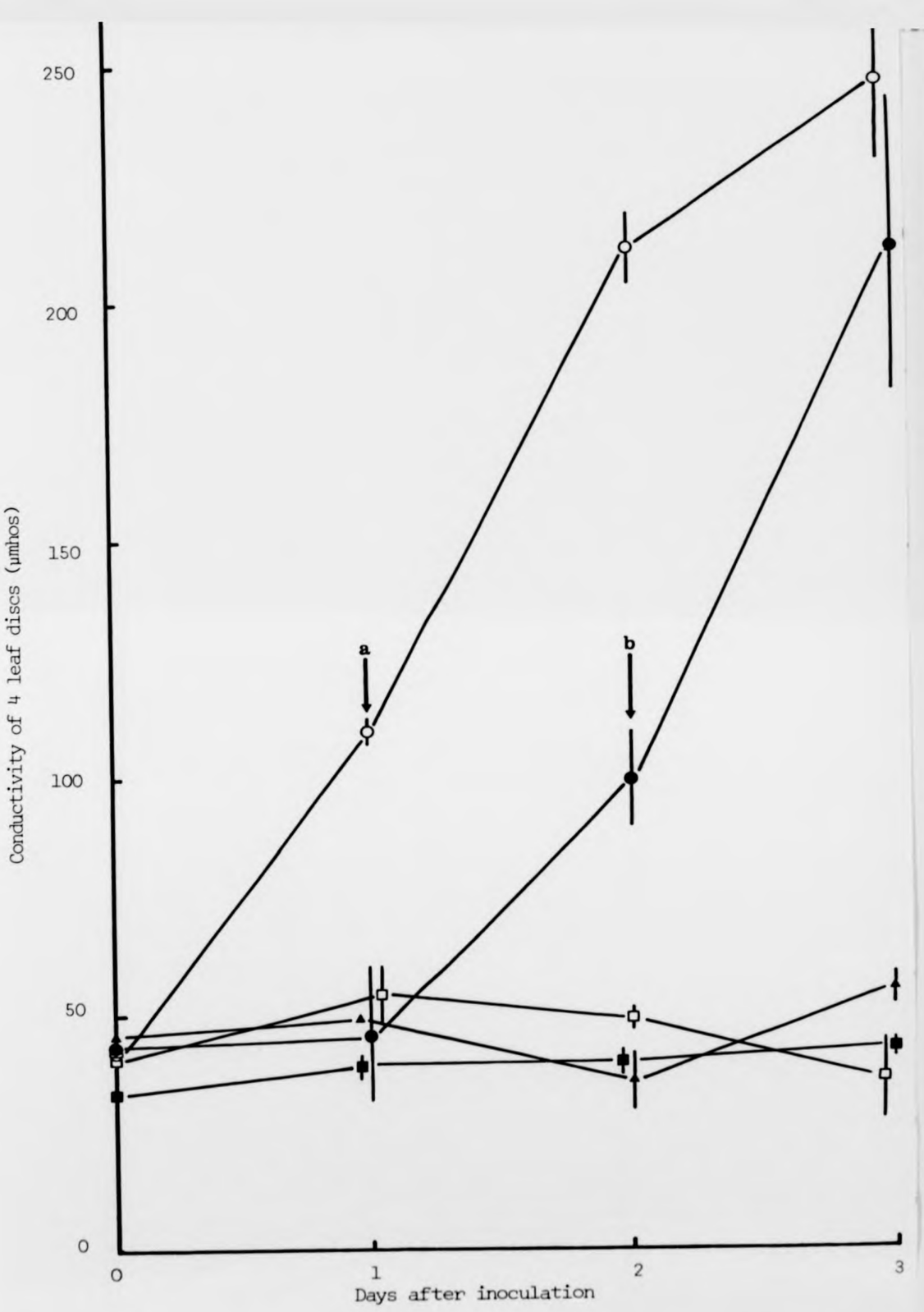

250
200
150
100
50
0
Conductivity of 4 leaf discs (µmhos)
a
b
0
1
2
3
Days after inoculation

FIG. 1.10

FIGURE 1.11 Electrolyte leakage from leaf discs of oat cv. Milford infiltrated with a suspension of heat-killed cells of *P. coronafaciens* var. *atropurpurea* ($5x10^9$ cells/ml) 24h prior to challenge with live *P. coronafaciens* ($10^8$ cells/ml) (o) or sterile distilled water (●). Points are offset to accommodate bars giving range of means from 2 repeated experiments.

iscs of
h a
of
rea
hallenge
cells/ml)
(●).
e bars
peated

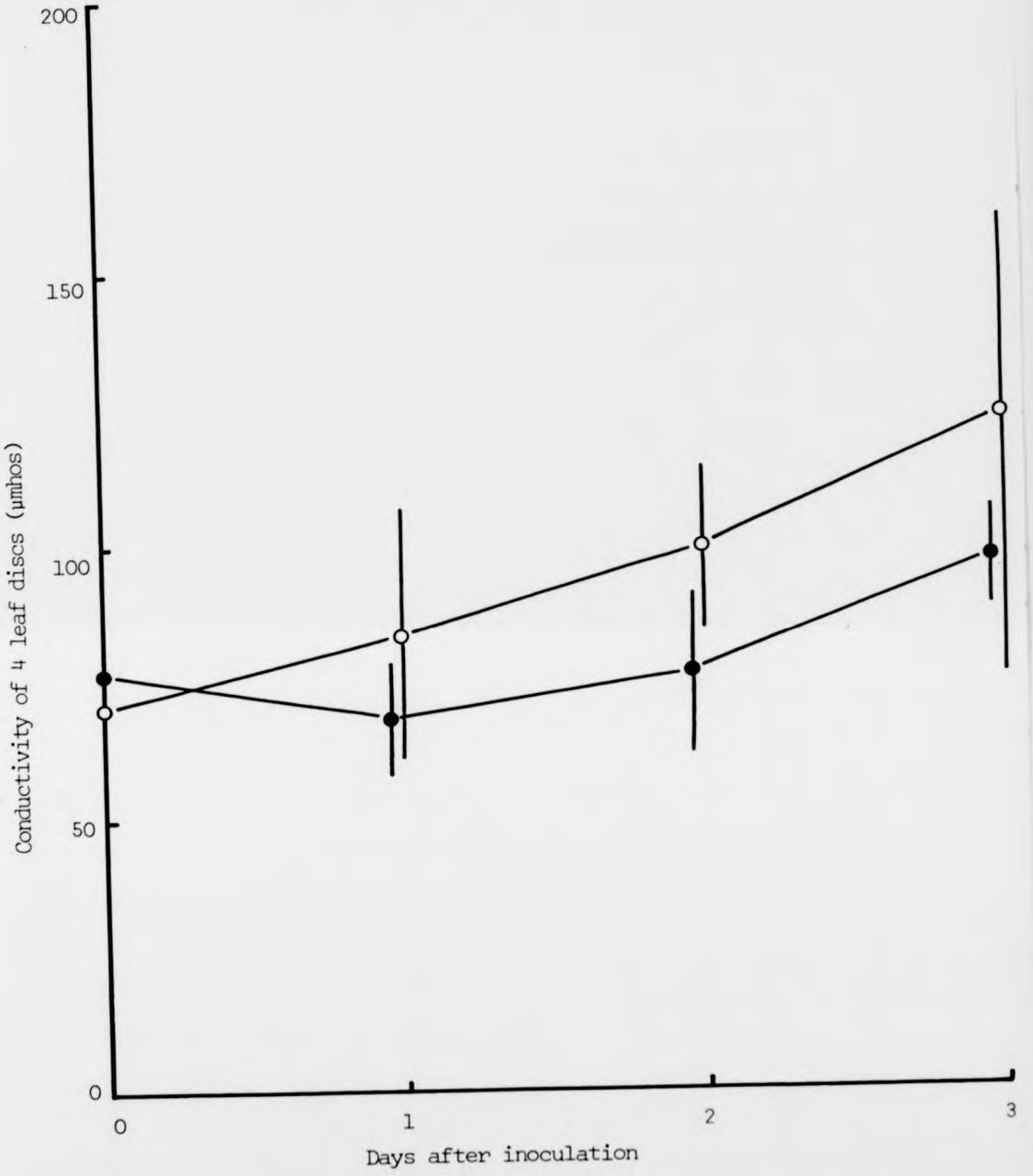



FIG. 1.11

# CHAPTER 2

## ULTRASTRUCTURAL STUDIES ON THE INTERACTIONS BETWEEN OAT LEAVES AND PSEUDOMONADS

### 1. Comparison of fixation and staining methods and preliminary observations

Preliminary experiments were carried out to select fixation and staining methods most suitable for quantitative studies on the attachment of bacteria to oat cell walls. In addition to providing comparative data these experiments allowed preliminary observation of features of oat/bacterium interactions.

Oat leaves infiltrated with various bacteria and sterile distilled water were sampled after incubation for 24h and fixed in 2% glutaraldehyde/$OsO_4$ prior to dehydration. Comparisons were made on tissues fixed by infiltrating the glutaraldehyde under vacuum or by rotation. The latter technique was examined following the recent report by Hildebrand et al (1980) that improved preservation of fine detail of bacteria/host cell wall interactions could be achieved by infiltrating tissue by rotation, rather than by the physically more damaging method of infiltration under vacuum.

Ultrathin sections were stained with uranyl acetate/lead citrate, or by one of the cytochemical stains described below, prior to examination in the transmission electron microscope.

As carbohydrates, either of bacterial (EPS) or plant origin (cell walls) have been reported to have a regulatory role in bacteria/plant interactions (Rudolph, 1980;

Sequira, 1979), the use of cytochemical stains to allow the localization of carbohydrates at the EM level was examined. Two staining techniques were applied; (a) silver proteinate, a stain for polysaccharides with free glycol groups (Roland, 1978) and (b) ruthenium red, a stain for acidic polysaccharides (Luft, 1971).

Sections of oat cv. Milford leaves inoculated 24h previously with P. coronafaciens or P. coronafaciens var. atropurpurea were stained with silver proteinate. Optimal staining occurred when the TCH treatment lasted between 24 and 48h (see plate 2.3). The staining controls were effective in that poor staining (especially cell walls) was achieved with no periodic acid or $H_2O_2$ treated sections. No staining was achieved if either TCH or silver proteinate were omitted (Plate 2.3iii). Additional, subsequent staining with uranyl acetate/lead citrate did not enhance resolution.

Tissues infiltrated with P. coronafaciens, P. coronafaciens var. atropurpurea, P. fluorescens or P. tabaci were stained with ruthenium red. Although no further staining was recommended, post-staining of sections in uranyl acetate/ lead citrate greatly improved contrast and enhanced resolution of plant cell organelles (see Plates 2.4i and ii).

A. P. coronafaciens

Good fixation was achieved by vacuum infiltration of glutaraldehyde; cellular detail was easily recognisable. Abundant bacteria were found in intercellular spaces, some were in close association with mesophyll cells but there was no evidence of bacterial encapsulation onto cell walls or

obvious signs of attachment to walls by fibrillar material (Plate 2.1i).

Oat cells appeared healthy, the tonoplast remaining intact in the majority of cells. The plasmalemma was often highly convoluted and chloroplast lamellae occasionally appeared swollen in comparison with water infiltrated controls (Plate 2.1ii). Host cell walls were occasionally slightly swollen and rough in appearance where in contact with bacteria (Plate 2.1iii).

Rotary infiltration enabled good fixation and several fine details absent from vacuum infiltrated infected tissues were revealed. P. coronafaciens was seen to be held in a fibrillar matrix within the intercellular spaces. This matrix was usually associated with large groups of dividing bacteria and appeared to be bacterial in origin (Plate 2.2). The matrix was very similar to that described as extracellular polysaccharide (EPS) in other interactions (Ayers et al, 1979; El-Banoby, Rudolph and Mendgen, 1981; Politis and Goodman, 1980). P. coronafaciens was not attached to oat cell walls except through connection with EPS. Preservation of plant cell cytoplasm was similar to that achieved in vacuum infiltrated tissue.

The most striking feature of sections stained with silver proteinate was the enhancement of oat cell wall detail, in particular the middle lamella between mesophyll cells was darkly stained (Plate 2.3). Diffusely stained bacteria were confined to intercellular spaces and were often seen to contain dark staining bodies

Although some of these bacteria were associated with a

fibrillar matrix (EPS), the staining of this matrix was poor in contrast to conventional staining. Bacteria were not attached to oat cell walls but host cell wall swelling was easily discernible when stained with silver proteinate. Individual organelles within host cells were not always clear.

Ruthenium red staining of pathogen-inoculated tissue combined with rotary infiltration of fixative revealed that the bacterial masses of P. coronafaciens within intercellular spaces were almost always immersed in a fibrillar matrix (EPS). The EPS was intensely stained with ruthenium red, suggesting that it contained polysaccharides. An electron lucent halo was also evident around each bacterium within the EPS (Plate 2.4). Staining of the fibrillar matrix was further enhanced by the application of uranyl acetate/lead citrate stain (Plate 2.4ii). Host cell wall and cytoplasmic detail were also improved by this additional staining. Bacterial membranes were characteristically stained as described by Dinsdale et al (1978) and Pate and Ordal (1967). Attachment of bacteria to oat cell walls, except by virtue of EPS, was not noted. In general, the staining of tissues with ruthenium red was a marked improvement on the results achieved either with silver proteinate or without cytochemical staining.

B. P. coronafaciens var. atropurpurea

Vacuum infiltration of glutaraldehyde into hypersensitive tissues enabled satisfactory fixation and examination of oat tissues 24h after inoculation with P. coronafaciens var. atropurpurea and revealed that host cell damage was extensive with marked membrane disruption. Host cell walls

were also markedly affected (Plate 2.5i). Many bacteria were found in the intercellular spaces and some seemed to be trapped between collapsed mesophyll cells. Few bacteria were seen to be dividing.

A small proportion of bacteria were closely associated with host cell walls. Of these, a few were apparently immobilized in that they were attached by stalks or encapsulated (Plate 2.5ii). It was not possible to discern the origin (bacterial or host) of stalk or encapsulating material but localized disruption of the plant cell wall was apparent at many attachment sites.

Similar observations were noted for tissues fixed by rotary infiltration of glutaraldehyde. Bacteria were again observed to be attached to host cell walls in some instances, the fine nature of the attachment structures being preserved in greated detail. No EPS was associated with bacteria.

Although bacteria were seen close to cell walls, silver proteinate staining did not reveal the presence of attachment structures but host wall disruption at potential sites of attachment was more clearly visible (Plate 2.6). Ruthenium red staining of rotary-fixed tissue allowed the visualization of well-stained encapsulating material surrounding some bacteria (Plate 2.7). The absence of a mesh of fine fibrils distinguished the encapsulating material from the EPS seen to be associated with the pathogen, P. coronafaciens (Plate 2.4). EPS was not associated with P. coronafaciens var. atropurpurea free in the intercellular spaces.

C. P. fluorescens

Examination of tissue fixed under vacuum showed that cells of P. fluorescens were mostly free in the intercellular spaces between well preserved mesophyll cells. Some, but not all, bacteria were attached to host cell walls by enveloping structures (Plate 2.8i). As with P. coronafaciens var. atropurpurea, the delicate nature of these attachment structures were better preserved by infiltration of fixative by rotation (Plate 2.8ii). Ruthenium red staining improved resolution of attachment structures and demonstrated that more bacteria were attached to walls than was apparent using other stains (Plate 2.9). Symptomless host cells were well stained and bacteria free in the intercellular spaces were not associated with EPS.

D. P. tabaci

As a result of earlier observations, tissue inoculated with P. tabaci was examined following fixation by rotary infiltration of glutaraldehyde. As with the saprophyte, P. fluorescens, host cells were little affected by P. tabaci, cause of the resistant symptom in oat leaves. Bacteria were either free in the intercellular spaces or attached to host cell walls (Plate 2.10i), EPS was not observed. Similar results were recorded when ruthenium red was incorporated into the fixative (Plate 2.10ii).

The rotation method of infiltration also allowed detection of the physical entrapment of bacteria in oat leaves. The term physical entrapment was used by Hildebrand et al (1980) to describe the way in which bacteria can be localized within intercellular spaces of leaves following

infiltration (see Introduction). Fibrillar deposits derived from the re-deposition of dissolved host wall material were observed to surround and apparently to trap bacteria at sites within bean leaves (Hildebrand et al, 1980). Physical entrapment of bacteria was also noted in oat leaves inoculated with bacteria (Plate 2.10iii). The polysaccharide nature of the fibrillar crosses which localized the bacteria was inferred from staining of the deposits with both silver proteinate and ruthenium red (Plate 2.11).

E. Summary

The comparative observations made on fixation and staining are summarized in Table 2.1. Preservation of fine detail achieved by fixing tissue in glutaraldehyde by rotation was far superior to vacuum infiltration and also allowed good fixation of plant cells. Thus EPS associated with the pathogen and the attachment of incompatible bacteria were easily discernible in tissues fixed by rotation.

Silver proteinate confirmed the polysaccharide nature of host cell walls and fibrillar crosses (Plate 2.11i) associated with cell wall junctions but did not stain attachment structures or EPS.

Ruthenium red, especially in conjunction with uranyl acetate/lead citrate, allowed excellent visualization of the association between bacteria and oat cell walls. The staining procedure gave considerably improved resolution when compared with conventional technique and clearly demonstrated the polysaccharide nature of EPS associated with the pathogen, physical entrapment structures and

TABLE 2.1 Comparison of staining methods used to resolve components of bacteria/oat leaf interactions

| Ultrastructural feature [b] | Resolution with stains used after fixation by rotation [a] | | | |
|---|---|---|---|---|
| | Uranyl acetate/ lead citrate | Silver proteinate | Ruthenium red | Ruthenium red + uranyl acetate/lead citrate |
| Bacterium | + | + | + | ++ |
| Host cytoplasm | + | - | + | ++ |
| Host cell wall | + | ++ | + | + |
| EPS | + | - | + | ++ |
| Attachment | + | - | + | ++ |
| Physical entrapment | + | + | + | + |

[a] Infiltration with glutaraldehyde by rotation allowed visualization of EPS, attachment of bacteria to walls and also physical entrapment, none of which were clearly seen when tissues were fixed under vacuum. Visualization of features denoted as + good, ++ very good, - poor.

[b] As described in 2.1 A-E.

encapsulating material surrounding bacteria attached to cell walls. Ruthenium red staining of tissue fixed by rotation was therefore employed in the following ultrastructural studies.

2. Time course studies

Oat leaves infiltrated with P. coronafaciens, P. coronafaciens var. atropurpurea, P. fluorescens and P. tabaci were sampled 2.5, 12, 18 and 24h after inoculation and examined under the electron microscope following ruthenium red staining. A final sampling time for each interaction was also chosen to coincide with maximal development of macroscopic symptoms. Thus tissues were sampled at 48h after inoculation with P. coronafaciens, 36h for P. coronafaciens var. atropurpurea and 96h for P. tabaci and P. fluorescens. Effects of infection on host cells were estimated by comparison with tissue injected with sterile distilled water.

A. Susceptible interaction leading to normosensitive tissue collapse

P. coronafaciens was associated with EPS by 2.5h after inoculation (Plate 2.12i). Bacteria were found within the fibrillar matrix at each time after inoculation; the density of EPS staining increasing with time. By 48h after inoculation, abundant dividing bacteria were noted within collapsed mesophyll (Plate 2.12iv). No obvious attachment of bacteria to host cell walls was recorded but a few examples of physical entrapment were seen (Plate 2.12iii).

Oat leaf cells remained apparently unaffected by P. coronafaciens until 12-18h after inoculation when

vesiculation of the plasmalemma and swelling of the chloroplast lamellae were evident, especially adjacent to bacteria. In some host cells, dark staining appositions had formed inside their walls opposite bacteria (Plate 2.12iii). The mesophyll cells of the host tissue remained viable up to 48h after inoculation by which time previously intact cytoplasmic membranes were ruptured (especially the tonoplast) and cytoplasmic contents were barely recognisable within dead cells. Cell wall rupture, possibly due to damage during preparation of tissue for microscopy was inferred from observations of cytoplasmic debris within intercellular spaces in a few sections.

B. Hypersensitive response (HR)

Attachment to cell walls and physical entrapment of some cells of P. coronafaciens var. atropurpurea had occurred by 2.5h after inoculation (Plates 2.13i and ii). There was little sign of EPS production by the bacterium. Similar observations were recorded at each time interval until 36h when few bacteria were seen in the intercellular spaces and although attachment of bacteria to cell walls was evident, the majority of bacteria were free or trapped between collapsed mesophyll cells.

Twelve hours after inoculation the cytoplasm of many mesophyll cells had become highly vesiculated and there were occasional examples of the formation of vesicles from the otherwise intact plasmalemma of responding cells (Plate 2.13iii). Chloroplast granal lamellae were swollen slightly and rupture of the tonoplast had occurred in some cells (Plate 2.13iv). Where bacteria were encapsulated onto cell walls, the plant wall showed signs of loosening and

swelling (Plates 2.13i, iii and iv). An increase in these symptoms appeared to precede complete disorganization of the cytoplasm and collapse of dead cells. Most mesophyll cells had collapsed by 36h after inoculation (Plate 2.13v).

C. Resistant symptom

In tissues inoculated with P. tabaci, the slight swelling of chloroplast granal lamellae seen at 24h had become more pronounced after 96h when vesiculation and occasionally rupture of the plasmalemma was observed (Plates 2.14iii). The extensive cytoplasmic disorganization and death of host cells associated with the HR was not observed.

Many cells of P. tabaci were localized by fine attachment structures (Plate 2.14ii) and physical entrapment was observed as early as 2.5h after inoculation (Plate 2.14i). Increasing numbers of localized and attached bacteria were observed with time. No bacterial cells were associated with EPS and few dividing bacteria were present.

D. Saprophyte (symptomless) response

In accordance with the lack of macroscopic symptoms, P. fluorescens elicited little or no change in the ultrastructure of host cells (Plate 2.15). The only differences noted for these tissues compared with controls (Plate 2.16) was a slight swelling of chloroplast granal lamellae and some vesiculation of the plasmalemma in mesophyll cells.

Cells of P. fluorescens were not associated with EPS but were frequently entrapped and encapsulated onto cell walls by 2.5h after inoculation (Plates 2.15i-v). Little bacterial division was detected but attachment to mesophyll

cell walls appeared to increase with time. Attachment was usually associated with localized erosion of the plant cell wall (Plate 2.15v).

3. Quantitative investigation into the attachment of bacteria to oat cell walls

A quantitative examination of the observed attachment of pseudomonads to oat cell walls was conducted. At least 100 bacteria were counted from single sections (to avoid repetition of counts) of oat tissues previously infiltrated with P. coronafaciens, P. coronafaciens var. atropurpurea, P. tabaci and P. fluorescens. Tissues were examined 2.5, 12 and 24h after inoculation. The experiment was repeated with different oat plants and bacterial suspensions. Bacteria were assigned to categories on the basis of their attachment to cell walls or production of EPS; the grading scheme used is summarized in Fig. 2.1. Differentiation between physical entrapment and attachment of bacteria to cell walls was made on the basis [illegible] that the latter involved some wall modification.

The results obtained confirmed that the pathogen, P. coronafaciens, was never attached but that its cells were nearly always associated with EPS (Table 2.2). By contrast, P. coronafaciens var. atropurpurea, P. tabaci and P. fluorescens were attached at each time interval but, except in the case of P. fluorescens, 24h after inoculation, this attachment rarely exceeded 30% of bacteria. Non-pathogenic bacteria were not associated with EPS production.

The frequency of attachment to oat cell walls increased

FIGURE 2.1 Assignment of bacteria to categories for quantitative assessment of their attachment to plant cell walls and production of EPS

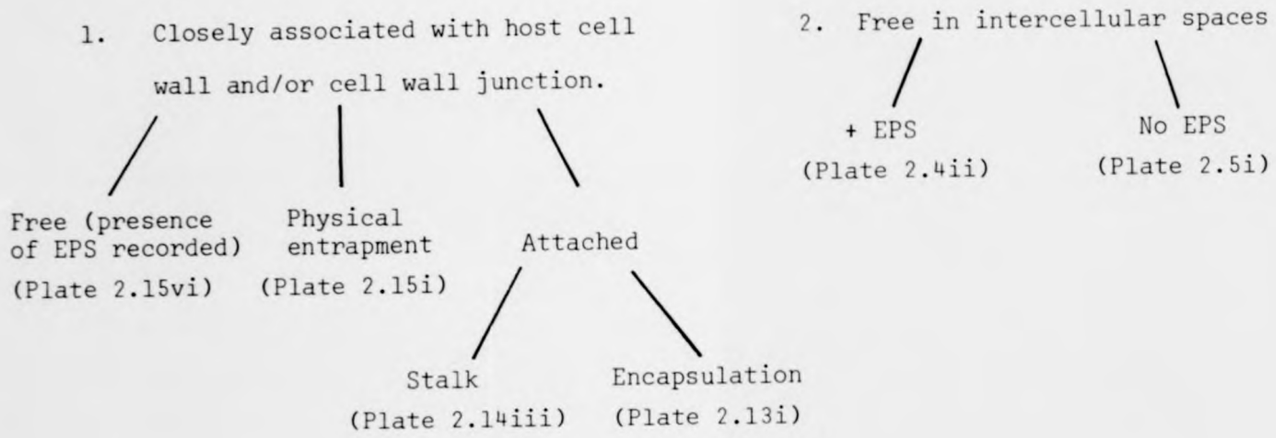


TABLE 2.2 Quantitative analysis of bacteria/cell wall interactions in oat leaves [a]

| Time after inoculation (h) | Bacterium | Percentage of bacteria [b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Close to wall or junction | | | | | | Free in intercellular spaces | |
| | | Free | Physical entrapment | Attached | | | | | |
| | | | | Stalk | | Encapsulation | | +EPS [c] | No EPS |
| 2.5 | P. coronafaciens | 25.5 [c] (12-39) | 23.2 [c] (-) | - | (-) | - | (-) | 41.4 (30-53) | 9.5 (7-12) |
| | P. coronafaciens var. atropurpurea | 42.4 (40-45) | 9.9 (6-14) | 4.4 | (2-7) | 9.9 | (5-15) | 2.0 (0-4) | 31.5 (29-36) |
| | P. fluorescens | 52.0 (35-69) | 20.1 (15-25) | 3.3 | (2-5) | 4.2 | (2-6) | - (-) | 20.1 (7-33) |
| | P. tabaci | 37.6 (24-52) | 21.8 (16-28) | - | (-) | 10.4 | (0-21) | - (-) | 30.7 (28-33) |
| 12 | P. coronafaciens | 43.0 [c] (38-48) | 4.2 [c] (0-9) | - | (-) | - | (-) | 51.4 (48-53) | 1.4 (0-3) |
| | P. coronafaciens var. atropurpurea | 6.8 (5-9) | 29.5 (28-32) | 1.4 | (1-2) | 22.3 | (21-24) | 15.0 (0-30) | 25.0 (13-37) |
| | P. fluorescens | 16.3 (11-22) | 41.3 (30-53) | 4.8 | (4-6) | 24.0 | (20-28) | - (-) | 13.5 (2-25) |
| | P. tabaci | 12.8 (11-15) | 30.8 (18-44) | 4.6 | (4-6) | 30.3 | (17-44) | - (-) | 21.5 (21-22) |
| 24 | P. coronafaciens | 31.1 [c] (27-35) | - (-) | - | (-) | - | (-) | 59.2 (54-64) | 9.7 (9-11) |
| | P. coronafaciens var. atropurpurea | 23.0 (10-36) | 3.3 (0-7) | 2.9 | (0-6) | 27.3 | (19-36) | 2.4 (0-5) | 41.1 (32-50) |
| | P. fluorescens | 16.0 (12-20) | 15.5 (12-18) | 2.6 | (2-3) | 49.0 | (42-56) | - (-) | 17.0 (16-18) |
| | P. tabaci | 28.5 (25-32) | 12.0 (8-16) | 11.0 | (-) | 16.3 | (9-24) | - (-) | 33.1 (26-40) |

[a] See Fig. 2.1 for further detail of categories described.

[b] Counts from single sections of at least 100 bacteria from 2 repeated experiments. Counts were made at intervals after inoculation of oat cv. Milford leaves with suspensions of $10^8$ bacteria/ml. The range between replicate counts a given in parentheses.

[c] With extracellular polysaccharide (EPS).

significantly with time for each of the non-pathogenic bacteria (Table 2.3; $\chi^2$, p = 0.05). This increase was significant between 2.5-12h for P. coronafaciens var. atropurpurea and P. tabaci. Increases in attachment of P. fluorescens to cell walls were also significant between 12 and 24h after inoculation. Comparisons between different species showed, however, that there was no significant difference ($\chi^2$, p=0.05) in the percentage of attachment recorded for P. coronafaciens var. atropurpurea, P. fluorescens or P. tabaci at each time after inoculation.

Table 2.2 also shows that physical entrapment occurred in each of the four interactions examined. The lack of physical entrapment of P. coronafaciens apparent 24h after inoculation may be attributed to the masking of physical entrapment structures by the presence of extensive EPS associated with the bacterial groups in the intercellular spaces (see Plate 2.4ii).

4. Ultrastructural observations on the infection of tobacco by P. coronafaciens and P. tabaci

An outstanding feature of the results of the ultrastructural study of the injection of oats by bacteria was the production of EPS by the oat halo blight bacterium P. coronafaciens but not by non-pathogens such as P. tabaci. It was therefore of interest to examine P. coronafaciens and P. tabaci growing within tobacco leaves. Sites in tobacco infiltrated with these bacteria were prepared for electron microscopy (using ruthenium red) 24h after inoculation.

Results obtained were closely comparable to those obtained with oats. The pathogen P. tabaci was not

TABLE 2.3 The relationship between attachment of pseudomonads to oat cell walls and time after inoculation

| Bacterium[a] | Percentage attachment [b] | | |
|---|---|---|---|
| | 2.5h | 12h | 24h |
| *P. coronafaciens* | - | - | - |
| *P. coronafaciens* var. *atropurpurea* | 14.3 | 23.6* | 30.1 |
| *P. fluorescens* | 7.5 | 28.8* | 51.5* |
| *P. tabaci* | 10.4 | 34.9* | 27.3 |

[a] Oat cv. Milford leaves infiltrated with $10^8$ bacteria/ml sterile distilled water suspensions.

[b] Percentage attachment (stalk and encapsulation) determined from counts of 100 bacteria from single sections of oat tissues (2 repeated experiments) at 2.5, 12 and 24h after inoculation.

* Significantly different from previous time interval ($\chi^2$, $p = 0.05$).

attached to cell walls but was always associated with extensive production of EPS (Plate 2.17). By contrast, P. coronafaciens did not appear to produce EPS in tobacco, instead many cells were attached to mesophyll cell walls (Plate 2.18).

Key to abbreviations used in electron micrographs

a, dark staining apposition; b, bacterium;
c, chloroplast; cl, chloroplast lamellae;
e, enveloping structure; er, endoplasmic reticulum;
f, fibrillar cross; fm, fibrillar matrix;
g, Golgi body; h, electron lucent halo; hc, host cell; ic, intercellular space; m, mitochondrion;
ml, middle lamella; n, nucleus; pl, plasmalemma;
s, stalk attachment; t, tonoplast; v, vesiculation;
w, plant cell wall.
Bars represent 0.5µm.

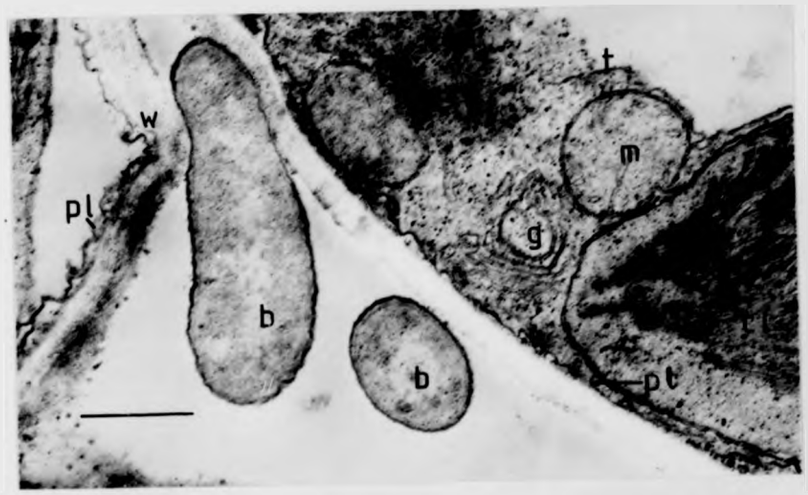
PLATE 2.1 Oat leaves fixed by vacuum infiltration and stained with uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens.

(i) Note the good resolution of organelles in the mesophyll cells and the convolution of the plasmalemma close to the bacteria.

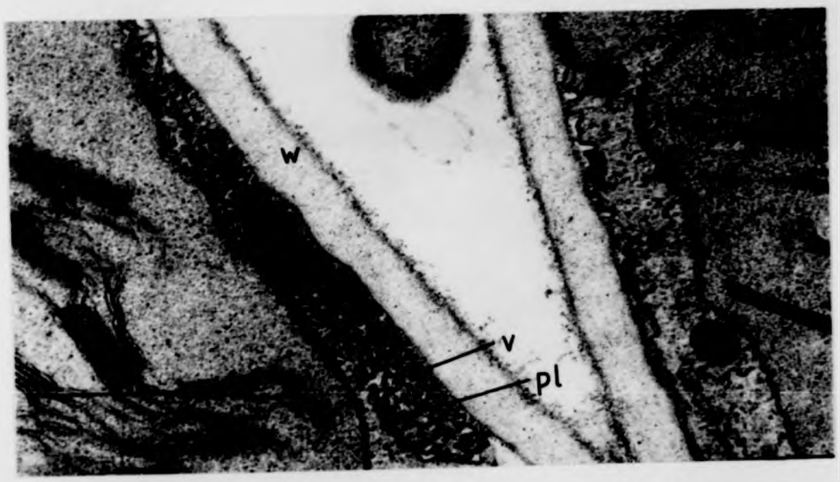
(ii) Vesicles (arrowed) accumulating between the plasmalemma and cell wall of a mesophyll cell close to intercellular bacteria.

(iii) Bacterium apparently in contact with mesophyll cell walls but not associated with plant wall erosion or marked swelling.

(i)



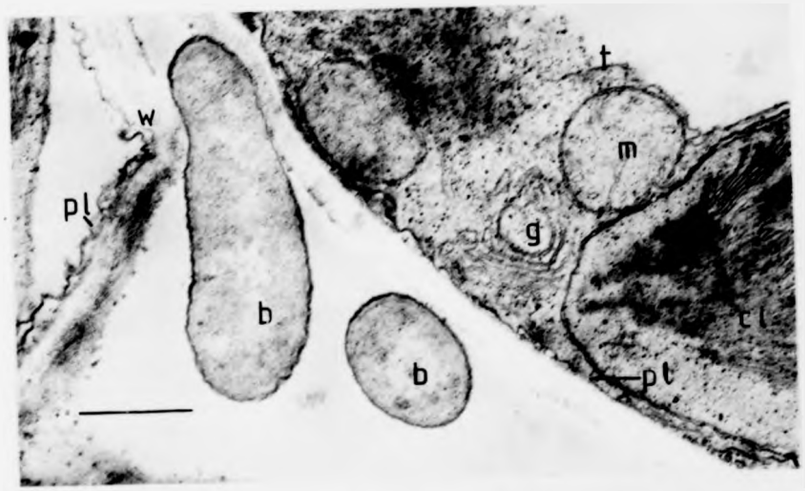

(ii)



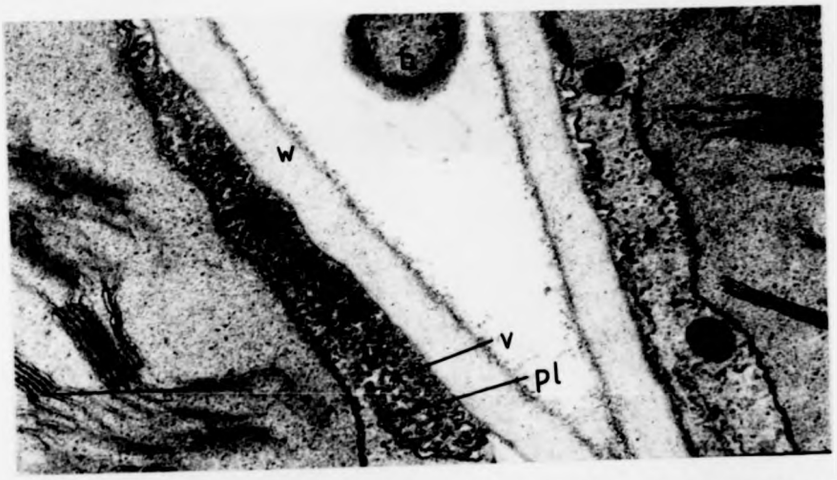

(iii)


PLATE 2.1

(i)




(ii)




(iii)

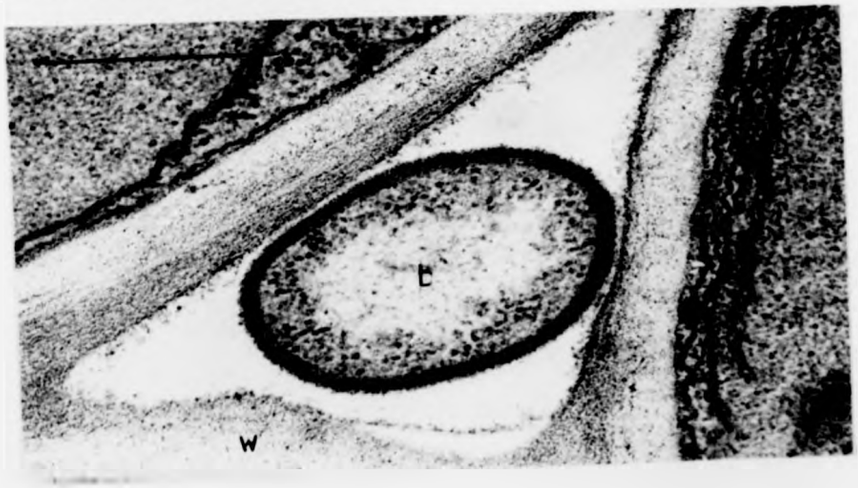



PLATE 2.1


PLATE 2.2 Oat leaf fixed by rotation and stained with uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens. Note the bacterial cells in a fibrillar matrix.


PLATE 2.2 Oat leaf fixed by rotation and stained with uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens. Note the bacterial cells in a fibrillar matrix.

PLATE 2.3 Oat leaves fixed by rotation and stained with silver proteinate 24h after inoculation with P. coronafaciens.

(i) 24h TCH treatment; note dense staining of middle lamella and also the apposition within the host cell adjacent to the bacteria. Bacteria occur within a poorly stained fibrillar matrix.

(ii) 72h TCH treatment; dark staining deposits on inner host cell walls are easily discernible. Host cell organelles are poorly stained.

(iii) TCH treatment omitted; note that the tissue is poorly stained with only the 'ghosted' outlines of bacteria and host cells apparent.

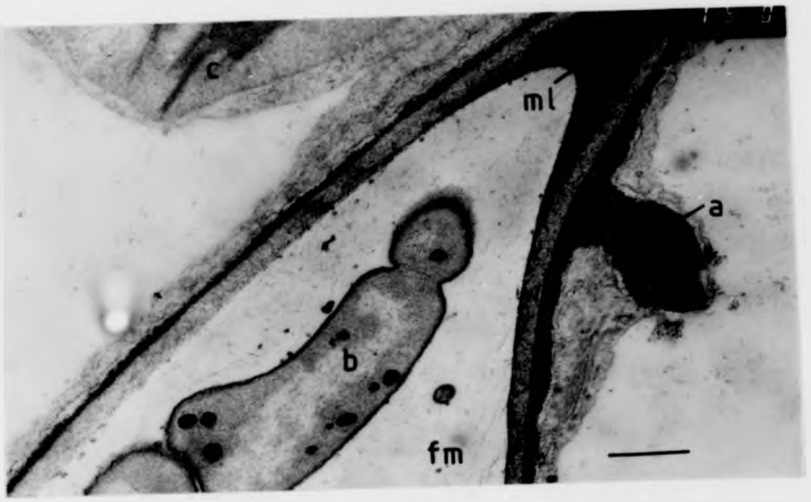
ained
oculation

staining
sition
e bacteria.
ned

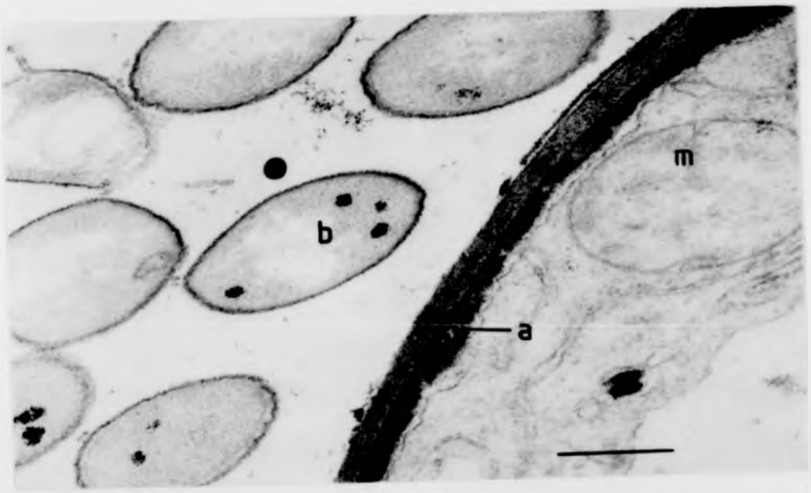
ning
re easily
are

that
nly the
host cells

(i)




(ii)




(iii)

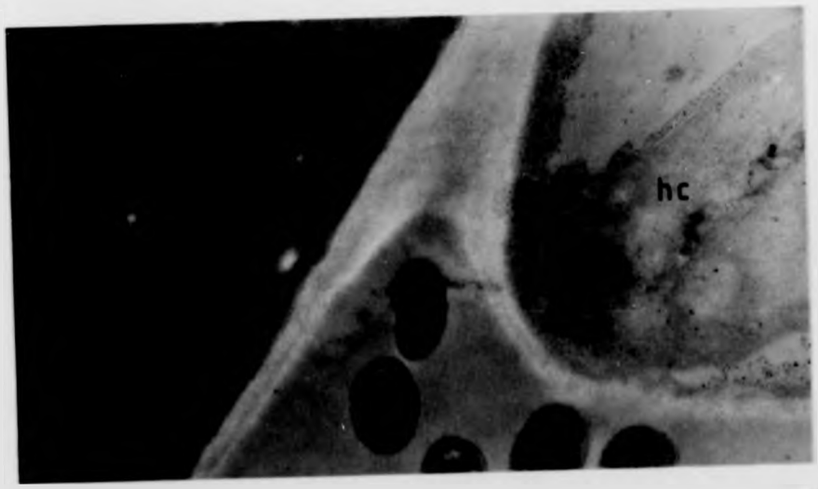
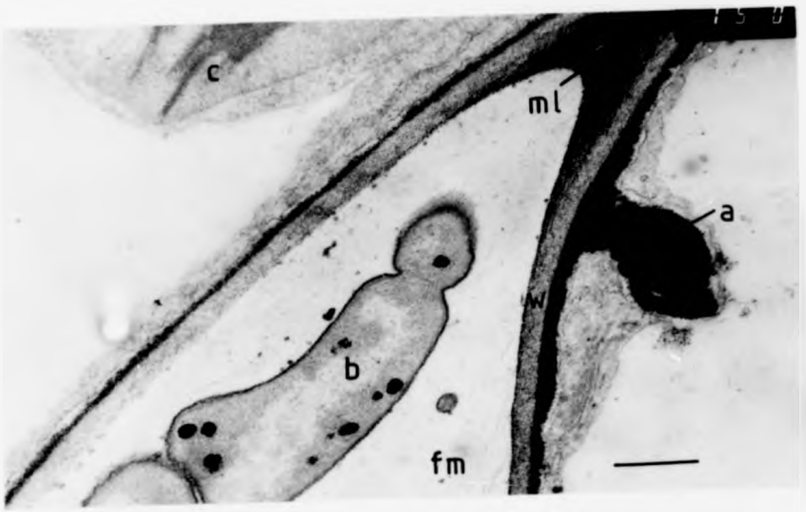
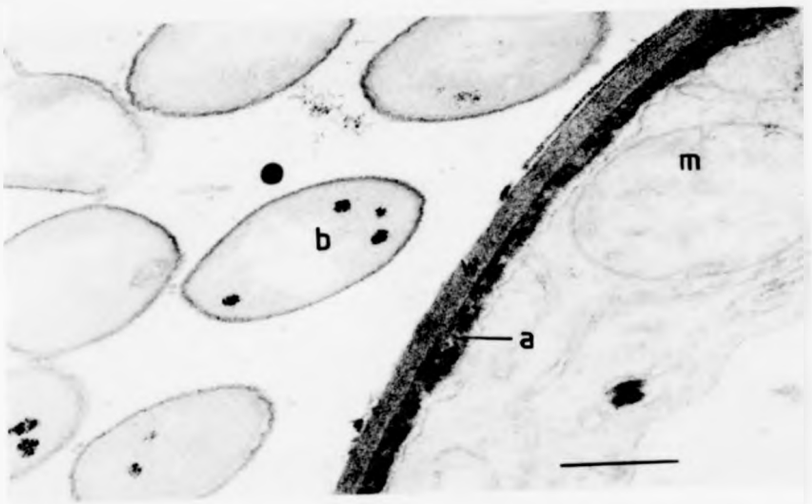



PLATE 2.3

(i)




(ii)




(iii)

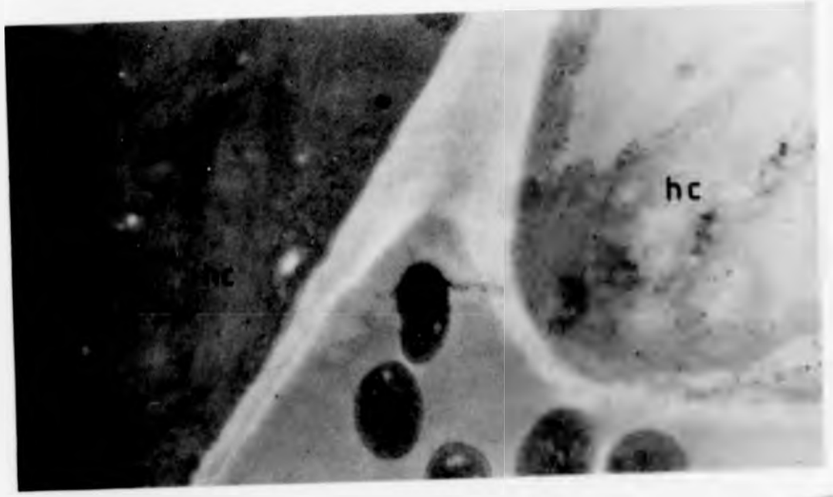


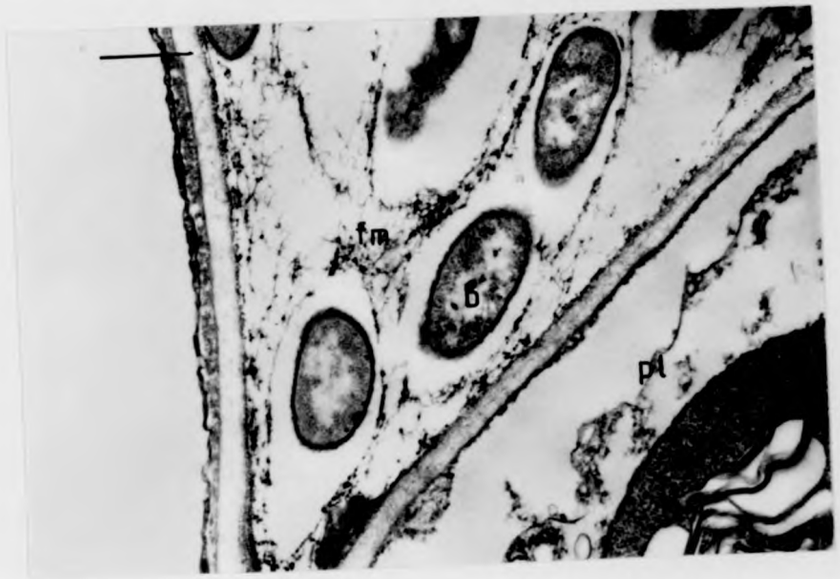

PLATE 2.3

PLATE 2.4 Oat leaves fixed by rotation and stained with ruthenium red 24h after inoculation with *P. coronafaciens*.

(i) No post-stain; bacteria associated with fibrillar matrix within intercellular spaces. Note vesiculation of plasmalemma and swelling of chloroplast lamellae within host cell.

(ii) Post-stained in uranyl acetate/lead citrate; bacteria, fibrillar matrix and host cell detail are accentuated.

(i)




(ii)

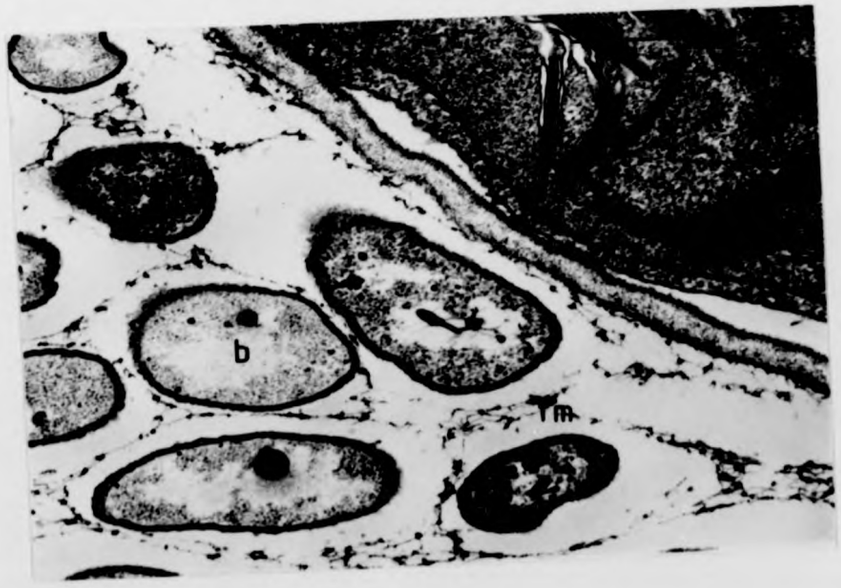
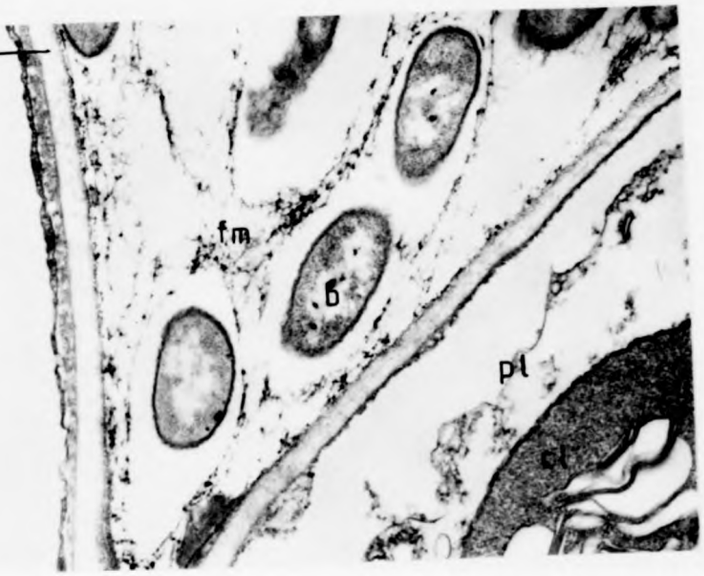



PLATE 2.4

(i)




(ii)

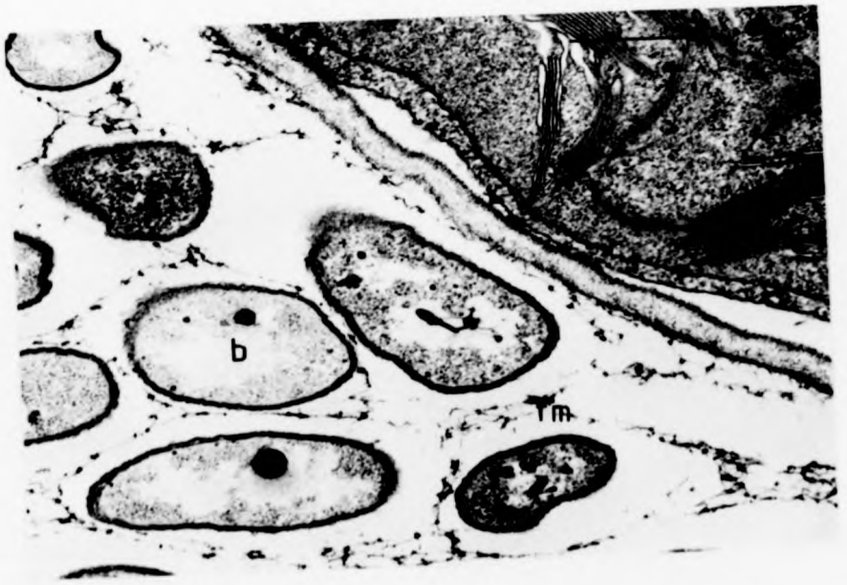



PLATE 2.4

PLATE 2.5 Oat leaves fixed by vacuum infiltration and stained with uranyl acetate/lead citrate 24h after inoculation with *P. coronafaciens* var. *atropurpurea*.

(i) Note bacteria apparently free in intercellular spaces between oat mesophyll cells. Vesiculation of the host plasmalemma is apparent but the tonoplast is intact. Host cell organelles are well stained, note the slight distortion of host cell wall.

(ii) Group of bacteria attached to host wall by enveloping structure. Host cell wall alteration can be detected close to bacteria.

PLATE 2.5 Oat leaves fixed by vacuum infiltration and stained with uranyl acetate/lead citrate 24h after inoculation with *P. coronafaciens* var. *atropurpurea*.

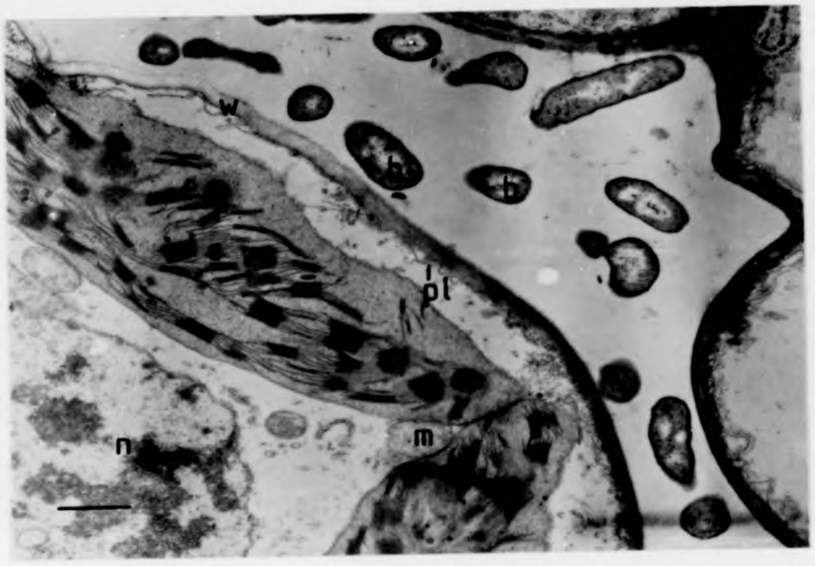
(i) Note bacteria apparently free in intercellular spaces between oat mesophyll cells. Vesiculation of the host plasmalemma is apparent but the tonoplast is intact. Host cell organelles are well stained, note the slight distortion of host cell wall.

(ii) Group of bacteria attached to host wall by enveloping structure. Host cell wall alteration can be detected close to bacteria.

tration and
citrate 24h
faciens var.

ee in
mesophyll
t plasma-
ast is intact.
ained, note
ll wall.
to host wall
cell wall
to bacteria.

(i)




(ii)

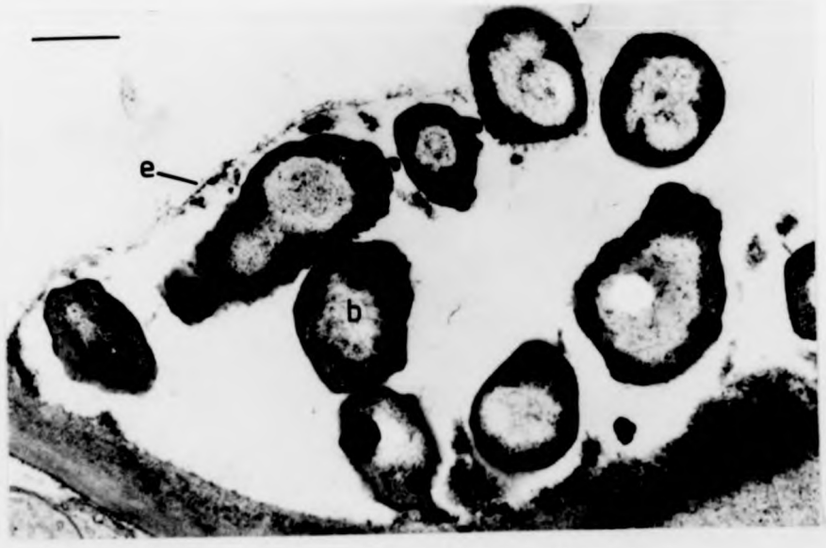
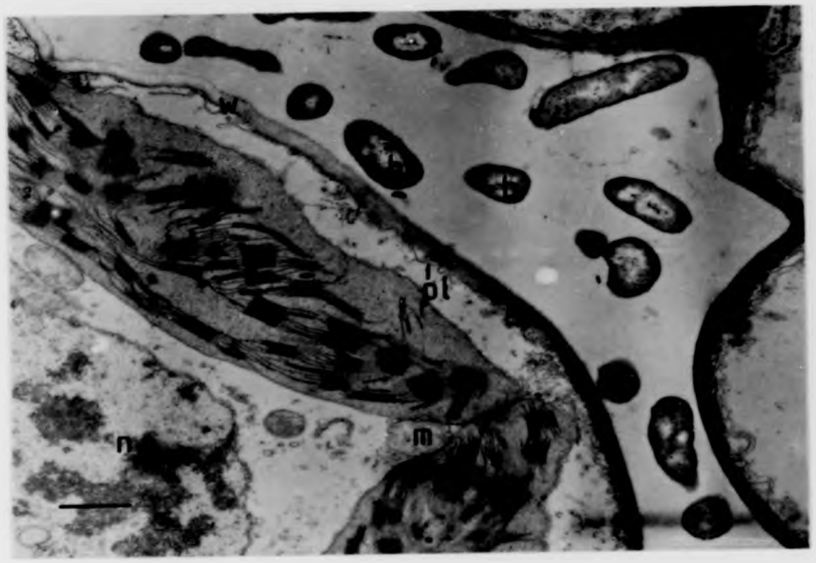



PLATE 2.5

ration and
citrate 24h
aciens var.

e in
mesophyll
plasma-
st is intact.
ined, note
l wall.
to host wall
ell wall
to bacteria.

(i)




(ii)


PLATE 2.5


PLATE 2.6 Oat leaves fixed by rotation and stained with silver proteinate 24h after inoculation with P. coronafaciens var. atropurpurea. Bacteria are in close contact with mesophyll cell walls but there is only slight indication of plant wall swelling and no enveloping structures are visible. Note that the plasmalemma (arrowed) has come away from the cell wall.

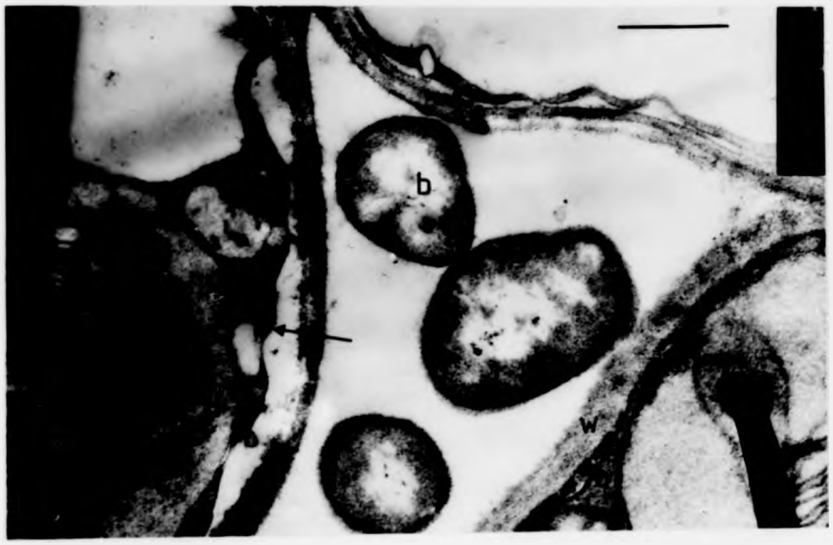



PLATE 2.6 Oat leaves fixed by rotation and stained with silver proteinate 24h after inoculation with P. coronafaciens var. atropurpurea. Bacteria are in close contact with mesophyll cell walls but there is only slight indication of plant wall swelling and no enveloping structures are visible. Note that the plasmalemma (arrowed) has come away from the cell wall.

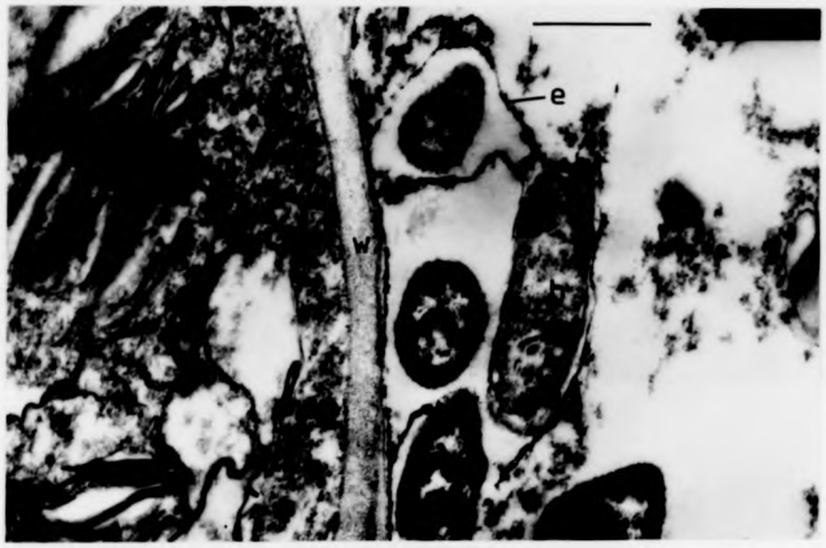



PLATE 2.7 Oat tissue fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens var. atropurpurea. Bacteria are attached to host mesophyll cell wall by an enveloping structure.

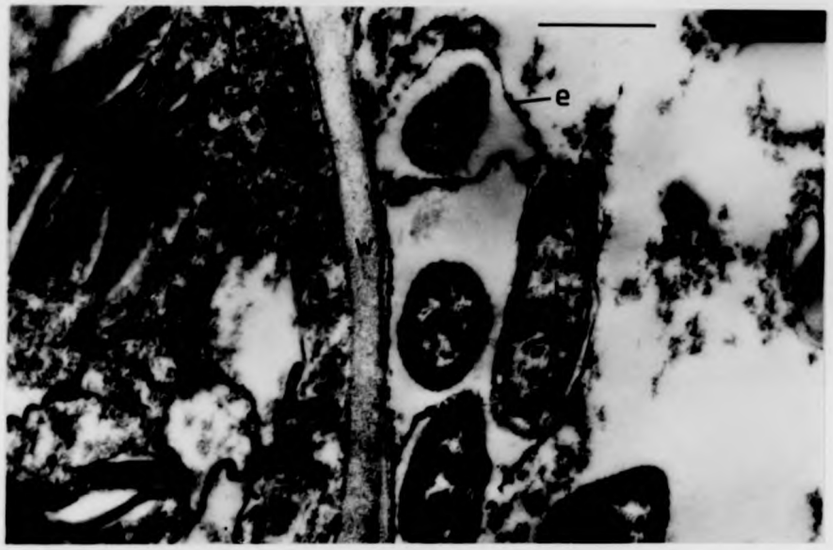



PLATE 2.7 Oat tissue fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens var. atropurpurea. Bacteria are attached to host mesophyll cell wall by an enveloping structure.

PLATE 2.8 Oat leaves fixed by (i) vacuum infiltration or (ii) by rotation and stained in uranyl acetate/lead citrate 24h after inoculation with P. fluorescens.

(i) Note the fibrillar attachment structures emanating from collars (arrowed) on adjacent mesophyll cell walls.

(ii) Bacteria encapsulated onto mesophyll cell wall by fine fibrils.

iltration

uranyl

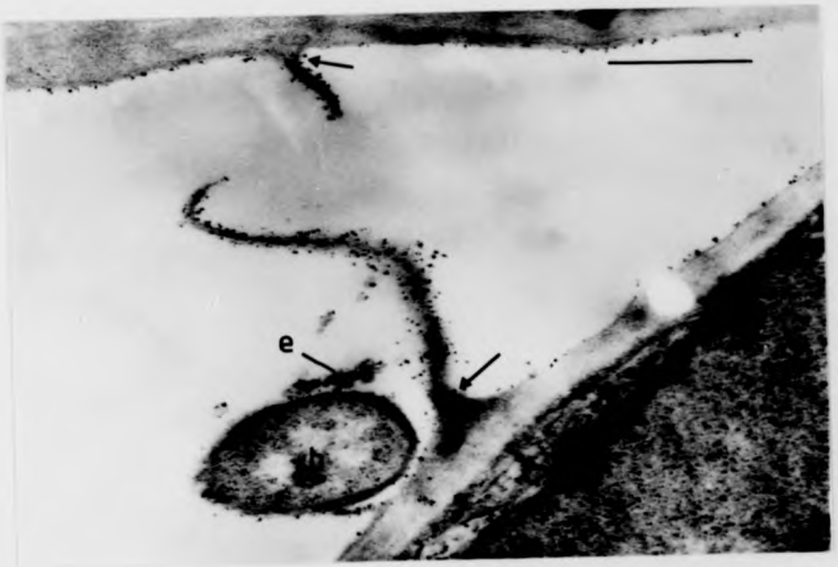
culation

structures

n adjacent

esophyll

(i)




(ii)

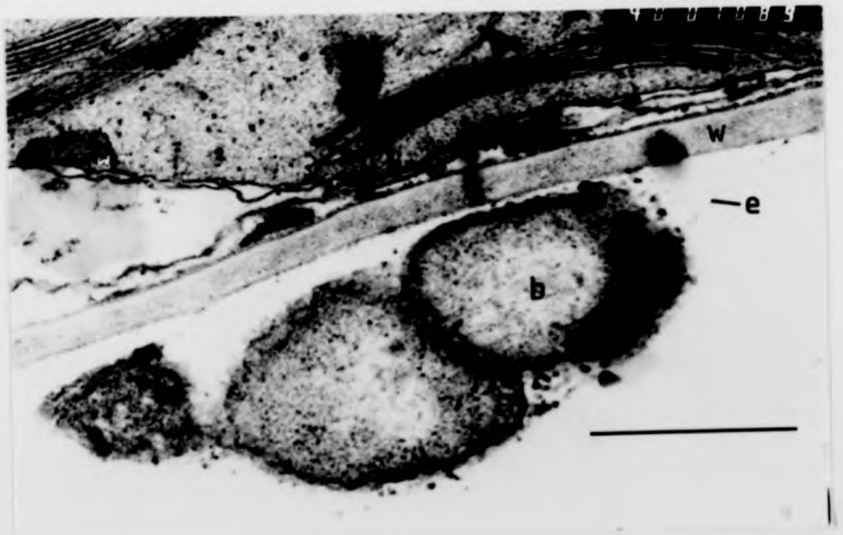
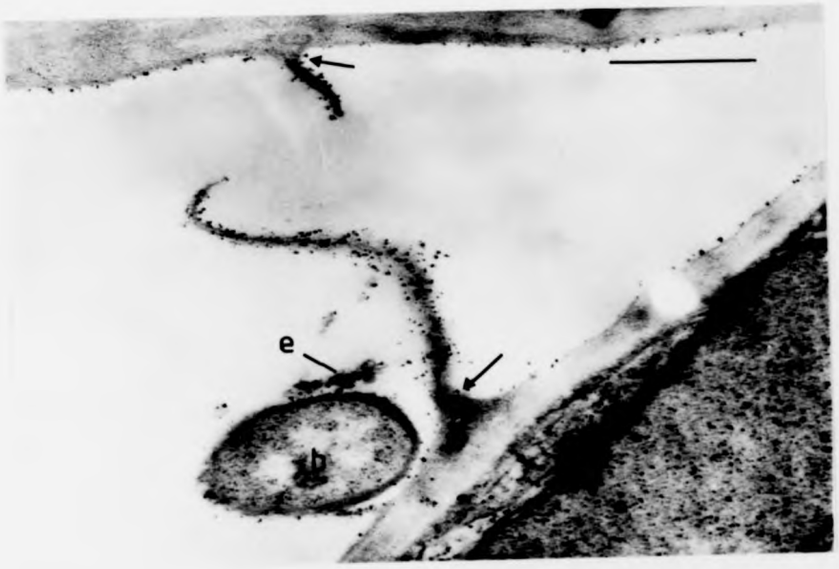



PLATE 2.8

(i)




(ii)

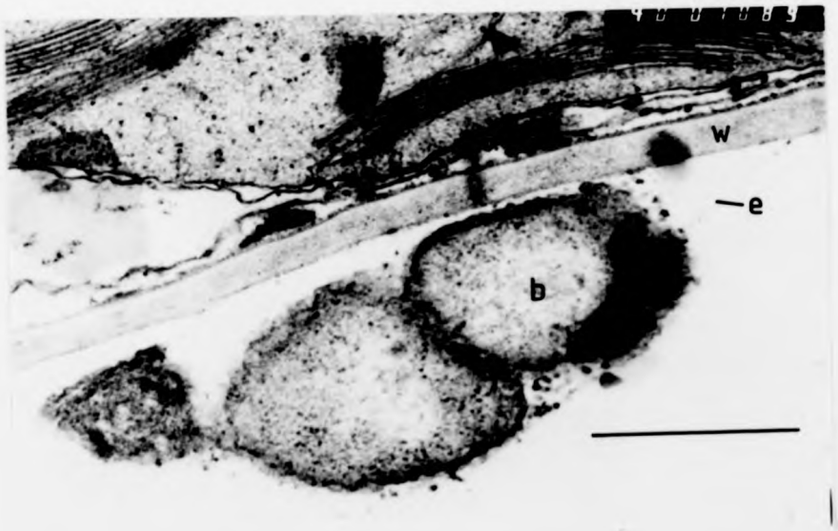



PLATE 2.8


PLATE 2.9 Oat leaf fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. fluorescens. The structure enveloping the bacteria and attaching them to the mesophyll cell wall appears to be associated with plant wall modification (arrowed). Note the formation of vesicles and a dark staining apposition within host cells between their plasmalemma and cell wall.


PLATE 2.9 Oat leaf fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. fluorescens. The structure enveloping the bacteria and attaching them to the mesophyll cell wall appears to be associated with plant wall modification (arrowed). Note the formation of vesicles and a dark staining apposition within host cells between their plasmalemma and cell wall.

PLATE 2.10 Oat leaves fixed by rotation and stained in uranyl acetate/lead citrate 24h after inoculation with *P. tabaci*.

(i) Bacterium attached by collar (arrowed) at the junction between mesophyll cells. Note the vesicles accumulating in the host cell between the plasmalemma and cell wall.

(ii) Ruthenium red staining; attachment of bacteria to plant mesophyll cell wall by an enveloping structure which involves clear modification of the cell wall. The host cell plasmalemma is still intact.

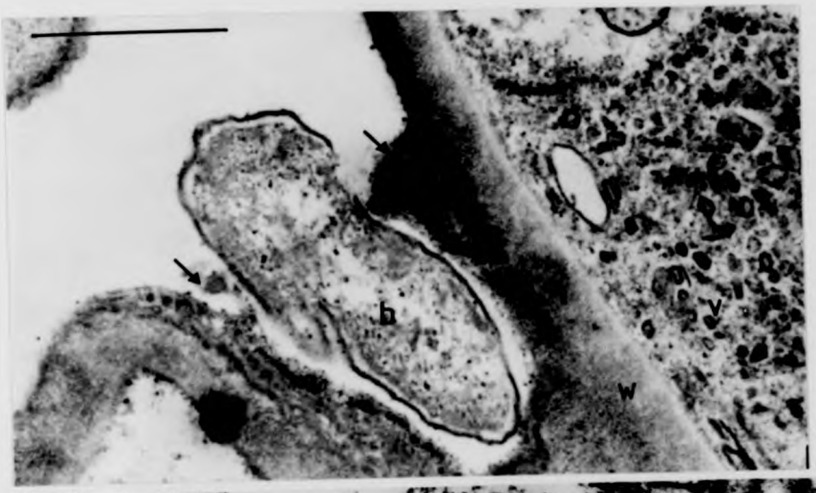
(iii) Bacterium and fibrillar cross between mesophyll cells.

tained in
ter

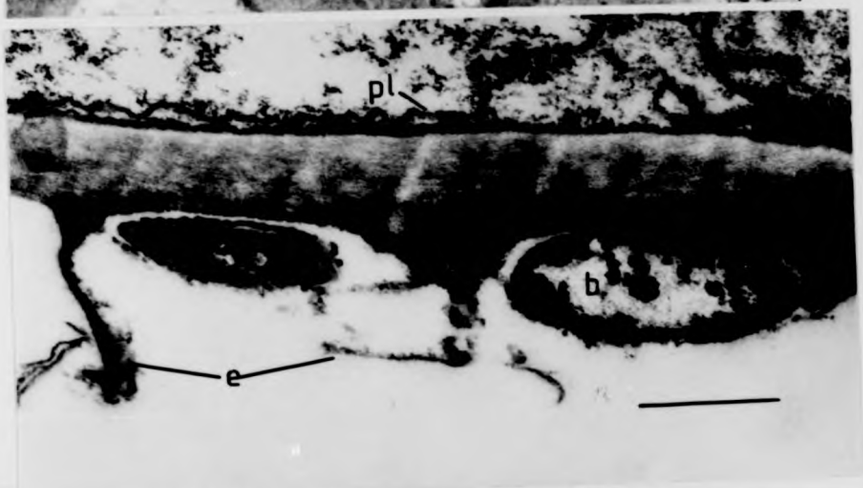
(arrowed)
cells.
the host
ell wall.
achment of
all by an
s clear
he host

ss between

(i)




(ii)




(iii)

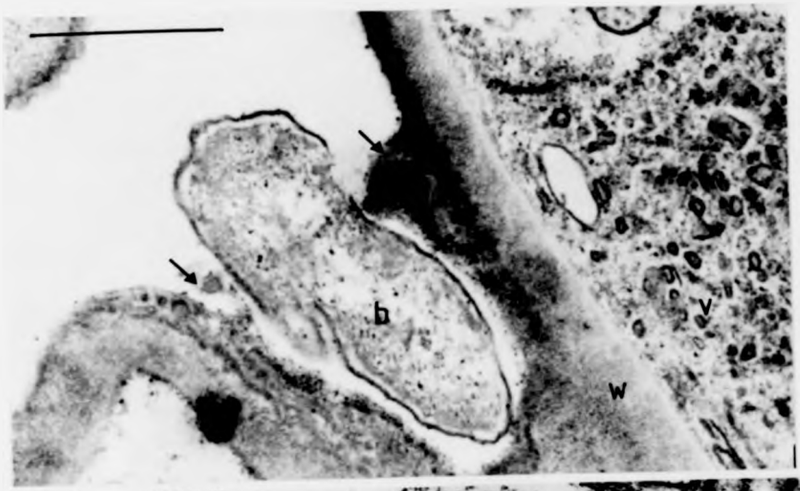

PLATE 2.10

stained in
fter

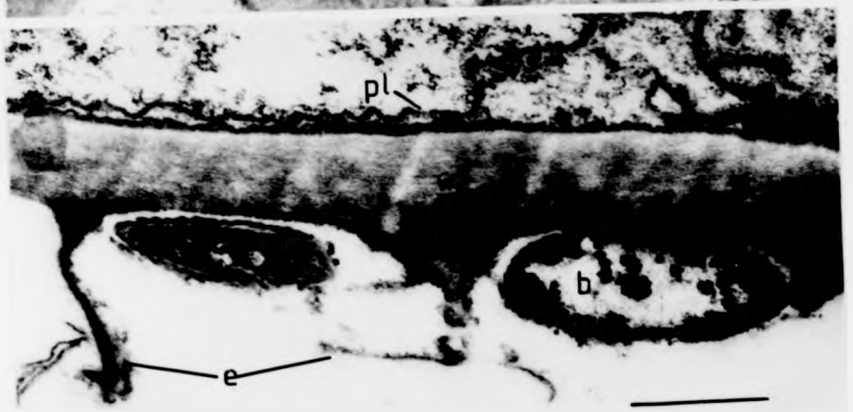
(arrowed)
cells.
the host
cell wall.
tachment of
wall by an
es clear
The host

oss between

(i)




(ii)




(iii)

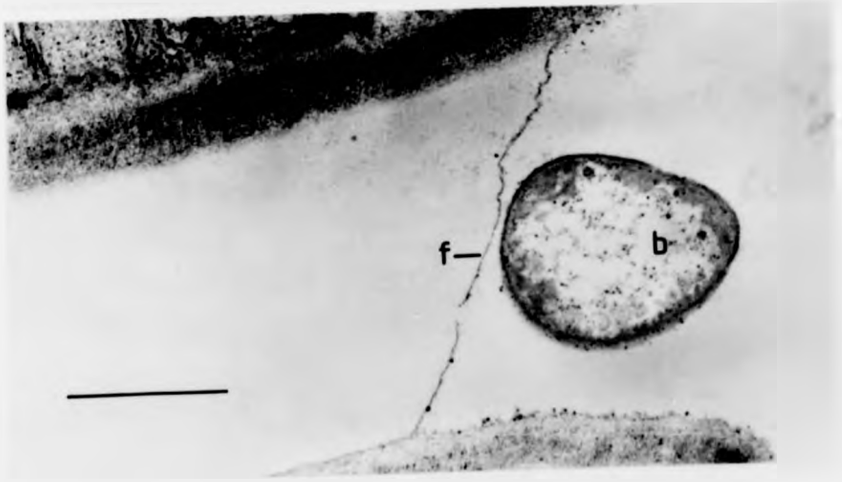



PLATE 2.10

PLATE 2.11 Oat leaves fixed by rotation and stained with (i) silver proteinate or (ii) ruthenium red 24h after inoculation with sterile distilled water alone.

(i) Note well-stained fibrillar cross at junction of mesophyll cells. Host cell detail is poorly defined except for the cell wall and in particular the middle lamella between adjacent cells.

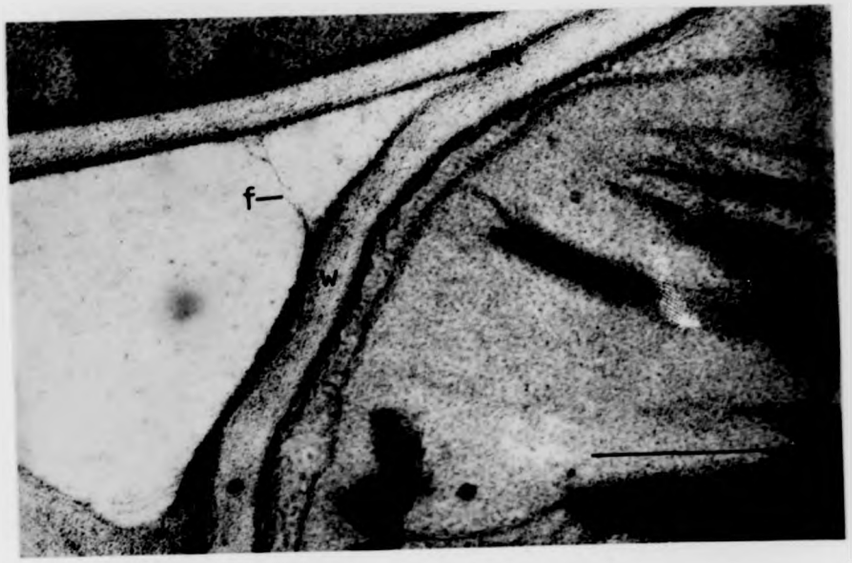
(ii) Fibrillar cross at a junction between mesophyll cells with granular deposit on the exterior of walls within the fibril. Note that there is good resolution of organelles within mesophyll cells.

tained with
enium red
distilled

ross at
t cell
r the cell
lamella

n between
sit on the
il. Note
rganelles

(i)




(ii)

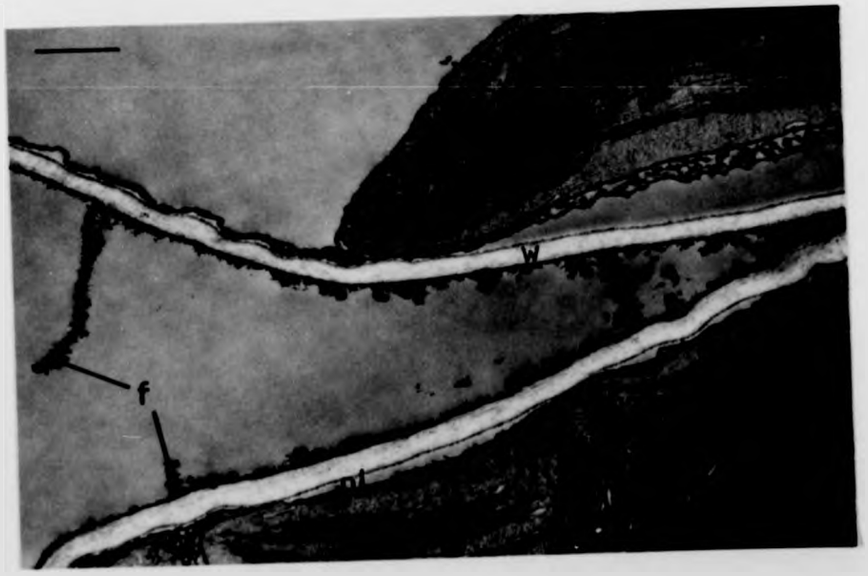



PLATE 2.11

stained with
henium red
e distilled

cross at
st cell
or the cell
lamella

on between
osit on the
il. Note
organelles

(i)


(ii)


PLATE 2.11

PLATE 2.12 Oat leaves fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate at intervals after inoculation with *P. coronafaciens*.

(i) 2.5h; bacteria in dense fibrillar matrix within intercellular spaces. Note that the organelles of mesophyll cells appear to be unaffected by the infection.

(ii) 12h; vesiculation of plant cell plasmalemma is now apparent close to bacteria.

(iii) 18h; host plasmalemma vesiculation has increased from that observed at 12h. Note the appearance of a dark staining apposition between the plasmalemma and cell wall of the mesophyll cell close to the bacteria. The bacteria appear to be physically entrapped by a fibrillar cross distinguishable from the EPS by its lightly stained appearance.

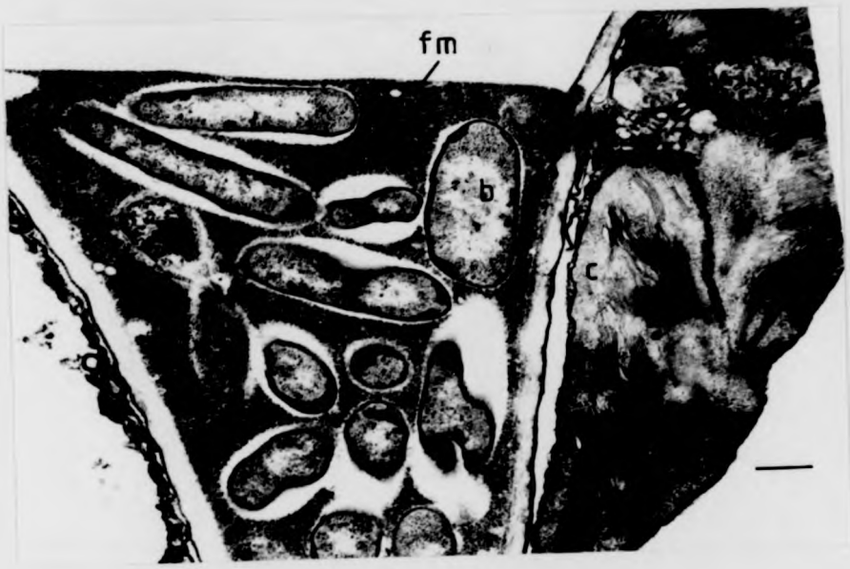
(iv) 48h; death of mesophyll cells is indicated by membrane disruption. Bacteria are associated with a densely staining fibrillar matrix. Note the electron lucent halo around each bacterium.

tained with
ead citrate
h

illar
. Note
ells appear

cell plasma-
teria.
iculation has
?h. Note
apposition
wall of the
ria. The
entrapped by
from the EPS
.
lls is
Bacteria
ining
tron lucent

(i)




(ii)

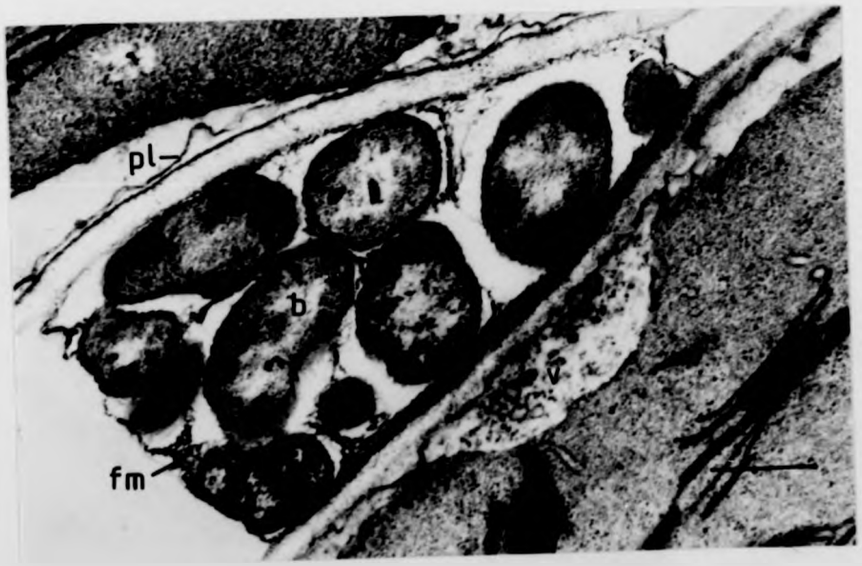


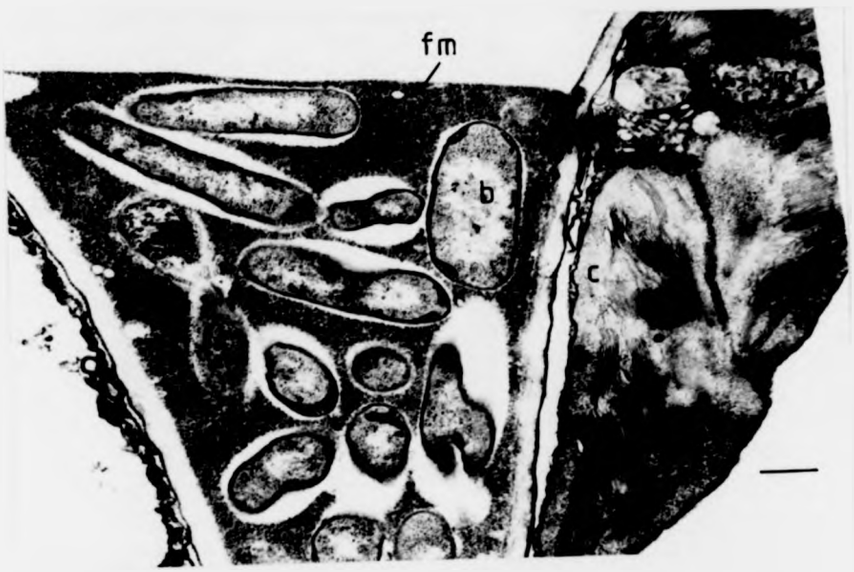

PLATE 2.12

tained with
lead citrate
h

rillar
s. Note
cells appear

cell plasma-
cteria.
iculation has
2h. Note
apposition
wall of the
ria. The
entrapped by
from the EPS
.
lls is
Bacteria
ining
tron lucent

(i)




(ii)

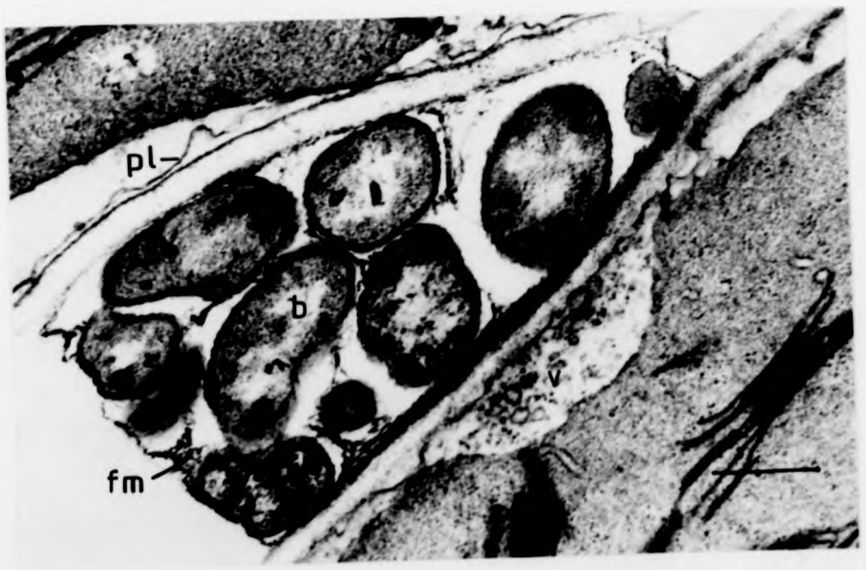
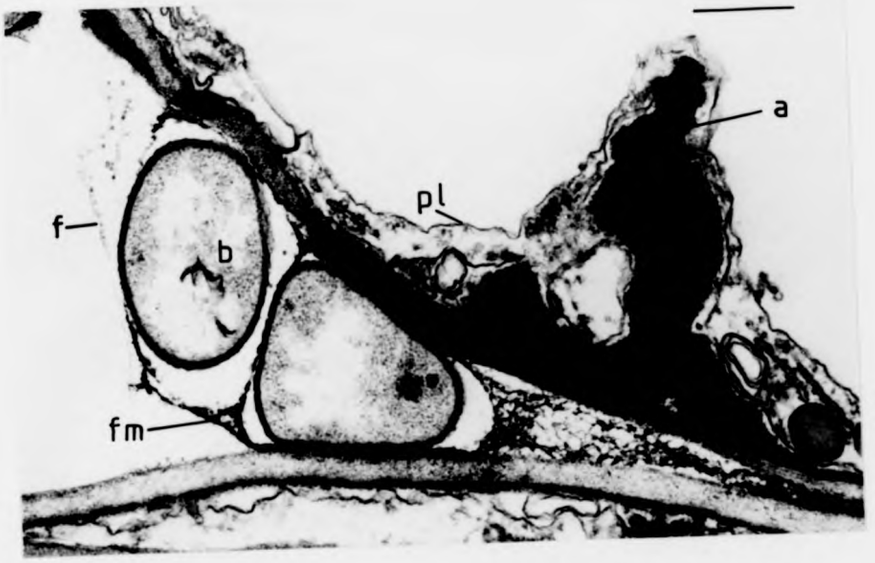



PLATE 2.12

(iii)




(iv)

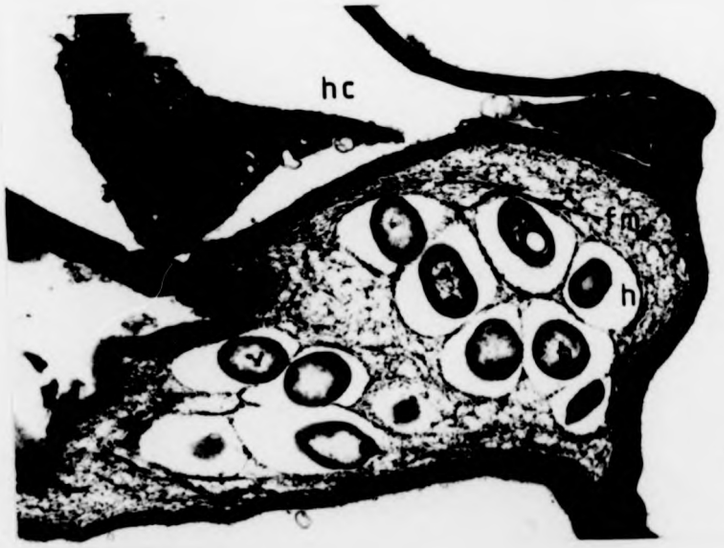
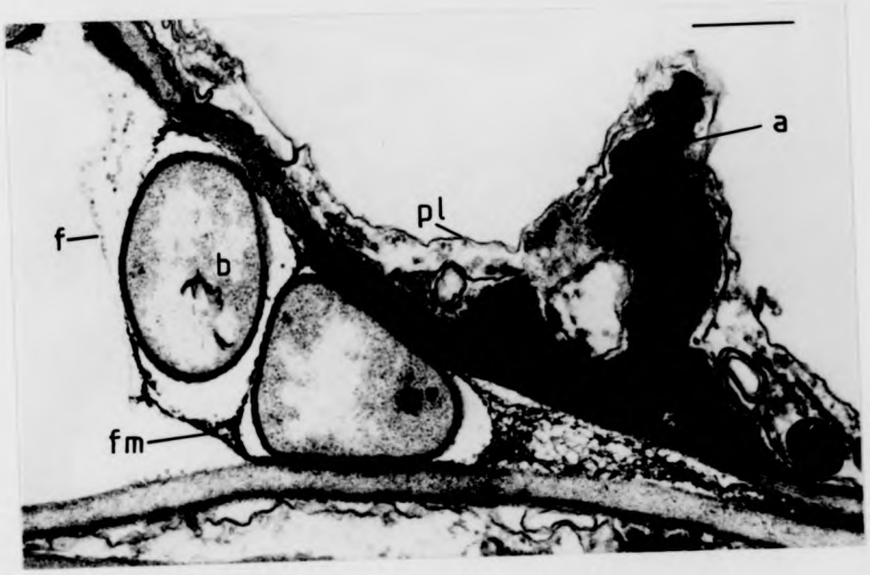



PLATE 2.12

(iii)




(iv)

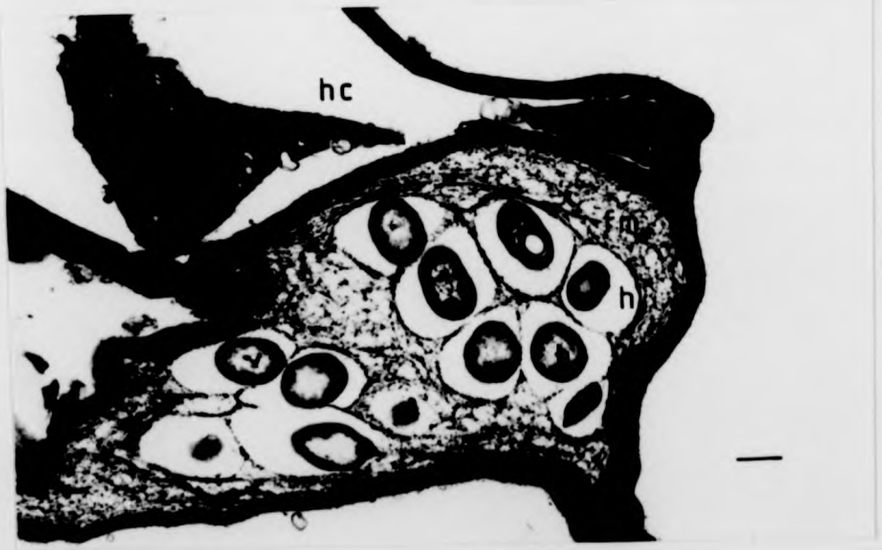



PLATE 2.12

PLATE 2.13 Oat leaves fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate at intervals after inoculation with *P. coronafaciens* var. *atropurpurea*.

(i) 2.5h; bacterium encapsulated onto mesophyll cell wall by fibrillar structure. Note localized dissolution of plant cell wall (arrowed).

(ii) 2.5h; bacteria physically entrapped by fibrillar cross at junction of mesophyll cells, there is apparently no effect on the host wall.

(iii) 12h; vesiculation of the plant cell plasmalemma adjacent to an enveloped bacterium. Note that the cell wall is smooth outwith the attachment structure (arrowed).

(iv) 18h; group of bacteria attached to oat cell wall. The plasmalemma and chloroplast lamellae of the host are disorganized.

(v) 36h; hypersensitive host cell death. Note the attachment of bacteria (arrowed) to mesophyll cell walls.

stained with
lead citrate
th
a.
onto
structure.
nt cell

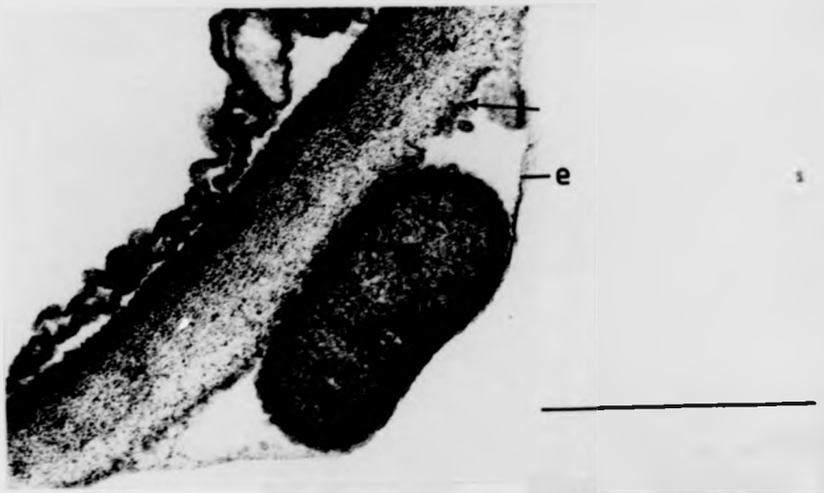
entrapped
mesophyll
ect on

plant
nveloped
all is
ucture

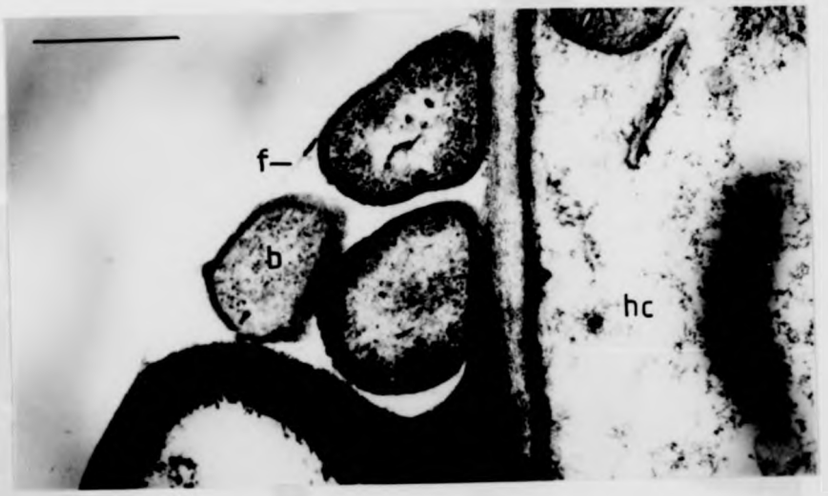
ached to
and
are

ll death.
arrowed)

(i)




(ii)




(iii)


PLATE 2.13

stained with
lead citrate
th
a.
onto
structure.
nt cell

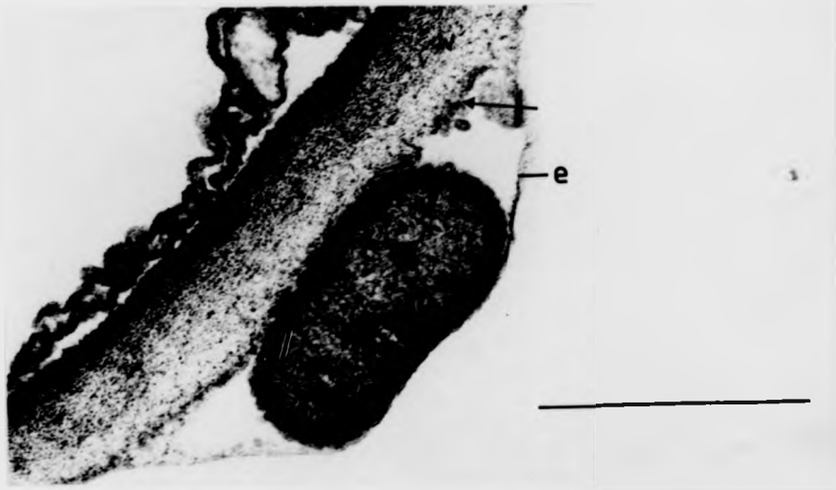
entrapped
mesophyll
fect on

plant
nveloped
wall is
ucture

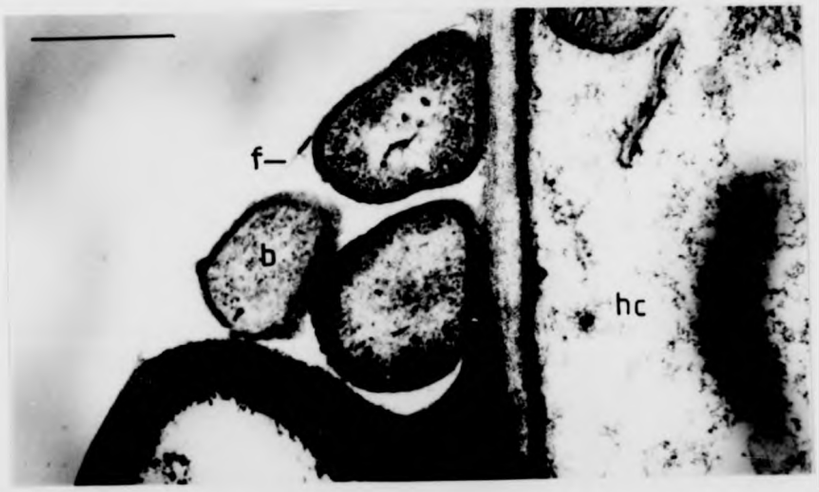
tached to
and
are

ell death.
(arrowed)

(i)



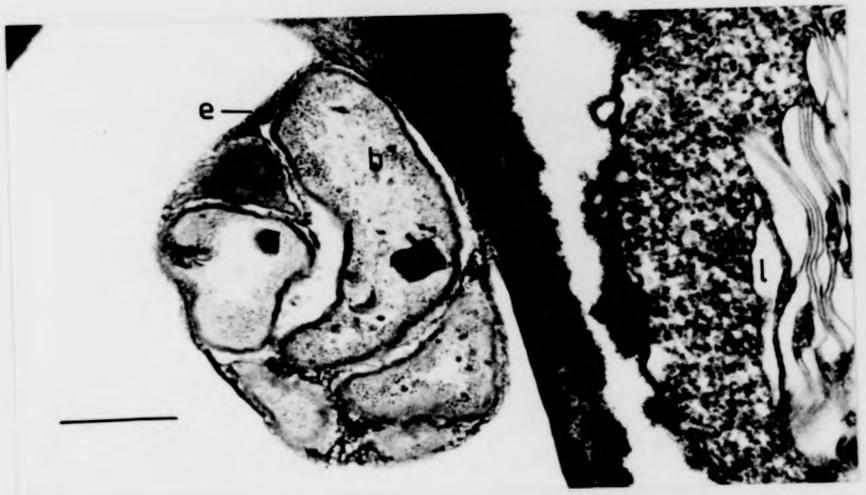

(ii)




(iii)


PLATE 2.13

(iv)




(v)


PLATE 2.13

PLATE 2.14 Oat leaves fixed by rotation and stained with ruthenium red and uranyl acetate/ lead citrate at intervals after inoculation with *P. tabaci*.

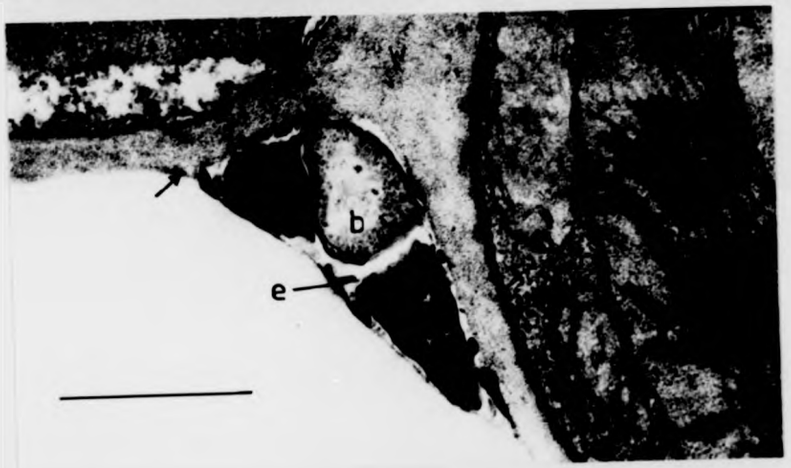
(i) 2.5h; physical entrapment of bacteria by a fibrillar cross.

(ii) 12h; mesophyll cell wall alteration can be detected (arrowed) in association with the enveloping structure attaching bacteria to the plant cell wall.

(iii) 96h; shrunken mesophyll cell in which slight swelling of chloroplast lamellae can be observed. A bacterium is close to the host cell wall but it is unattached.

(i)


(ii)




(iii)

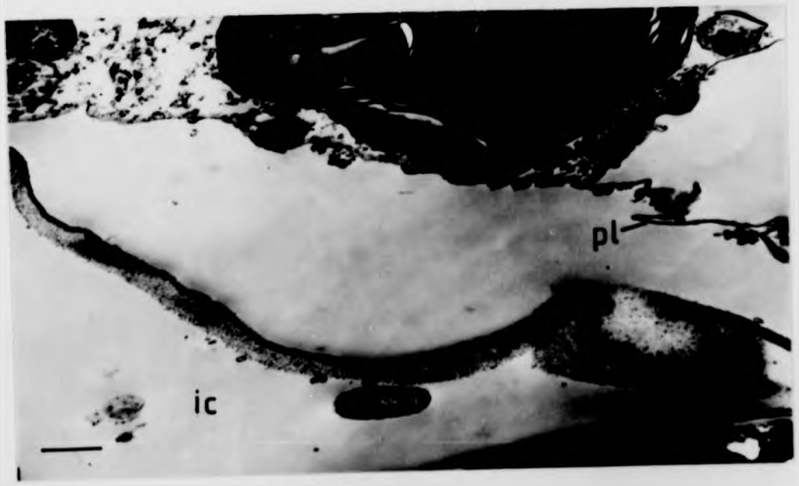



PLATE 2.14

stained
tate/
noculation

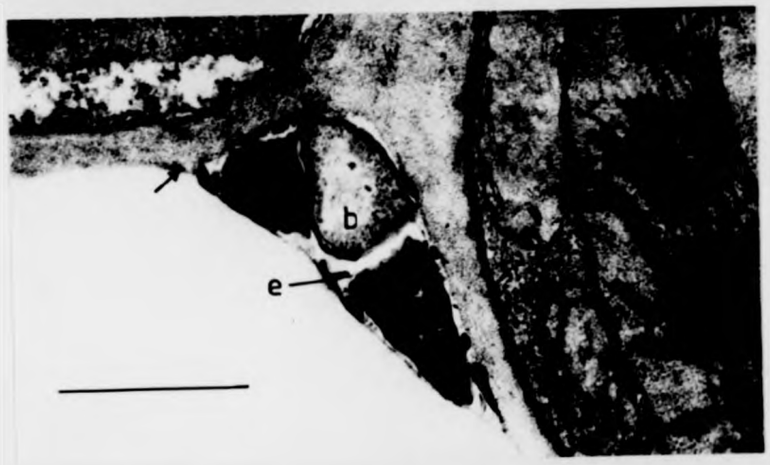
bacteria

teration
ciation
aching

l cell in
ast
terium is
t is

(i)


(ii)




(iii)

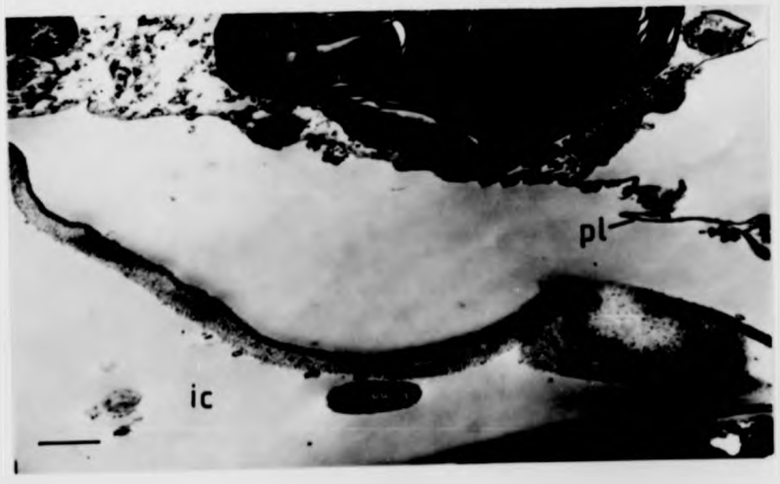



PLATE 2.14

PLATE 2.15 Oat leaves fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate at intervals after inoculation with P. fluorescens.

(i) 2.5h; physical entrapment of bacteria at mesophyll cell junction. Note the good resolution of healthy plant cell organelles.

(ii) 2.5h; bacteria attached between healthy oat mesophyll cells. Note the endoplasmic reticulum adjacent to the plasmalemma in the host cell. The tonoplast of the mesophyll cell is intact.

(iii) 12h; bacterium attached to host cell by a stalk-like protruberance from the plant cell wall.

(iv) 12h; encapsulation of bacteria onto the wall of a healthy mesophyll cell.

(v) 96h; pitting (arrowed) of host cell wall below a bacterium which is firmly attached to the mesophyll cell by encapsulating polysaccharide.

(vi) 96h; mesophyll cell exhibiting slight plasmolysis and swollen chloroplast lamellae. A bacterium close to the plant cell wall remains unattached.

tained
tate/lead
tion

bacteria
the good
rganelles.
tween
te the
the
e tonoplast

host cell
n the plant

ria onto
ell.
st cell wall
attached
ating

ing slight
st lamellae.
ll wall

(i)


(ii)


(iii)


PLATE 2.15

stained
tate/lead
ation

bacteria
e the good
organelles.
tween
te the
the
he tonoplast

host cell
m the plant

ria onto
ell.
st cell wall
attached
ating

ing slight
st lamellae.
ll wall

(i)

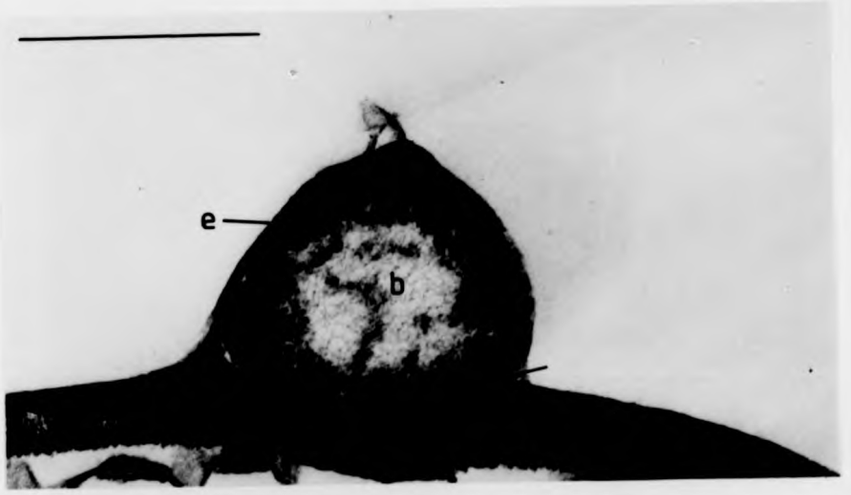

(ii)


(iii)


PLATE 2.15

(iv)


(v)

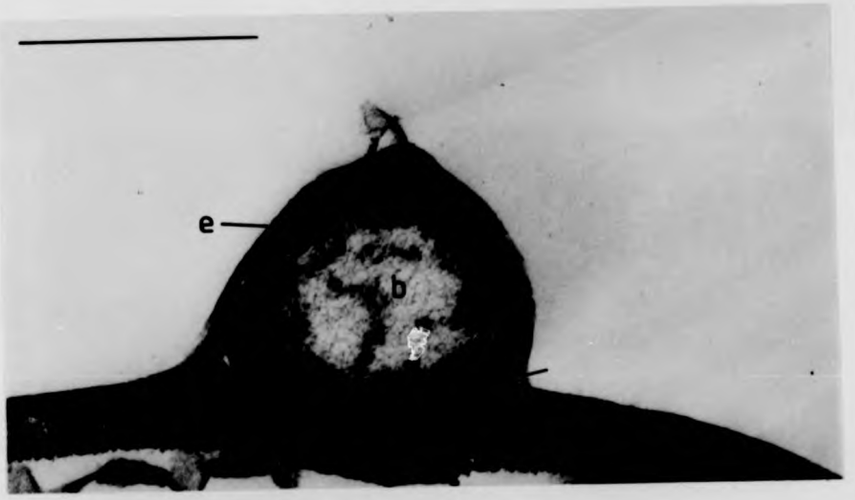



(vi)


PLATE 2.15

(iv)


(v)



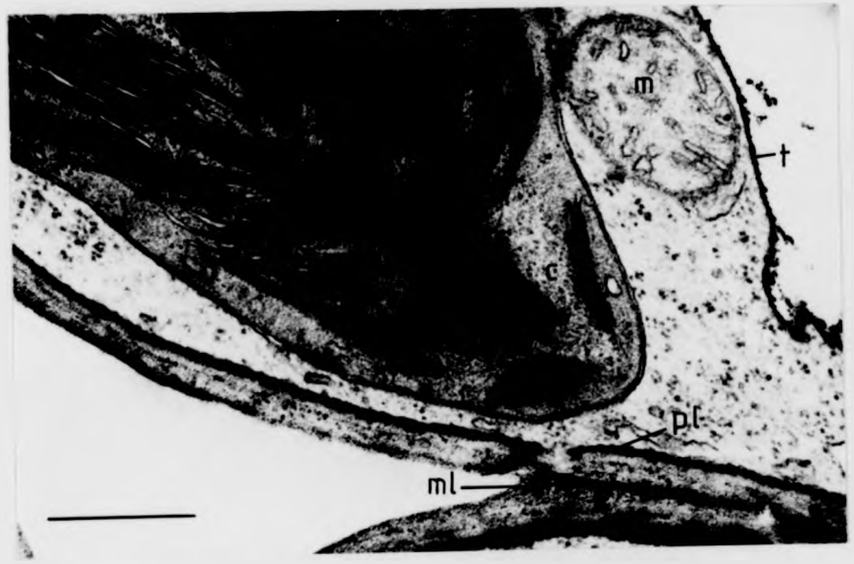

(vi)


PLATE 2.15

PLATE 2.16 Oat leaves fixed by rotation and stained with uranyl acetate/lead citrate (i) 18h and (ii) 96h after inoculation with sterile distilled water. Note the good preservation of organelles.

stained
(i) 18h
ith
the

(i)




(ii)

PLATE 2.16

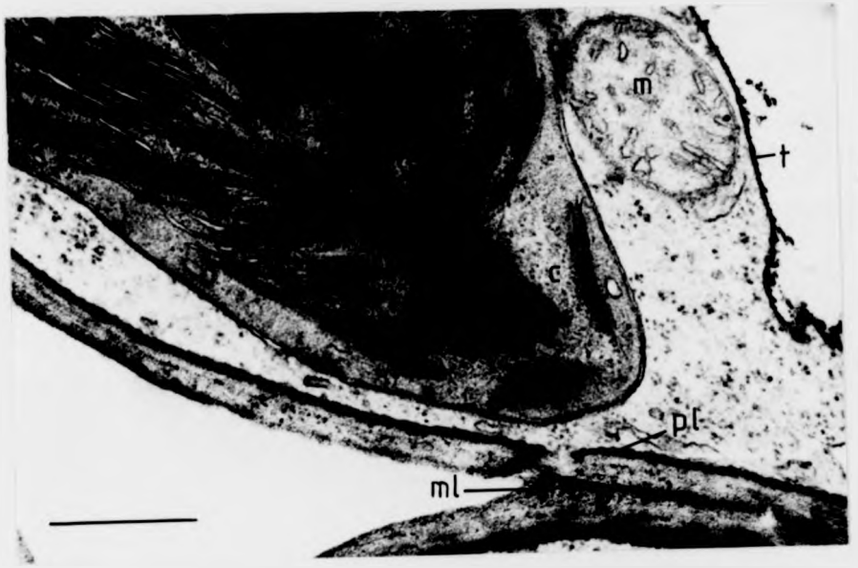
stained

(i) 18h

ith

the

(i)




(ii)

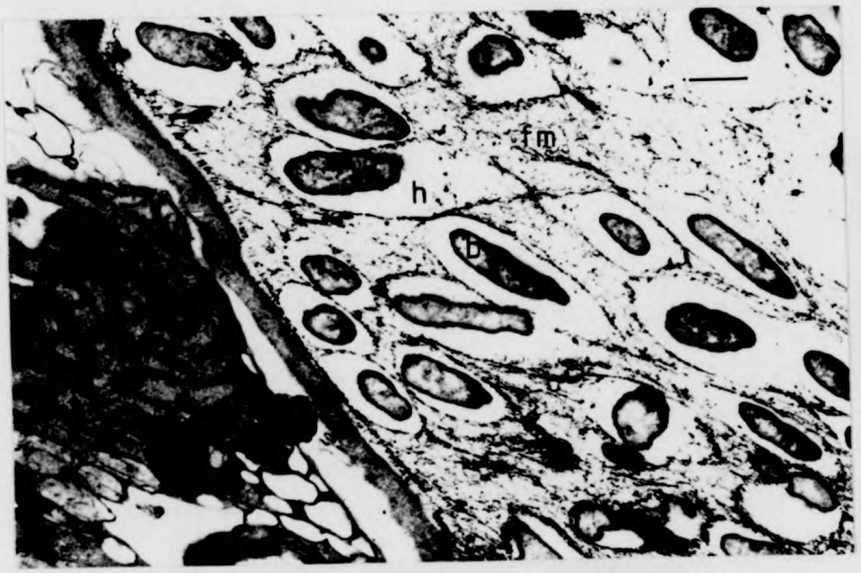

PLATE 2.16

PLATE 2.17 Tobacco leaves fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. tabaci.

(i) Bacteria embedded in a fibrillar matrix within intercellular spaces. Note disorganization of the cytoplasm of the mesophyll cell.

(ii) Fibrillar matrix surrounding a single bacterium. The densely stained matrix is beaded in appearance in this micrograph. Note the electron lucent halo between the bacterium and matrix.

(i)




(ii)

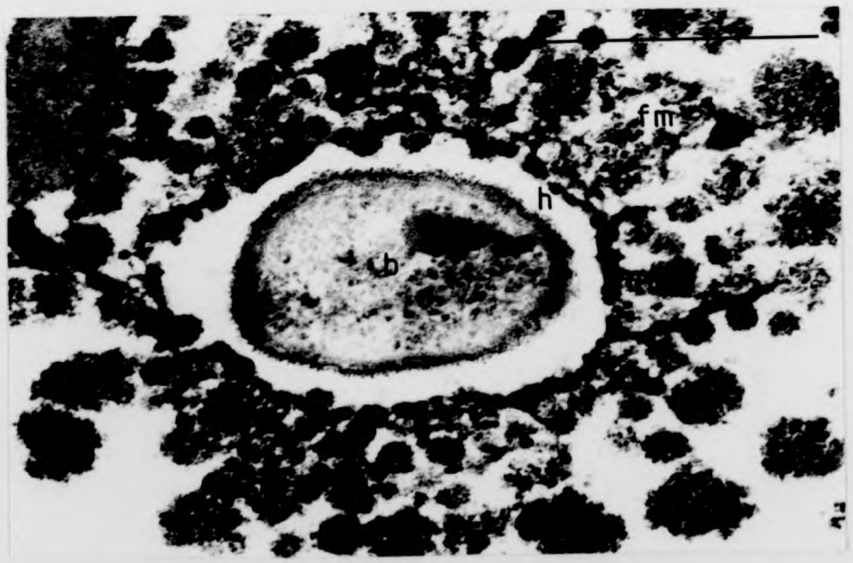
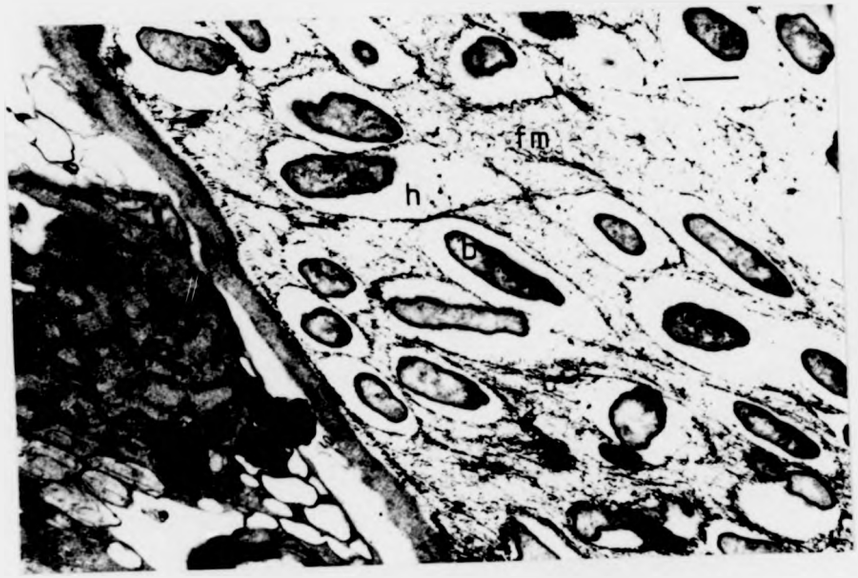



PLATE 2.17

and stained
etate/lead
th P. tabaci.
illar
es. Note
of the

ng a single
matrix is
rograph.
tween the

(i)




(ii)

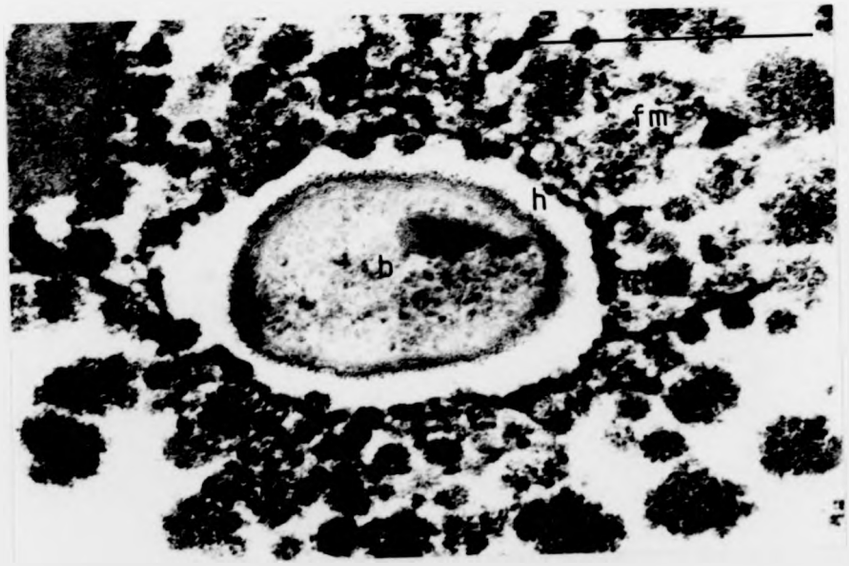



PLATE 2.17

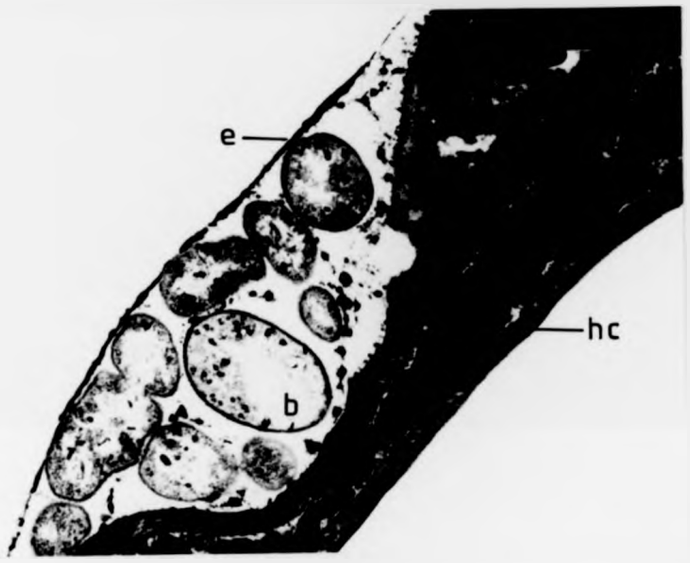



PLATE 2.18 Tobacco leaf fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens. Note the attachment of bacteria to the walls of dead mesophyll cells.

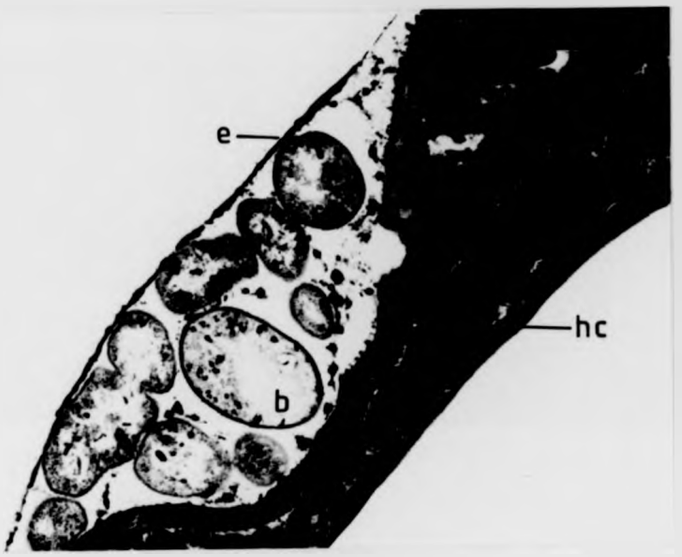



PLATE 2.18 Tobacco leaf fixed by rotation and stained with ruthenium red and uranyl acetate/lead citrate 24h after inoculation with P. coronafaciens. Note the attachment of bacteria to the walls of dead mesophyll cells.

CHAPTER 3

# THE PRODUCTION OF ANTIMICROBIAL COMPOUNDS BY OAT LEAVES INOCULATED WITH PHYTOPATHOGENIC BACTERIA

In an attempt to identify a chemical component of the resistance of oat leaves to bacterial infection, extracts from artificially inoculated oat leaves were tested for antimicrobial activity using a variety of assays.

## 1. Preliminary assays with extracts from oat leaves undergoing the hypersensitive response

Earlier work with bacteria/plant interactions has demonstrated the accumulation of antibacterial compounds in French bean and soybean leaves undergoing hypersensitive reactions (Bruegger and Keen, 1979; Gnanamanickam and Patil, 1977a; Lyon and Wood, 1975). Leaves undergoing the HR therefore provided a likely source of inhibitors if such compounds were to be found in oats. Extracts from leaves undergoing the HR 48h after their inoculation with P. coronafaciens var. atropurpurea were therefore used to test the sensitivity of various assay methods.

### A. Seeded agar plate bioassay

Both crude ethyl acetate and aqueous phases of extracts from oat leaves (cv. Milford) were assayed against P. coronafaciens var. atropurpurea and the Gram positive bacterium Bacillus megaterium.

Antibiotic assay discs impregnated with the ethyl acetate phase (50µl, representing the extract from 0.2g fresh tissue) gave no indication of activity against

P. coronafaciens var. atropurpurea or B. megaterium (Table 3.1). However, the direct droplet technique (10µl, representing the extract from 0.04g fresh tissue) demonstrated the presence of inhibitors in this fraction, activity being more noticeable against B. megaterium (Plate 3.1a). The aqueous phase produced a very slight zone of inhibition around the antibiotic assay disc placed on cultures of P. coronafaciens var. atropurpurea but the direct droplet assay gave no indication of activity. The aqueous phase applied by both techniques considerably enhanced growth of B. megaterium (Plate 3.1b).

B. Thin layer chromatography (TLC) plate bioassay

Both phases of extracts of oat leaves undergoing the HR (100µl, representing the extract from 0.04g fresh tissue) were applied to TLC plates over 4.0cm origins and developed in hexane/acetone (2:1) prior to bioassay with P. coronafaciens var. atropurpurea, B. megaterium and the fungus C. herbarum.

One zone of antimicrobial activity was detected first above the origin in the chromatograms of the ethyl acetate phase assayed with each organism (Plate 3.2). On plates sprayed with bacteria the zone of inhibition was more marked with B. megaterium (Plate 3.2a) than with P. coronafaciens var. atropurpurea (Plate 3.2b). The zone of inhibition produced with C. herbarum was particularly striking (Plate 3.2c). Four bands of fluorescence were noted in chromatograms of the ethyl acetate phase examined under UV radiation (366nm) but none of these corresponded closely with zones of inhibition. No inhibitors were detected in chromatograms

TABLE 3.1 Seeded agar plate bioassay of ethyl acetate and aqueous phases of extracts from oat leaves undergoing the hypersensitive response

| Phase of extract | Test bacterium [a] | Assay [b] | Diameter of inhibition zone (mm) [c] |
|---|---|---|---|
| Ethyl acetate | 2397 | AA | - |
| | 2397 | DD | 17.0 ± 0.32 |
| | *B. megaterium* | AA | - |
| | *B. megaterium* | DD | 21.0 ± 0.63 |
| Aqueous | 2397 | AA | 10.2 ± 2.58 |
| | 2397 | DD | - |
| | *B. megaterium* | AA | - [d] |
| | *B. megaterium* | DD | - [d] |

[a] 2397, *P. coronafaciens* var. *atropurpurea*

[b] AA = Antibiotic assay disc, DD = direct droplet

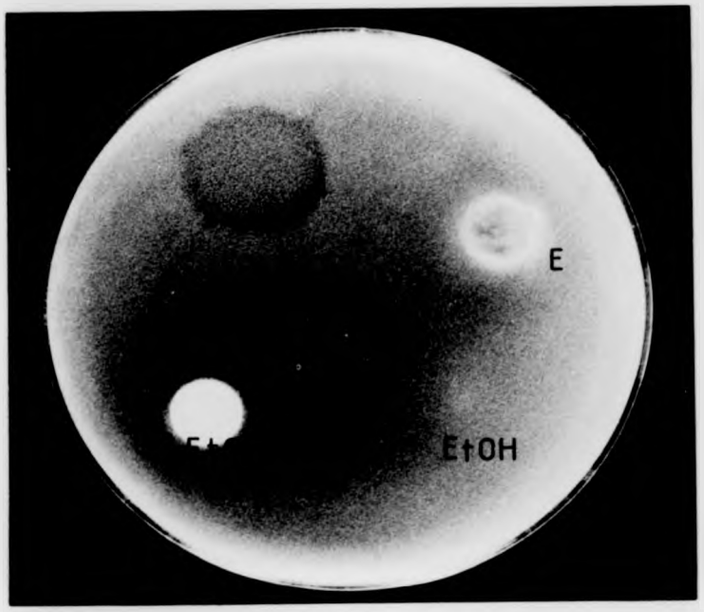
[c] Mean of 5 replicate plates ±SEM, - denotes lack of inhibition

[d] Zones of bacterial stimulation 31.8 ± 0.49 and 25.8 ± 0.49mm in diameter were recorded beneath antibiotic assay discs or sites of droplet addition respectively.

PLATE 3.1 Seeded agar plate bioassays of extracts from 0.04g fresh tissue (direct droplet) and 0.2g fresh tissue (antibiotic assay disc) from oat leaves undergoing the HR (a) Ethyl acetate phase and (b) aqueous phase. The test bacteria was B. megaterium. A zone of inhibition (darker than the bacterial lawn) is present where the ethyl acetate phase was applied directly to the plate (I) in (a), the antibiotic disc with extract (E) or EtOH, and direct addition of EtOH had no effect on bacterial growth. Zones of stimulation (S) are apparent with both antibiotic assay disc and direct droplet of aqueous phase in (b). Sterile distilled water alone had no effect.

cts from
and 0.2g
from oat
acetate
est
of
l lawn)
hase was
n
t (E) or
d no
of
oth anti-
t of
tilled

(a)




(b)

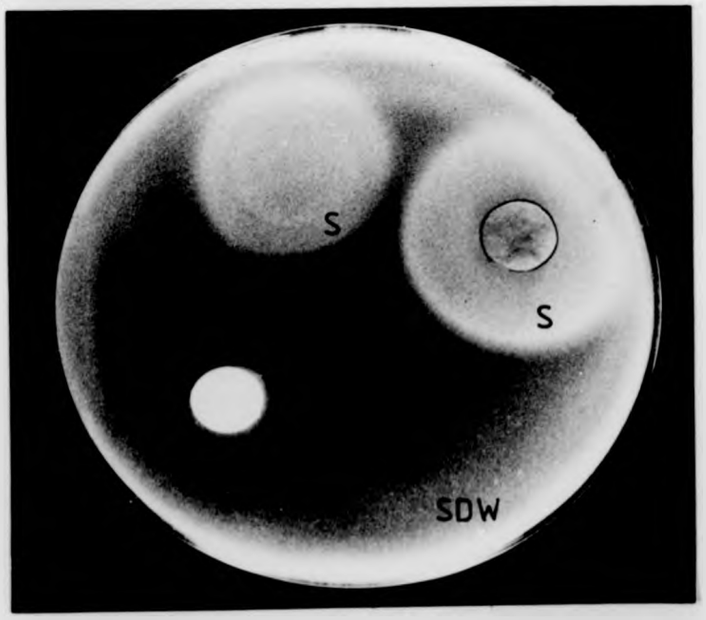
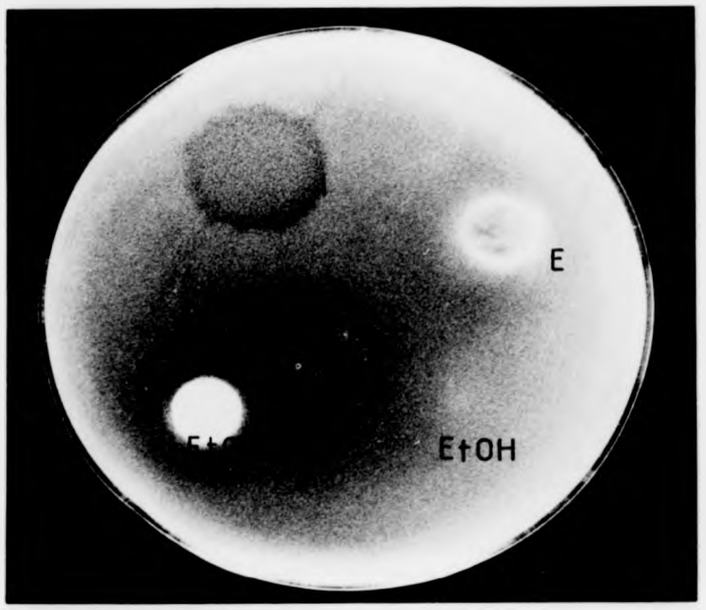



PLATE 3.1

(a)




(b)

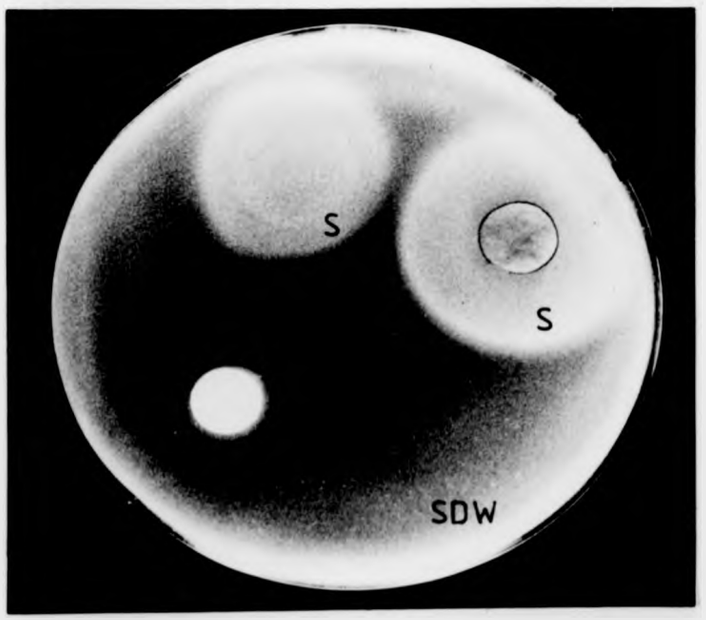
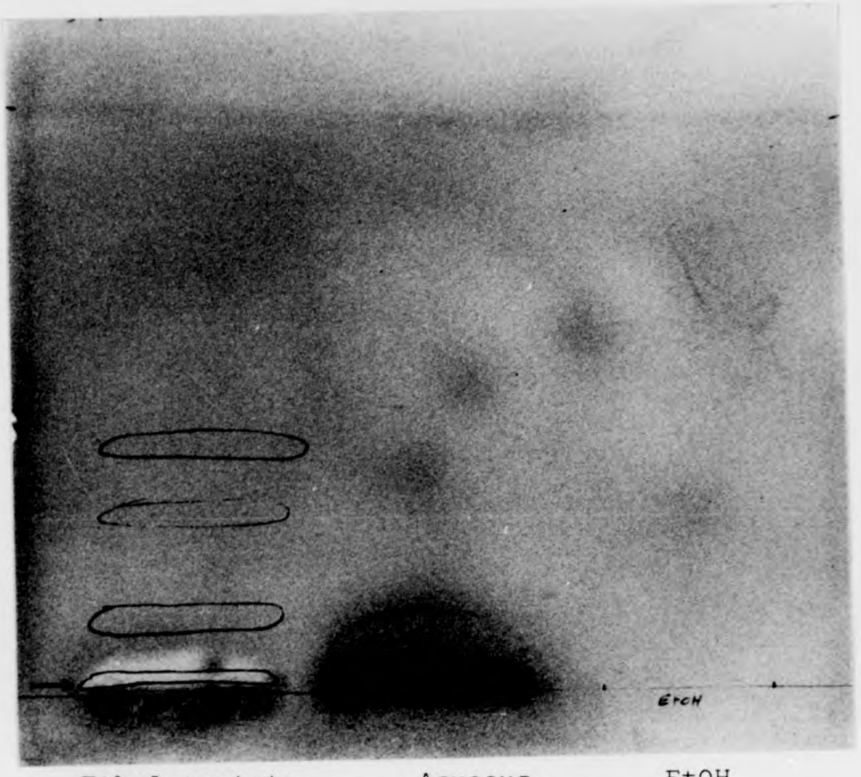



PLATE 3.1

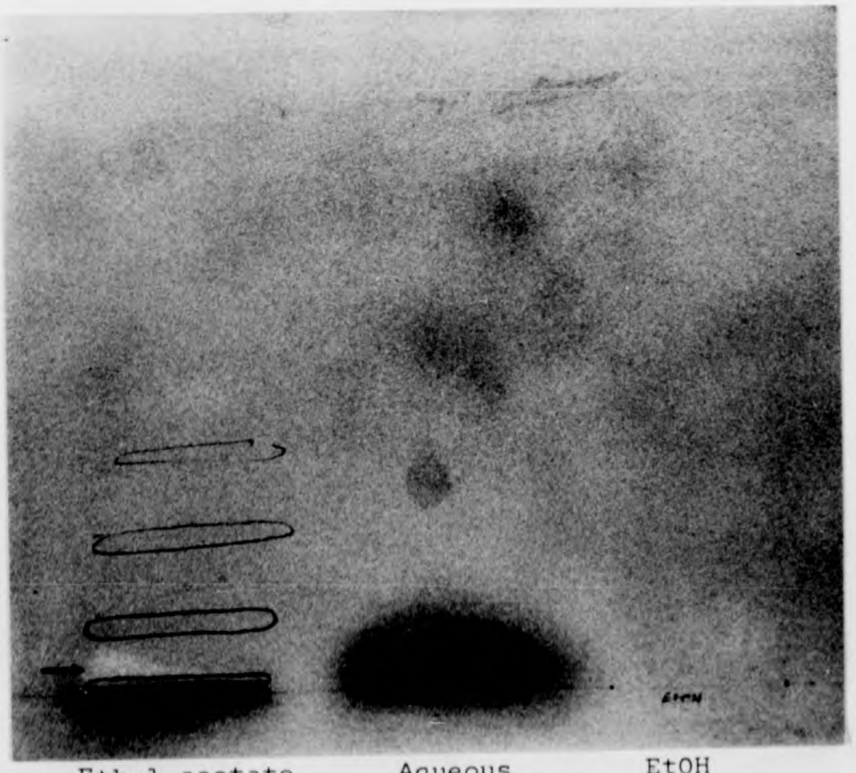
PLATE 3.2 TLC plate bioassays of ethyl acetate and aqueous phases of extracts from oat leaves undergoing the HR. 100µl, representing the extract from 0.4g fresh tissue was spotted onto 4.0cm origins and chromatographed in hexane/acetone (2:1) prior to bioassay against (a) B. megaterium, (b) P. coronafaciens var. atropurpurea and (c) C. herbarum. One zone of antimicrobial activity (arrowed) is seen on each plate just above the origin of the chromatogram of the ethyl acetate fraction.

(a)




Ethyl acetate Aqueous EtOH

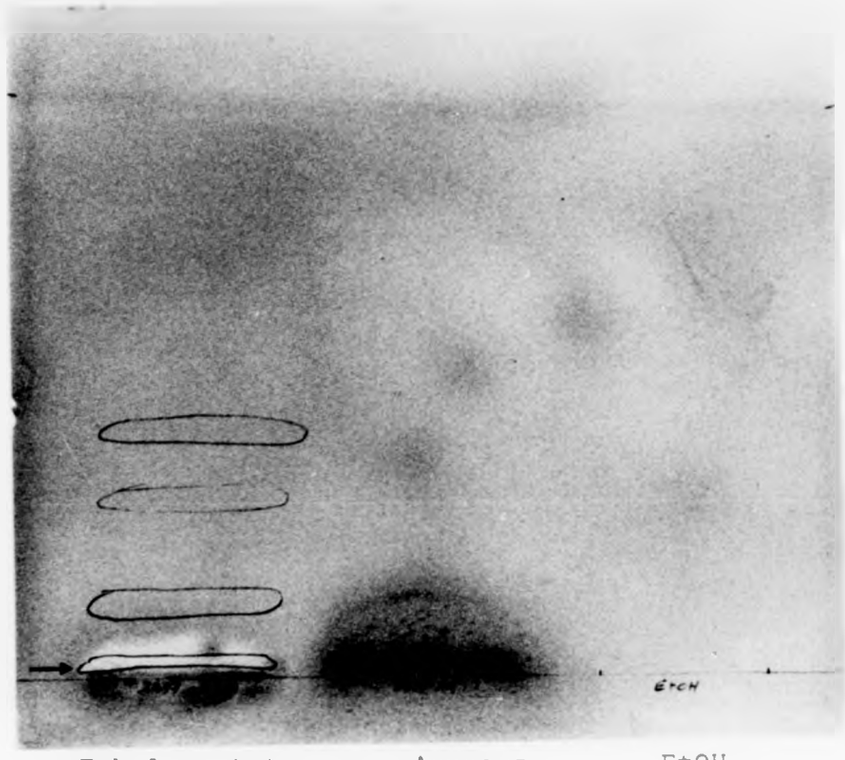
(b)




Ethyl acetate Aqueous EtOH

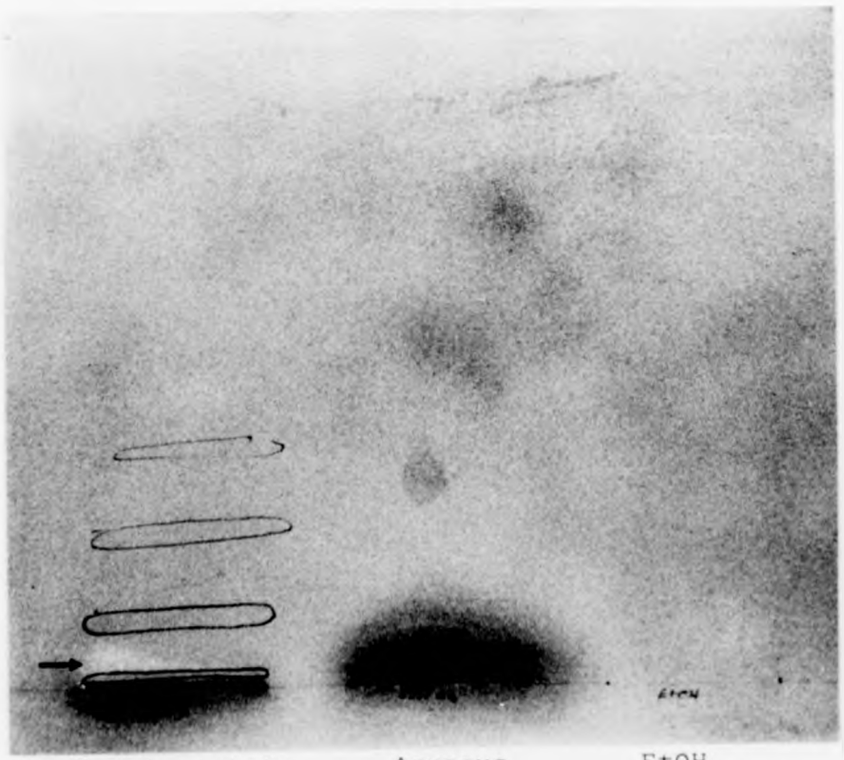
PLATE 3.2

(a)




Ethyl acetate Aqueous EtOH

(b)




Ethyl acetate Aqueous EtOH

PLATE 3.2

(c)

Ethyl acetate Aqueous EtOH

PLATE 3.2

(c)

Ethyl acetate Aqueous EtOH

PLATE 3.2

of the aqueous phase of the extracts.

C. Liquid culture bioassays

Results of liquid culture bioassays of aqueous and ethyl acetate phases of extracts (at a concentration of the extract from 1g/ml) confirmed the presence of antibacterial factors. A marked decline in bacterial numbers was recorded for both phases assayed against P. coronafaciens var. atropurpurea but only the ethyl acetate fraction affected the viability of B. megaterium (Table 3.2). Neither test bacterium was affected by the presence of the carrier solvent EtOH (2%). Bacterial numbers, particularly those of B. megaterium, increased rapidly in peptone water alone.

Although each assay gave positive results, liquid culture was the most sensitive indicator of antibacterial activity in oat leaf extracts. This assay also enabled a higher concentration (250µl, representing the extract from 1g fresh tissue) of extract to be tested. Direct droplet addition and antibiotic assay discs on seeded agar assays were conducted with lower concentrations of extract (volumes representing 0.04 and 0.2g fresh weight respectively) in order to maintain discrete droplets on the agar surface and to avoid over-saturation of the antibiotic assay disc respectively. Similarly, volumes representing extracts from only 0.4g fresh tissue could be applied to 4cm origins on TLC plates without overloading the chromatograms.

TABLE 3.7 Antibacterial activity of extracts from oat leaves undergoing the hypersensitive response demonstrated by the liquid culture bioassay

| Phase of Extract | Test Bacterium[a] used in bioassay | No. viable bacteria/ml [b] 0h | 24h | Difference | Difference as percentage of original number |
|---|---|---|---|---|---|
| Ethyl acetate | 2397 | $10^9 \times 7.8 \pm 0.9$ | $10^7 \times 1.5 \pm 0.9$ | $-10^9 \times 7.8$ | -99 |
| | B. megaterium | $10^8 \times 3.7 \pm 1.5$ | $10^5 \times 1.3 \pm 1.0$ | $-10^8 \times 3.7$ | -99 |
| Aqueous | 2397 | $10^9 \times 8.5 \pm 0.7$ | $10^8 \times 6.0 \pm 0.7$ | $-10^9 \times 7.9$ | -92 |
| | B. megaterium | $10^8 \times 2.3 \pm 0.9$ | $10^8 \times 4.2 \pm 0.9$ | $+10^8 \times 1.9$ | +82 |
| 2% EtOH | 2397 | $10^8 \times 6.3 \pm 2.1$ | $10^{10} \times 2.4 \pm 0.7$ | $+10^{10} \times 2.4$ | +3809 |
| | B. megaterium | $10^7 \times 3.0 \pm 0.3$ | $10^9 \times 2.3 \pm 0.8$ | $+10^9 \times 2.3$ | +7666 |
| No EtOH | 2397 | $10^9 \times 3.9 \pm 0.4$ | $10^9 \times 6.5 \pm 1.6$ | $+10^9 \times 2.6$ | +66 |
| | B. megaterium | $10^7 \times 3.2 \pm 0.7$ | $10^9 \times 4.4 \pm 0.8$ | $+10^9 \times 4.4$ | +1375 |

[a] 2397, P. coronafaciens var. atropurpurea or B. megaterium

[b] Determined by dilution plating, mean of 6 replicate plates ±SEM.

2. Comparison of the antimicrobial activities of extracts from oat leaves undergoing hypersensitive, susceptible and resistant symptoms

A. Liquid culture bioassays

In view of their sensitivity, liquid culture bioassays were used to compare the antibacterial activity of extracts prepared from leaves collected 48h after inoculation with P. coronafaciens var. atropurpurea (HR), P. coronafaciens (susceptible), P. tabaci (resistant symptom) and also sterile distilled water.

(i) Leaves undergoing the HR

Extracts from leaves undergoing the HR in response to P. coronafaciens var. atropurpurea were further assayed by liquid culture against P. coronafaciens and P. tabaci. The ethyl acetate soluble phase of the extract again proved to be a potent inhibitor of growth of both bacteria (Table 3.3). By contrast, the aqueous phase had little effect. Good growth was recorded for both P. coronafaciens and P. tabaci in 2% EtOH in 0.1% peptone water and 0.1% peptone water alone. The ethyl acetate phase had therefore been demonstrated to contain inhibitors active against P.coronafaciens, P. coronafaciens var. atropurpurea, P. tabaci and B. megaterium.

(ii) Leaves undergoing normosensitive collapse

The ethyl acetate soluble fraction contained compounds which were strongly inhibitory to P. coronafaciens var. atropurpurea whereas P. tabaci and P. coronafaciens remained little affected (Table 3.4). By contrast, the aqueous phase inhibited both P. coronafaciens and P. coronafaciens

TABLE 3.3 Antibacterial activity of extracts from oat leaves undergoing the HR demonstrated against P. coronafaciens (1348) and P. tabaci (2706) by the liquid culture bioassay

| Phase of Extract | Test Bacterium used in bioassay | No. viable bacteria/ml[a] 0h | 24h | Difference | Difference as percentage of original number |
|---|---|---|---|---|---|
| Ethyl acetate | P. coronafaciens | $10^6 \times 4.0 \pm 1.7$ | 0 | $-10^6 \times 4.0$ | -100 |
| | P. tabaci | $10^5 \times 3.5 \pm 1.0$ | 0 | $-10^5 \times 3.5$ | -100 |
| Aqueous | P. coronafaciens | $10^8 \times 6.3 \pm 1.5$ | $10^9 \times 1.0 \pm 0.1$ | $+10^8 \times 3.8$ | +60 |
| | P. tabaci | $10^9 \times 1.5 \pm 0.2$ | $10^9 \times 1.7 \pm 0.2$ | $+10^8 \times 1.7$ | +13 |
| 2% EtOH | P. coronafaciens | $10^8 \times 7.9 \pm 1.5$ | $10^9 \times 8.2 \pm 0.2$ | $+10^9 \times 7.5$ | +949 |
| | P. tabaci | $10^9 \times 3.1 \pm 0.3$ | $10^{10} \times 2.9 \pm 0.7$ | $+10^{10} \times 2.5$ | +806 |
| No EtOH | P. coronafaciens | $10^8 \times 3.5 \pm 0.6$ | $10^{10} \times 1.7 \pm 0.2$ | $+10^{10} \times 1.6$ | +4571 |
| | P. tabaci | $10^9 \times 6.6 \pm 1.8$ | $10^{10} \times 7.3 \pm 0.7$ | $+10^{10} \times 6.6$ | +1000 |

[a] Determined by dilution plating, mean of 6 replicate plates ±SEM

TABLE 3.4 Antibacterial activity of extracts from oat leaves undergoing the normosensitive collapse following inoculation with the pathogenic bacterium P. coronafaciens demonstrated by liquid culture bioassay

| Phase of Extract | Test bacterium[a] used in bioassay | No. viable bacteria/ml [b] 0h | 24h | Difference | Difference as percentage of original number |
|---|---|---|---|---|---|
| Ethyl acetate | 2397 | $10^9 \times 3.6 \pm 0.4$ | $10^6 \times 6.7 \pm 3.3$ | $-10^9 \times 3.6$ | -99 |
| | P. coronafaciens | $10^5 \times 8.3 \pm 1.7$ | $10^6 \times 1.2 \pm 0.8$ | $+10^5 \times 5.0$ | +60 |
| | P. tabaci | $10^9 \times 1.4 \pm 0.3$ | $10^9 \times 1.0 \pm 0.2$ | $-10^8 \times 4.0$ | -28 |
| Aqueous | 2397 | $10^9 \times 2.4 \pm 0.2$ | $10^8 \times 2.6 \pm 0.5$ | $-10^9 \times 2.1$ | -87 |
| | P. coronafaciens | $10^9 \times 1.5 \pm 0.2$ | $10^8 \times 1.6 \pm 0.4$ | $-10^9 \times 1.5$ | -98 |
| | P. tabaci | $10^9 \times 2.7 \pm 0.5$ | $10^9 \times 5.5 \pm 1.0$ | $+10^9 \times 2.8$ | +103 |

[a] 2397, P. coronafaciens var. atropurpurea

[b] Determined by dilution plating, mean of 6 replicate plates ±SEM

var. atropurpurea but had no effect against P. tabaci

(iii) Leaves undergoing the resistant symptom

The ethyl acetate soluble phase was inhibitory to P. coronafaciens, P. tabaci and P. coronafaciens var. atropurpurea (Table 3.5). Only P. coronafaciens was strongly inhibited by components in the aqueous phase of the extract, P. coronafaciens var. atropurpurea and P. tabaci were little affected.

(iv) Sterile distilled water infiltrated leaves

The ethyl acetate phase of the extract showed some signs of antibacterial activity against P. coronafaciens, P. coronafaciens var. atropurpurea and most markedly against P. tabaci (Table 3.6). This activity was not as pronounced as that of extracts from leaves undergoing the hypersensitive reaction. The aqueous phase was only effective in preventing the growth of P. coronafaciens var. atropurpurea; both P. tabaci and P. coronafaciens remained unaffected.

(v) Summary

Results obtained using the liquid culture bioassay are summarized in Table 3.7. In general, compounds in the ethyl acetate phases of each extract were more inhibitory than components of aqueous phases. The HR eliciting P. coronafaciens var. atropurpurea was the most sensitive bacterium tested, responding to aqueous as well as ethyl acetate phases of extracts. By contrast, P. tabaci was least affected. Extracts from each tissue tested differed in their activities. In general, however, least

TABLE 3.5 Antibacterial activity of extracts from oat leaves undergoing the resistant symptom demonstrated by liquid culture bioassay

| Phase of Extract | Test bacterium[a] used in bioassay | No. viable bacteria/ml[b] 0h | 24h | Difference | Difference as percentage of original number |
|---|---|---|---|---|---|
| Ethyl acetate | 2397 | $10^9 \times 3.6 \pm 1.1$ | $10^8 \times 4.5 \pm 0.8$ | $-10^9 \times 3.2$ | -87 |
| | P. coronafaciens | $10^8 \times 4.7 \pm 1.3$ | $10^7 \times 2.7 \pm 1.0$ | $-10^8 \times 4.4$ | -94 |
| | P. tabaci | $10^9 \times 4.3 \pm 0.4$ | $10^8 \times 1.5 \pm 0.3$ | $-10^9 \times 4.1$ | -95 |
| Aqueous | 2397 | $10^9 \times 1.6 \pm 0.3$ | $10^9 \times 1.3 \pm 0.3$ | $-10^8 \times 3.6$ | -22 |
| | P. coronafaciens | $10^9 \times 1.7 \pm 0.2$ | $10^8 \times 4.8 \pm 0.2$ | $-10^9 \times 1.2$ | -70 |
| | P. tabaci | $10^9 \times 2.7 \pm 0.3$ | $10^9 \times 3.4 \pm 0.6$ | $+10^8 \times 7.3$ | +27 |

[a] 2397, P. coronafaciens var. atropurpurea

[b] Determined by dilution plating, mean of 6 replicate plates ±SEM

TABLE 3.6 Antibacterial activity of extracts from oat leaves previously infiltrated with sterile distilled water demonstrated by liquid culture bioassay

| Phase of Extract | Test bacterium[a] used in bioassay | No.viable bacteria/ml [b] 0h | 24h | Difference | Difference as percentage of original number |
|---|---|---|---|---|---|
| Ethyl acetate | 2397 | $10^9 \times 1.2 \pm 0.1$ | $10^8 \times 4.3 \pm 0.7$ | $- 10^8 \times 7.9$ | -65 |
| | P. coronafaciens | $10^8 \times 8.7 \pm 1.2$ | $10^8 \times 4.6 \pm 2.1$ | $- 10^8 \times 4.1$ | -47 |
| | P. tabaci | $10^9 \times 2.9 \pm 0.5$ | $10^8 \times 5.1 \pm 1.3$ | $- 10^9 \times 2.4$ | -82 |
| Aqueous | 2397 | $10^9 \times 3.4 \pm 1.0$ | $10^8 \times 5.1 \pm 0.8$ | $- 10^9 \times 2.9$ | -85 |
| | P. coronafaciens | $10^8 \times 6.2 \pm 0.8$ | $10^9 \times 1.3 \pm 0.1$ | $+ 10^8 \times 7.0$ | +112 |
| | P. tabaci | $10^9 \times 1.8 \pm 0.3$ | $10^9 \times 3.7 \pm 0.3$ | $+ 10^9 \times 1.9$ | +105 |

[a] 2397, P. coronafaciens var. atropurpurea

[b] Determined by dilution plating, mean of 6 replicate plates ±SEM

TABLE 3.7 Antibacterial activities of ethyl acetate and aqueous phases of extracts prepared from oat leaves following inoculation with phytopathogenic bacteria or sterile distilled water and assayed by liquid culture

| Inoculum | Phase of Extract [a] | Activity against test bacterium [b] | | | |
|---|---|---|---|---|---|
| | | *P. coronafaciens* var. *atropurpurea* | *P. coronafaciens* | *P. tabaci* | *B. megaterium* |
| *P. coronafaciens* var. *atropurpurea* | Ethyl acetate | +++ | +++ | +++ | +++ |
| | Aqueous | ++ | - | - | ++ |
| *P. coronafaciens* | Ethyl acetate | +++ | - | + | NT[c] |
| | Aqueous | ++ | +++ | - | NT |
| *P. tabaci* | Ethyl acetate | +++ | +++ | +++ | NT |
| | Aqueous | + | ++ | - | NT |
| Sterile distilled water | Ethyl acetate | ++ | ++ | + | NT |
| | Aqueous | ++ | - | - | NT |

[a] Ethyl acetate and aqueous phases of extracts from oat leaves inoculated 48h previously with *P. coronafaciens* var. *atropurpurea* (HR), *P. coronafaciens* (normosensitive), *P. tabaci* (resistant symptom) and sterile distilled water.

[b] Activity recorded as - no activity, +++ strongly inhibitory. Based on comparison of cell number at 24h with cell number at 0

[c] NT = not tested.

activity was detected in phases of extracts from leaves infiltrated with sterile distilled water.

B. Thin layer chromatography (TLC) plate bioassays

Further comparisons of the antimicrobial activities of extracts were made by the TLC plate assay method. Drops (100µl, the extract from 0.4g fresh tissue) of ethyl acetate and aqueous phases recovered from extracts of the range of tissues collected were applied to equidistant spots on TLC plates. The plates were not developed in a solvent system but assayed immediately against P. coronafaciens var. atropurpurea, B. megaterium and C. herbarum. All eight extracts could thus be tested collectively for antimicrobial activity. B. megaterium was included as it had already proved to be particularly suitable for TLC assay.

Components of the ethyl acetate phase of extracts from leaves previously inoculated with P. tabaci, P. coronafaciens var. atropurpurea and sterile distilled water were strongly antifungal (Plate 3.3a). Activity was greatest in the extract from leaves undergoing the HR. The ethyl acetate phase from susceptible oat leaves previously inoculated with the pathogen, P. coronafaciens exhibited little or no antifungal properties. All four aqueous phases of extracts promoted fungal growth.

Growth of B. megaterium was strongly inhibited only by the ethyl acetate phase of the extract from leaves undergoing the HR. This phase recovered from extracts of leaves undergoing the resistant symptom (P. tabaci inoculated) or inoculated with sterile distilled water alone proved to be only mildly inhibitory. The ethyl

(a)

PLATE 3.3 TLC plate bioassays of extracts from 0.4g fresh tissue collected 48h after inoculation with sterile distilled water (SDW), *P. tabaci* (2706), *P. coronafaciens* (1348) and *P. coronafaciens* var. *atropurpurea* (2397). The extracts were assayed against (a) *C. herbarum* and (b) *B. megaterium*, zone of inhibition arrowed.

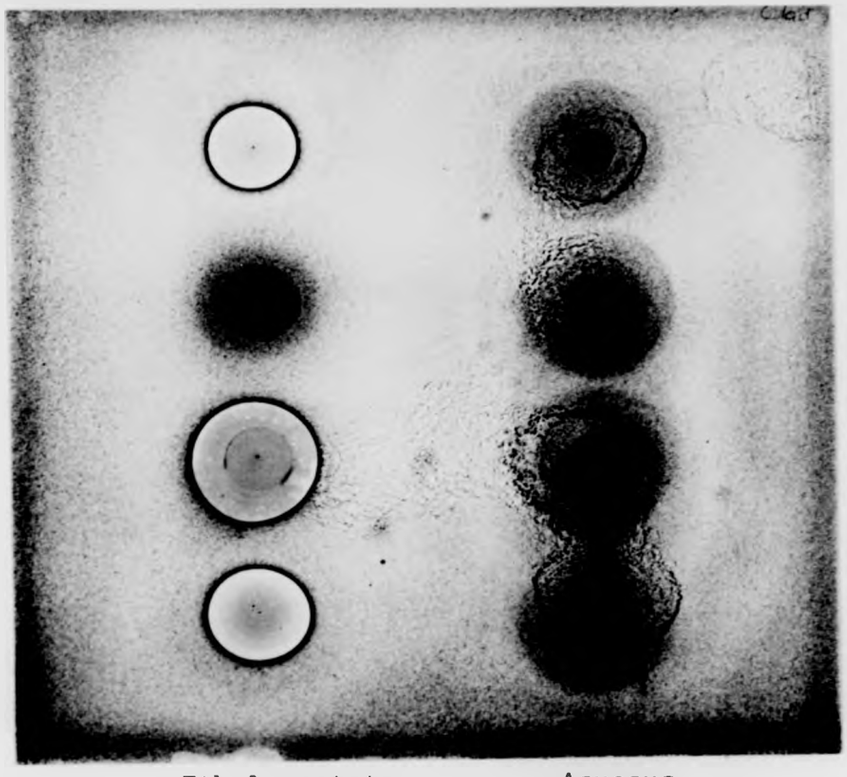
(b)

PLA

om 0.4g
noculation
, P. tabaci
d
(2397).

ium,

(a)




(b)

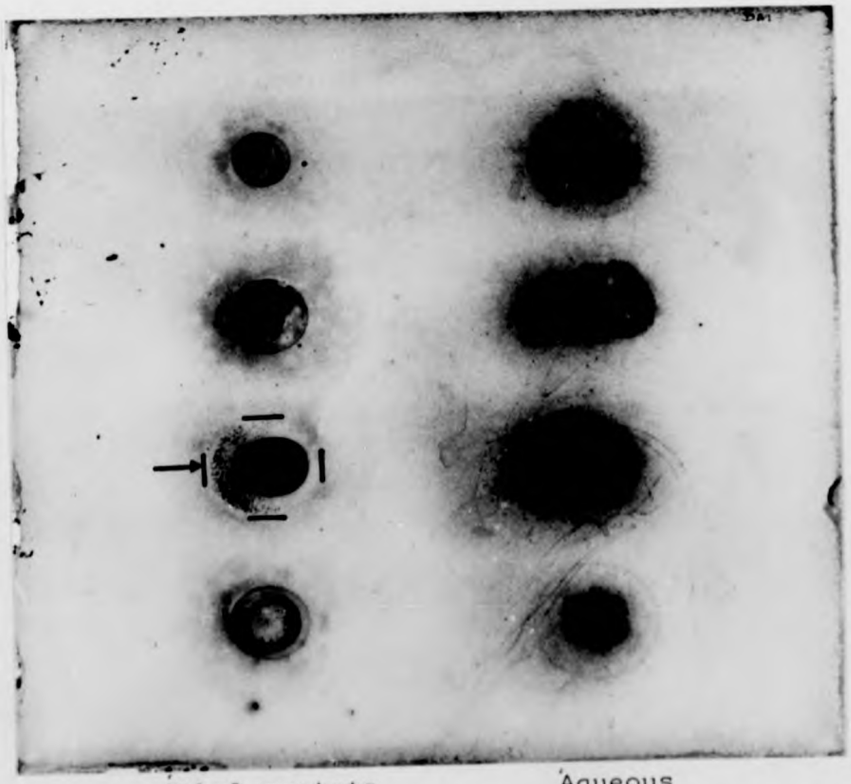
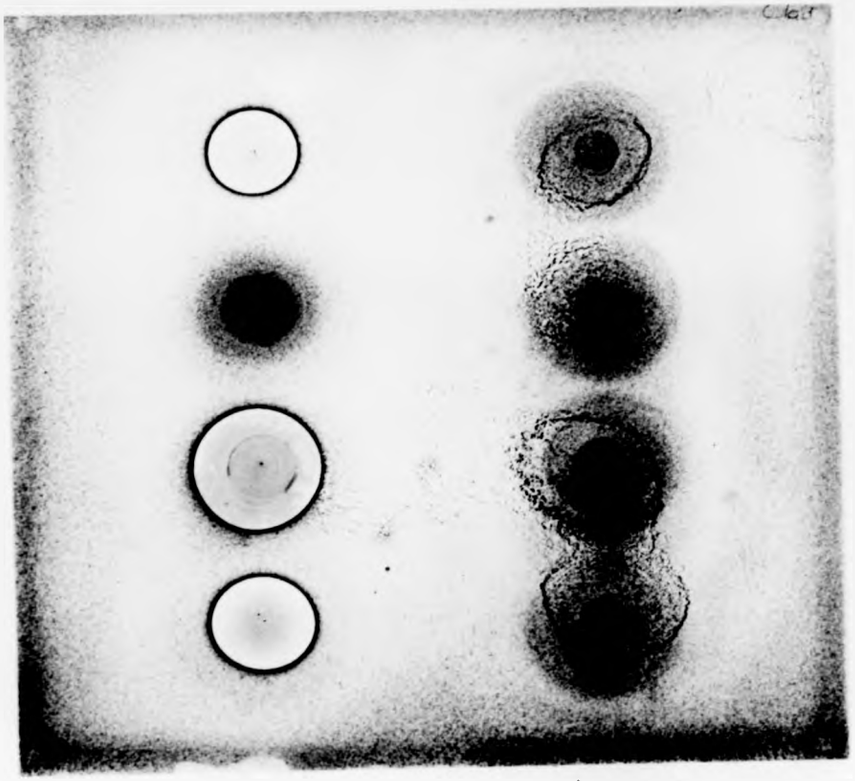



PLATE 3.3

om 0.4g
noculation
, P. tabaci
d
(2397).

ium,

(a)




(b)

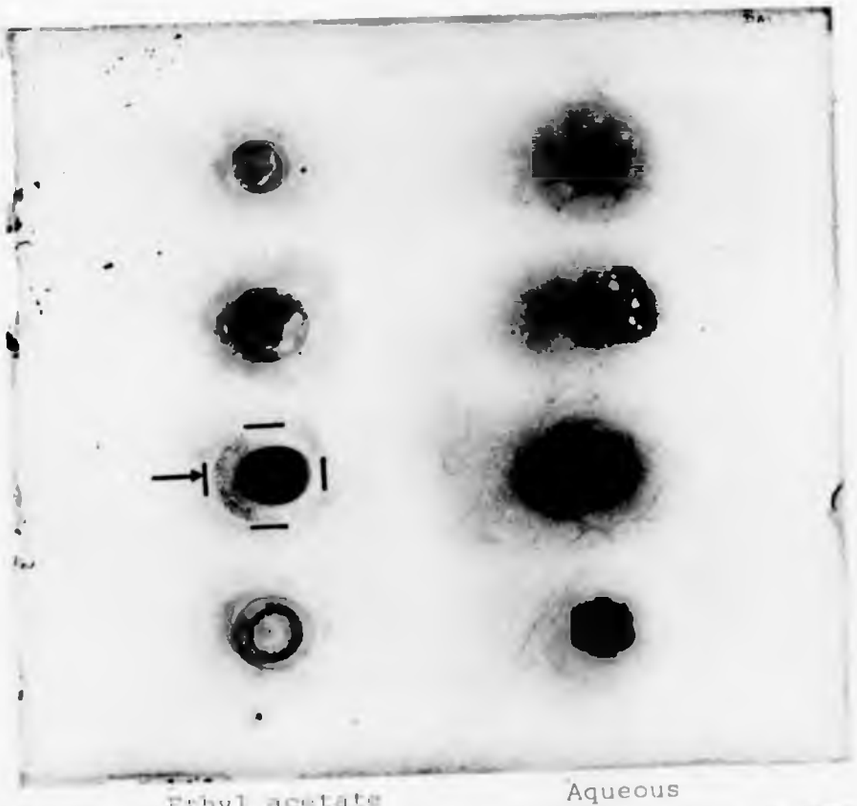



PLATE 3.3

acetate soluble fraction from the extract of susceptible (*P. coronafaciens* inoculated) leaves and all aqueous phases were not inhibitory but they did not stimulate growth (Plate 3.3b).

Growth of *P. coronafaciens* var. *atropurpurea* was not inhibited by any of the extracts compared in these bioassays.

Results obtained are summarized in Table 3.8. The antibacterial properties of each ethyl acetate phase of the extracts demonstrated by liquid culture assays was confirmed only by TLC plate bioassays carried out with *B. megaterium*. *P. coronafaciens* var. *atropurpurea*, although the most sensitive bacterium in the liquid culture assays, was not sensitive to the extracts when assayed by TLC. The strong activity of components of ethyl acetate phases from extracts of leaves undergoing the HR against *B. megaterium* as demonstrated by liquid culture was confirmed by TLC assay. Based on size of inhibition zone, the activity of this phase was the strongest of the extracts tested. The TLC plate bioassay confirmed the antifungal nature of the ethyl acetate phases as suggested previously. This phase of extracts from the susceptible interaction, however, were not strongly antifungal.

TABLE 3.8 Thin layer chromatography plate bioassay of extracts from 0.4g fresh weight of tissue collected 48h after its inoculation with sterile distilled water or suspensions of phytopathogenic bacteria

| Bacterium inoculated into leaf tissue | Phase of extract[a] | Test organism[b] | Inhibition zone diam. (mm) | Observation[c] |
|---|---|---|---|---|
| *P. tabaci* | Ethyl acetate | 2397 | - | - |
| | | *B. megaterium* | - | + |
| | | *C. herbarum* | 21 | ++ |
| | Aqueous | 2397 | - | - |
| | | *B. megaterium* | - | - |
| | | *C. herbarum* | - | S |
| *P. coronafaciens* | Ethyl acetate | 2397 | - | - |
| | | *B. megaterium* | - | - |
| | | *C. herbarum* | 20 | + |
| | Aqueous | 2397 | - | - |
| | | *B. megaterium* | - | - |
| | | *C. herbarum* | - | S |
| *P. coronafaciens* var. *atropurpurea* | Ethyl acetate | 2397 | - | - |
| | | *B. megaterium* | 22 | +++ |
| | | *C. herbarum* | 30 | +++ |
| | Aqueous | 2397 | - | - |
| | | *B. megaterium* | - | - |
| | | *C. herbarum* | - | S |
| None (Sterile distilled water only) | Ethyl acetate | 2397 | - | - |
| | | *B. megaterium* | - | + |
| | | *C. herbarum* | 23 | ++ |
| | Aqueous | 2397 | - | - |
| | | *B. megaterium* | - | - |
| | | *C. herbarum* | - | S |

[a] Extracts were prepared from oat leaves 48h after inoculation with *P. tabaci* (2706), *P. coronafaciens* var. *atropurpurea* (2397), *P. coronafaciens* (1348) and sterile distilled water.

(contd.)

[b] 2397, *P. coronafaciens* var. *atropurpurea*

[c] Qualitative scale based on - no inhibition to +++ strong inhibition,
S = stimulation of growth.

# DISCUSSION

## I THE RESPONSES OF CEREALS TO BACTERIAL INFECTION

### 1. Symptom expression

#### A. General observations

In susceptible oat cultivars the oat halo blight bacterium P. coronafaciens produced a disease syndrome similar to that previously described by Elliott (1920). There was no evidence for multiplicity of symptoms under experimental conditions as described for field infections (Cunfer and Schaad, 1976; Cunfer, Schaad and Morey, 1978; Roane and Kuriger, 1976). The symptoms were clearly defined by means of a disease symptom key constructed from symptom records. It should be borne in mind that the ratings of the symptom key describe artificially inoculated leaves kept in a controlled environment. The 'field situation' would include more environmental variables as well as lower inoculum concentrations of bacteria (see Goodman, 1976).

Twenty-five isolates of P. coronafaciens produced halo blight symptoms in oat cv. Milford but the severity of infection varied between isolates, perhaps reflecting differences in virulence. Barley and the resistant oat line Cc4146 were moderately susceptible to the 25 isolates of P. coronafaciens tested. Resistance of Cc4146 and barley was apparent only when low concentrations of inocula were used. The higher inoculum concentrations ($10^8$ bacteria/ml) may well overcome the resistance to halo blight evidently effective under field conditions (Elliott, 1920; Griffiths and Peregrine, 1964). Reducing inoculum concentrations of P. coronafaciens only succeeded in delaying symptom

development in the fully susceptible oat cv. Milford.

No bacterial isolate tested was pathogenic to wheat, P. coronafaciens isolates generally eliciting a resistant reaction in inoculated wheat leaves. One exception to this was the rye isolate of P. coronafaciens which evoked a clearly defined HR in each cereal tested. Thus, the expression of resistance in wheat leaves was not necessarily associated with the HR.

Oat and barley leaves also responded in a hypersensitive manner to P. coronafaciens var. atropurpurea but only developed yellowing (resistant symptom ) at sites inoculated with P. morsprunorum, P. tabaci and P. phaseolicola. These bacteria were all capable of producing the HR in other plants. Macroscopically, therefore, there appears to be some basic difference in resistance expression between cereals and for example tobacco. Although the HR is evident in cereal leaves infected with certain plant pathogenic pseudomonads, if it is playing an important part in resistance, it is clearly not the sole mechanism by which cereal leaves can defend themselves.

It is noteworthy that those pathogens which produced hypersensitivity in cereal leaves; P. coronafaciens (NCPPB 1327), P. coronafaciens var. atropurpurea, P. syringae, P. atrofaciens HR in barley (Hagborg, 1970) are all pathogens of the Gramineae (Cunfer and Schaad, 1976; Ribeiro et al, 1977; Sellam and Wilcoxson, 1976; Tominaga, 1968). These observations suggest that the factor eliciting the HR in cereals may also be associated with pathogenecity to the Gramineae.

The saprophyte, P. fluorescens, did not cause symptom development of any kind in cereal leaves, in keeping with previous observations on tobacco and other plants (Klement, Farkas and Lovrekovich, 1964; Lelliott et al, 1966; Lyon and Wood, 1976).

B. Relationship between bacterial populations and macroscopic symptom development

The pathogen, P. coronafaciens, multiplied rapidly within oat leaves at rates similar to those reported for P. phaseolicola and P. tabaci in their respective hosts (Diachun and Troutman, 1954; Ercolani and Crosse, 1966; Gnanamanickam and Patil, 1976; Klement and Lovrekovich, 1961; Lyon and Wood, 1976; Young, 1974). Progressive symptom development in susceptible oat leaves for each inoculum concentration of P. coronafaciens closely matched bacterial multiplication with peak numbers corresponding with maximal symptom development within the infiltrated area (Fig. 1.1).

Multiplication of P. coronafaciens in the moderately susceptible oat line Cc4146 and also in barley was less rapid than in oat cv. Milford but the appearance of symptoms was again closely associated with the multiplication of bacteria to critical numbers, i.e. $>4 \times 10^6$ bacteria/ $cm^2$ leaf. The delay in bacterial multiplication and concomitant symptom expression in barley is clearly not due to bacteriocidal activity within injected leaves as viable bacteria were recovered 3 weeks after inoculation. Localized bacteriostasis may be important in this context as described for cotton by Essenberg et al (1979).

The localized yellowing response (resistant symptom ) of wheat to P. coronafaciens was confirmed as being associated with resistance by the observation that bacterial populations remained constant following infiltration. However, as numbers did not decline markedly and as viable bacteria were recovered from wheat 4 weeks after inoculation, it is again unlikely that bacteria are killed by the defence mechanism acting during the resistant symptom . Similarly, the levels of P. phaseolicola in oats, wheat and barley and P. tabaci in oat supported the view that the resistant symptom was generally associated with constant and not declining bacterial numbers, the one exception proving to be P. morsprunorum in oat.

The saprophyte P. fluorescens maintained a constant population within all cereal leaves. Similar results were obtained by Klement (1972) working with tobacco.

The occurrence of the HR in cereal leaves was associated with a rapid decline in bacterial numbers (Fig. 1.3). A similar decline was recorded by Goodman (1972) and Sequeira and Hill (1974) for tobacco leaves. Desiccation, following tissue collapse, has often been implicated as the major cause of the drop in numbers of recoverable bacteria during the HR (Cook and Stall, 1968; Goodman, 1968; 1972; Kelman and Sequeira, 1972). In cereals, however, bacterial numbers had declined sharply by 1 day after inoculation, before visible HR expression and extensive desiccation of the infiltrated tissue. Thus, tissue collapse during the HR in cereal leaves may be

acting as a secondary determinant in resistance, contributing to the initial decline caused by some other mechanism. A possible candidate for this primary mechanism is the release of antibacterial compounds from challenged cells, before the onset of necrosis. On the basis of population data, therefore, the HR recorded in cereal leaves in response to certain plant pathogenic pseudomonads is associated with host resistance but its precise role remains undefined.

2. Hypersensitive and normosensitive collapse

A. Expression and specificity

It is arguable whether or not the macroscopically visible HR plays a role in plant resistance in the field because inocula of less than $5x10^6$ bacteria/ml of P. coronafaciens var. atropurpurea failed to elicit confluent necrosis in oat leaves. Inoculum concentrations in the field are likely to be well below this critical number. Microscopically, however, only 1 bacterium per host cell is required for cell death in tobacco (Turner and Novacky, 1974). Similarly, Essenberg et al (1979) demonstrated that resistant cotton responded to X. malvacearum by necrotic flecking upon infiltration of low doses of inocula. Conceivably, therefore, the collapse of individual cells may contribute to resistance even in the absence of confluent necrosis. The hypersensitive response will almost certainly be affected by light, temperature and humidity (Giddix, Lukezic and Pell, 1978; Holliday et al, 1981; Klement and Goodman, 1967; Lyon and Wood, 1976).

Living bacterial cells are clearly essential for the induction of the HR and normosensitive collapse in cereal, French bean, broad bean and tobacco (see Table 1.6). This requirement was also noted by Klement and co-workers (Klement and Goodman, 1967; Klement, Hevesi and Sasser, 1978) working with tobacco. The HR induction periods and subsequent symptom appearance were generally longest in oat. This is perhaps more likely to indicate that the biochemical events leading to HR collapse in oat leaves require more time to reach completion than a reflection of a different process of HR induction since HR expression differs only in timing in each plant/bacteria interaction tested.

Normosensitive induction times were much longer than the HR induction times in oats, tobacco and French bean. Multiplication is an essential feature of susceptible interactions and thus P. coronafaciens has to reach a critical level of c. $4x10^6$ bacteria/$cm^2$ leaf before normosensitive collapse occurs. Klement et al (1978) postulated that the normosensitive induction time parallels bacterial proliferation to a critical number of bacteria per plant cell before collapse is elicited. Clearly, the number of compatible bacteria per host cell required to elicit normosensitive collapse will be much higher than the numbers of incompatible bacteria causing the HR. The need for bacterial multiplication prior to normosensitive collapse is further demonstrated by the observation that normosensitive collapse in oat cv. Milford after inoculation with low numbers of bacteria was delayed until populations of P. coronafaciens reached > $4x10^6$ bacteria/$cm^2$ leaf (Fig. 1.1).

In addition to timing, the processes of hypersensitive and normosensitive collapse are apparently quite different because P. coronafaciens var. atropurpurea is declining in numbers prior to HR expression whereas multiplication appears to be essential to normosensitivity.

Results of experiments with streptomycin suggested that the HR was not induced by a diffusible elicitor, for example a toxic metabolite produced by bacteria in the intercellular spaces. When infiltrated tissues were flushed with antibiotic solution tissue collapse was always confined to the initial inoculation sites; spreading necrosis was never observed. If a toxic metabolite responsible for the HR was produced by the bacterium, the HR would have been expected to develop in the surrounding tissue; the elicitor but not the bacteria being dispersed by flushing with streptomycin. It is likely, therefore, that the inducer of the HR is bound to the live bacterial cell. This view supports the contention of Klement (1977) that the contact between bacterium and host cell is necessary for recognition of incompatibility and subsequent triggering of the HR. The importance of cell contact in the induction of the HR has also been demonstrated for cotton (Essenberg et al, 1979) and pepper (Cook and Stall, 1977; Stall and Cook, 1979).

Electrolyte leakage from oat leaves mirrored macroscopic symptom development. Where tissue collapse (HR and normosensitive) was observed, this coincided with a massive increase in conductivity of bathing solutions (Fig. 1.10). Electrolyte leakage is indicative of changes in host cell

permeability mediated through membrane disruptions. Ultrastructural observations on oat tissues undergoing hypersensitive or normosensitive collapse indicate that the electrolyte leakage recorded can be attributed to plasmalemma disruption as reported for tobacco (Goodman, 1967; 1972) and pepper (Cook , 1975; Cook and Stall, 1968). In oat tissue inoculated with saprophytic and resistant reaction inducing bacteria, no electrolyte leakage was recorded, consistent with the observation that symptom expression in these instances is not accompanied by tissue collapse.

In cereals, the HR cannot be regarded as the usual response to incompatible phytopathogenic bacteria as those bacteria eliciting a resistant symptom clearly evoke an inducible HR in other plants. Based on symptom appearance, the resistant symptom is distinctly different from the HR. It is unlikely that the resistant symptom is merely a reduced HR since high concentrations of bacteria ($10^{10}$ bacteria/ml) still failed to evoke tissue collapse. The feint yellowing symptoms of infiltrated areas undergoing the resistant symptom is probably due to slight chloroplast disruption and very local cell collapse as indicated by electron microscopy. Where cell death occurred during the resistant symptom a much higher ratio of bacteria to host cell was required than the 1:1 ratio reported for single cell collapse in tobacco (Turner and Novacky, 1974). Many cells of the resistant symptom inducing P. tabaci were also found to be attached to living oat cells. It is possible that the elicitor of the HR is absent from bacteria causing the resistant symptom. Processes occurring

during the resistant symptom in cereal leaves are clearly very effective in restricting multiplication of incompatible plant pathogenic bacteria.

B. Prevention of the HR and normosensitive collapse

Heat-killed cells of P. coronafaciens var. atropurpurea and both heat-killed and live cells of P. tabaci and P. fluorescens protected oat leaves against hypersensitive and normosensitive collapse. If the lipopolysaccharide portion of the outer membrane of the bacterium is responsible for eliciting protection as suggested by Bonatti et al (1979), Graham et al (1977) and Mazzuchi and Pupillo (1976), the failure of P. coronafaciens to protect against tissue collapse suggests that variations in such membranes constituents of related bacteria may result in failure to elicit the protective response. If protection is caused by the induced accumulation of antibacterial compounds in the intercellular fluid as reported for tobacco by Rathmell and Sequeira (1975), it is possible that the pathogen P. coronafaciens fails to protect because it lacks specific elicitors of inhibitors. There is no evidence to suggest that an inhibitor from heat-killed bacteria is involved in the protection of oat leaves like that implicated in the prevention of the HR in barley (Hagborg, 1974).

Protection of oat leaves by heat-killed cells of P. coronafaciens var. atropurpurea also suppressed electrolyte leakage associated with normosensitive collapse. This indicates that the trigger for cell collapse and subsequent membrane damage is impeded in some way by the

protective response. Furthermore, if heat-killed cells of P. coronafaciens var. atropurpurea are responsible for the stimulation of host cells to produce antibacterial compounds which confer protection to oat leaves, this effect is not translocatable from the initial protected area nor is it light dependent. There are further differences from results obtained with tobacco leaves similarly protected in the experiments of Lozano and Sequeira (1970) and Sequeira (1976).

Although not excluded, mild or non-specific injury of leaf tissue found by Novacky and Hanchey (1976) to prevent the HR in tobacco, is unlikely to be the cause of protection in oat leaves. Protection, like the HR itself, appears to involve more specific processes.

## II ULTRASTRUCTURAL EVENTS DURING INTERACTIONS BETWEEN OAT LEAVES AND PSEUDOMONADS

### 1. General observations

Although they occurred at different times after inoculation, ultrastructural studies showed that cytoplasmic disorganization and cell collapse were very similar during hypersensitive and normosensitive responses. The changes in host cells in both instances were first apparent in the latent phases of infection and thus preceded macroscopically visible collapse. In keeping with the observations on the differences in the length of the HR induction and latent periods in oats and tobacco, the plasmalemma, tonoplast and cellular organelles of oat were not visibly affected until 12h after inoculation with the HR eliciting P. coronafaciens var. atropurpurea, 5h after the same changes were noted in

tobacco by Goodman and Plurad (1971). Alterations in the plasmalemma and chloroplast lamellae in oats inoculated with the pathogen did not occur until 18-24h after inoculation. Similarities in cell collapse during the HR and susceptible responses have also been reported in other systems (Daub and Hagedorn, 1980; Goodman and Burkowicz, 1970; Sigee and Epton, 1976). However, for reasons stated previously the processes leading to hypersensitive and normosensitive cell collapse in oat leaves are likely to be quite different. Furthermore, normosensitive tissue collapse does not impede multiplication of the pathogen. The importance of bacterial EPS in this context is discussed elsewhere in more detail (see Discussion II3).

Between 18 and 24h after inoculation with P. coronafaciens symptoms of chloroplast dysfunction increased in severity and occasionally host wall swelling and apposition formation were seen in host cells adjacent to bacteria. The significance of wall appositions in host cells is unclear since Politis and Goodman (1978) have implicated appositions in resistance in tobacco. As appositions appear in both hypersensitive and normosensitive oat tissues it is more likely that they are a non-specific host response to injury rather than a specific component of resistance. Nevertheless, it is interesting that appositions were normally found close to bacteria, perhaps indicating that localized apposition formation was elicited by some factor diffusing from bacteria close to the host cell wall.

Ultrastructural changes due to bacterial toxins such as those described by Jutte and Durbin (1979) were probably obscured by the severity of normosensitive collapse within

infiltrated areas of oat leaves. The cell wall swelling observed, however, may be due in part to the production of extracellular enzymes involved in the colonization of oat leaves by the pathogen. A role for enzymes in pathogenesis has also been inferred from the ultrastructural studies of Wallis et al (1973) and Fox (1972) but cell wall degrading enzymes are not generally thought to play a major role in the pathogenicity of leaf infecting pseudomonads.

2. Attachment of bacteria to oat cell walls

Attachment of bacteria to mesophyll cells involving host wall modification and encapsulation or stalk-like protuberances was observed in oat leaves inoculated with incompatible plant pathogenic or saprophytic bacteria (Table 2.2). By contrast the pathogen was never observed to be structurally attached to oat cell walls. However, it is unlikely that bacterial attachment is a resistance mechanism per se. Similar patterns of bacterial attachment have been noted by Goodman et al (1976; 1977), Sequeira et al (1977) and Sing and Schroth (1977) but these studies were qualitative observations on the interactions between plant and bacterial cells. The quantitative approach to this aspect presented in Chapter 2 enables a more critical assessment of the role of immobilization at the host cell surface in restricting bacterial numbers. As attachment of bacteria to oat cell walls rarely exceeded 30% in incompatible combinations, immobilization cannot account for the observed changes in populations of bacteria following inoculation. Furthermore, as attachment of the HR eliciting

P. coronafaciens var. atropurpurea does not increase beyond 12h attachment cannot explain the declining numbers still evident 5 days after inoculation.

The attachment of bacteria to host walls is more likely to be a pre-requisite for the induction of host resistance such as the HR and release of bacteriostatic compounds. However, attachment itself cannot be the process which leads to the HR since saprophytic bacteria are also immobilized within leaves. Perhaps cell contact would instead be vital to the transfer of inducer molecules, a view supported by the observation that attachment of P. coronafaciens var. atropurpurea occurs well before the end of the induction period. Similar conclusions were drawn by Sequeira et al (1977) for the process of recognition in tobacco. They proposed that interactions between specific constituents of the bacterial cell wall and binding sites on the host cell wall established the fate of incompatible bacteria.

If attachment occurs at specific sites on host cells then the large numbers of incompatible bacteria observed to be free in the intercellular spaces of oat leaves may indicate that these sites are full, thus resistance is triggered and the bacteria are localized within intercellular spaces rather than on cell walls. This hypothesis also infers that P. coronafaciens multiplies unimpeded within oat leaves because it fails to attach to host cells and therefore does not stimulate a resistant response.

A cytochemical approach is valuable in determining the nature of the structure encapsulating bacteria onto host cell

walls. Since ruthenium red staining enhances visualization of oat cell wall polysaccharide modification associated with attachment structures, it would appear that these structures are predominantly host derived in oat. However, a much wider range of ultracytochemical tests are required to determine the nature of attachment structures; for example, it is not clear if the structures are host wall or part bacteria and host wall derived in tobacco (Bonatti, Dargeni and Mazzuchi, 1979; Goodman, Huang and White, 1976).

Both incompatible and compatible bacteria were apparently localized within oat leaves by material released from cell walls during inoculation, as reported by Hildebrand et al (1980) for pseudomonads in bean leaves. The fibrillar crosses, often at oat cell junctions, which apparently trapped bacteria were polysaccharide in nature as indicated by cytochemical staining. Physical entrapment of bacteria did not appear to involve host cell wall modification in the manner associated with encapsulation.

The difficulty in differentiating between true attachment of bacteria to host walls and apparent localization by inoculation generated artefacts has been recently emphasised by Atkinson et al (1981). These workers suggest that the efficient adsorption of P. pisi onto plant cell walls is not essential to the induction of the HR in tobacco, cells of this bacterium were not seen to be enveloped on tobacco cell walls but physical entrapment was considered possible. However, my results suggest that both encapsulation and physical entrapment of bacteria

occur in oat leaves, the latter being indescriminate whereas only incompatible bacteria are attached by well defined wall modifications.

3. Bacterial extracellular polysaccharide (EPS) and pathogenicity

The densely staining fibrillar material observed around cells of P. tabaci and P. coronafaciens in susceptible leaves of tobacco and oat respectively is almost certainly capsular polysaccharide widely associated with Gram negative bacteria (Ayers, Ayers and Goodman, 1979; El-Banoby and Rudolph, 1980a). The strongly positive staining reaction of this extracellular polysaccharide (EPS) with ruthenium red indicates the presence of acidic polysaccharides as postulated by Politis and Goodman (1980) for the EPS of E. amylovora.

The appearance of P. tabaci and P. coronafaciens and their EPS in host tissues is similar to that reported by Fletcher and Floodgate (1973; 1976) who studied the adherence of marine Pseudomonas spp. to solid surfaces. The fibrillar matrix produced by marine pseudomonads was considered by Fletcher and Floodgate to be the polysaccharide responsible for adherence and the electron lucent halo around each bacterium represented a fine, primary polysaccharide produced to initiate adherence to solid surfaces. The fibrillar matrix or secondary polysaccharide was produced later to consolidate this adhesion and thus play a vital part in the survival of the marine bacteria. It is possible, therefore, that the observations on P. coronafaciens and P. tabaci in susceptible leaves

represent primary (halo) and secondary (fibrillar matrix) EPS production. In other words, the capsular EPS associated with these plant pathogenic pseudomonads is composed of at least two layers, only an outer, major water stable portion remaining visible after preparation for electron microscopy (Fig. D.1). Politis and Goodman (1980) also discuss the possibility of losing a water soluble portion of the EPS of E. amylovora during fixation and dehydration.

The fibrillar EPS of P. tabaci and P. coronafaciens is clearly associated with pathogenicity but what purpose does it serve in infected tissues? It is likely that these bacteria are able to multiply within the intercellular spaces of host tissues by virtue of their EPS which maintains an abundant water supply even after normosensitive cell collapse. Rudolph (1980) has pointed out that waterfilled intercellular spaces are essential to maintain bacterial multiplication within leaves. Thus, the pathogenecity of virulent P. phaseolicola isolates to bean leaves has been attributed to the maintenance of watersoaked leaves by a host specific principle identified as bacterial EPS (El-Banoby and Rudolph, 1979a; b; 1980a; El-Banoby, Rudolph and Hulterman, 1980; Rudolph, 1978). More recently El-Banoby, Rudolph and Mendgen (1981) have shown that purified EPS from P. phaseolicola was degraded following its injection into intercellular spaces of resistant bean plants. If such a role for EPS is important in oat and tobacco leaves, it infers that the EPS of P. coronafaciens and P. tabaci is hydrophillic in nature.

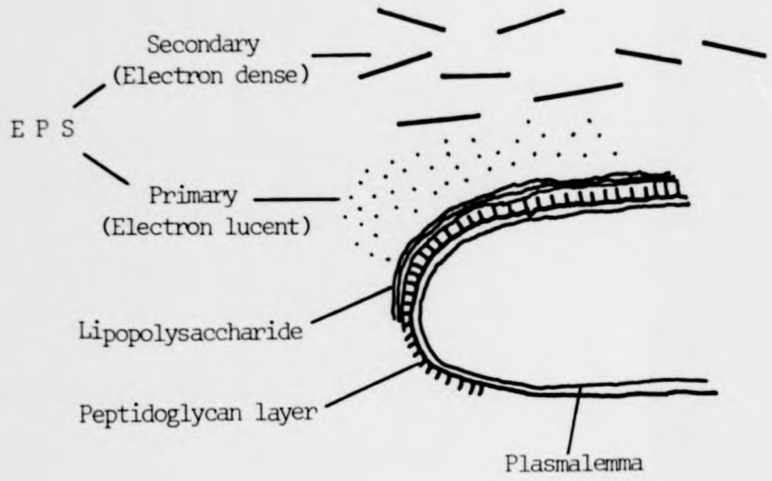



FIG. D.1 Ultrastructural model of plant pathogenic pseudomonad and relationship to extracellular polysaccharide (EPS) as determined by ruthenium red staining.

This offers an alternative interpretation of the relationship between bacterium and matrix observed in the electron microscope; fixation and dehydration of tissues causing contraction of the EPS away from the bacterial cell to leave the electron lucent halo.

As EPS was not observed in incompatible combinations, it is reasonable to assume that EPS production plays a direct part in the successful colonization of oat and tobacco by P. coronafaciens and P. tabaci respectively, similar to that reported by Ayers et al (1979) for E. amylovora in apple. The non-production of EPS by incompatible bacteria could therefore result in a failure of these bacteria to multiply due to desiccation of intercellular spaces.

If the lipopolysaccharide portion of bacterial membranes is that recognised by host cell lectins as a prelude to binding and elicitation of host response (see Introduction II3A) then the EPS of P. tabaci and P. coronafaciens produced in susceptible host tissues could prevent attachment and host recognition of the bacterial cell and subsequent trigger of resistance. Similarly, Graham and Sequeira (1977) and Sequeira and Graham (1977) showed that the failure of virulent cells of P. solanacearum to bind to potato lectin was due to the coat of EPS which was not evident for avirulent, lectin binding cells of the same bacteria. This hypothesis implies that EPS production must precede the triggering of resistance, that is, during the induction period. The EPS of P. coronafaciens is evident in oat tissues by 2.5h after inoculation, bacteria

which do not produce EPS e.g. P. coronafaciens var. atropurpurea are attached to host cells by this time and proceed to elicit resistance. My observations suggest that EPS production is host specific and thus the factors controlling EPS production in vivo could be the key to specificity. Further examination of EPS production in vitro and in vivo by plant pathogenic pseudomonads is required to substantiate these proposals.

## III ANTIBACTERIAL ACTIVITIES OF OAT LEAF EXTRACTS

The results of bioassays of extracts collected from oat leaves indicate that antimicrobial compounds may be involved in resistance. Inhibitory activity was confined predominantly to lipophilic material in the ethyl acetate phase. The bacteria tested differed in their sensitivity to the inhibitors present in the extracts. Similarly, there was no consistent pattern of inhibition in each of the bioassay techniques employed.

The fact that extracts from oat leaves inoculated with sterile distilled water possess antibacterial and antifungal activity may indicate the presence of pre-formed inhibitors within leaf tissues. As this activity was not as great as that recorded for extracts of leaves inoculated with bacteria, a compound which is mildly antimicrobial may be converted to a more active state upon infection as for the system described in maize by Hartman et al (1975). Such an inhibitor would be classed as an inhibitin by the definitions proposed by Ingham (1973).

Since P. coronafaciens is least affected by the

inhibitory principles within extracts from sterile distilled water inoculated leaves, the pathogen may be able to overcome the inhibitor by detoxification. Clearly the inhibitor is present in tissue undergoing the susceptible interaction as extracts from normosensitive leaves were inhibitory to other test bacteria but not to P. coronafaciens as indicated by liquid culture bioassay. If there is a role for constitutive chemical resistance in oat/bacteria interactions, the inhibitor is unlikely to be the well known inhibitor of fungal growth in oats, avenacin, since this saponin is not antibacterial in its action (Deacon, personal communication).

The differences noted in antibacterial activity of extracts in liquid culture bioassay could also indicate the presence of induced inhibitors (phytoalexins) within extracts of oat leaves previously inoculated with pseudomonads. This hypothesis is supported by the observation that greatest activity was detected in extracts from oat leaves undergoing the HR. The HR and phytoalexin accumulation are closely associated in French bean (Gnanamanickam and Patil, 1977a; Lyon and Wood, 1975) and soybean (Keen and Kennedy, 1974; Keen et al, 1981). Recent work has shown that previously elusive cereal phytoalexins have been implicated in the resistance of wheat and rice to fungal and bacterial pathogens (Callow, personal communication; Keen, 1978).

The failure of incompatible bacteria to multiply within oat leaves may be due to the presence of inhibitory compounds but it is not clear if this chemical resistance

is constitutive or induced. Clearly, to be effective these compounds must be present in the early stages of infection, especially in the case of P. coronafaciens var. atropurpurea which is declining in numbers prior to HR expression. Therefore, if phytoalexins are involved they are not, as Deverall (1977) suggests, necessarily synthesized from precursors in hypersensitive cells. Similarly, constitutive chemical barriers to infection would not necessarily require the HR as they are pre-existing. The timing of appearance of antibacterial compounds in oats and other plants inoculated with bacteria merits detailed examination, at present there are only limited data available on this important aspect of resistance.

Comparison of the bioassay techniques employed in this investigation highlight certain technical difficulties often encountered when testing plant extracts for anti-bacterial activity. For example, smaller amounts of extract were tested in agar plate and TLC plate bioassay than in liquid culture. Furthermore, liquid culture bioassay of oat extracts showed that there was no selective activity against the Gram positive B. megaterium as was apparent in agar plate and TLC bioassays. The low activity of recognised phytoalexins against Gram negative bacteria in agar plate bioassays has also been noted by Gnanamanickam and Smith (1980) and Gnanamanickam and Mansfield (1981). Although each assay must be considered valid in its own right, if the value of the assay in reproducing conditions similar to those encountered by

bacteria *in vivo* is to be considered then the liquid culture bioassay must be favoured since bacteria will be exposed *in vivo* to inhibitors released from host cells into the intercellular fluid. The use in liquid culture studies of intercellular fluids extracted from plants by the method of Klement (1965) may be useful in investigations of this type.

## IV GENERAL SUMMARY AND CONCLUSIONS

Broadly speaking, four types of resistance of cereal leaves to bacterial infection were distinguished on the basis of symptom appearance. These are: (a) delayed development of disease symptoms, as noted for barley and oat line Cc4146 in response to *P. coronafaciens*; (b) a hypersensitive response induced by plant pathogenic bacteria isolated from the Gramineae or with cereal pathotypes; (c) a resistant symptom in response to other plant pathogenic pseudomonads and (d) symptomless resistance following inoculation with the saprophyte *P. fluorescens*.

The success of *P. coronafaciens* as the oat halo blight pathogen is related to its ability to multiply and proliferate within the intercellular spaces of inoculated oat leaves. The production of EPS by the bacterium is likely to play a role in pathogenesis by (i) maintaining a watersoaked environment thus facilitating multiplication (Rudolph, 1980) and (ii) by obscuring the lipopoly-saccharide portion of the bacterial outer membrane and thus preventing host cell recognition of the bacteria and

elicitation of resistance as postulated for virulent strains of P. solanacearum by Graham and Sequeira (1977). The specific determinants of pathogenicity may be the factor controlling EPS production in vivo and later release of the bacterial toxin, tabtoxin. Pathogen tolerance of inhibitory, plant-derived compounds is also possible.

In contrast to other plants, HR expression in cereal leaves was not the general response to incompatible plant pathogenic bacteria. Thus resistance associated with the HR is unlikely to be the sole reason behind host specificity in oats, as has been suggested for tobacco by Klement (1972). The nature of the elicitors of the HR in cereals and other plants merits detailed comparative investigation.

The HR occurring in oat leaves is similar in its requirements and physiology to that noted for tobacco by Klement (1971) and Sequeira (1976). However, since P. coronafaciens var. atropurpurea is declining in numbers prior to HR collapse, the involvement of bacteriostatic compounds released from non-hypersensitive host cells may be a factor contributing to resistance. Desiccation of leaf tissue associated with the HR may play an independent, secondary role. The significance of the HR in imparting resistance in cereals and other plant/bacteria interactions requires further attention. In soybean and French bean, for example, hypersensitivity and phytoalexin accumulation are inseparable (Keen et al, 1981; Lyon and Wood, 1975).

The demonstration that the majority of incompatible

bacteria tested are prevented from multiplying in cereal leaves in the absence of hypersensitive necrosis lends further support to the hypothesis that processes other than desiccation during the HR confer resistance. Evidence is presented which could suggest that pre-formed or induced chemical resistance may be important. The involvement of phytoalexins with the HR cannot be ruled out. Clearly a more detailed study of this chemical component of the resistance of oats to bacterial infection is required.

Ultrastructural observations on the oat/bacteria interaction showed that changes in host cells precede macroscopic symptom development and in the case of incompatible bacteria, these changes are likely to be stimulated by factors released by bacteria attached to host cell walls. Quantitative analysis of the attachment phenomenon indicated that bacteria are not attached in sufficient numbers for immobilization per se to be considered as an important part in the localisation of bacteria within intercellular spaces.

Of the possible resistance mechanisms considered, therefore, the release of bacteriostatic compounds (constitutive or induced) seems likely to be the most important in restricting numbers of imcompatible bacteria within intercellular spaces of oat leaves. Cell contact between bacterium and host is probably intrinsic to this process. The protection phenomena observed may also be related to the accumulation of bacteriosatic compounds within the intercellular spaces, a view further supported

by the protective effects of live P. fluorescens and P. tabaci which are also unable to multiply in inoculated leaves.

Pathogenesis and resistance in cereal leaves infected with bacteria are clearly complex phenomena. It is evident that resistance and susceptibility depend upon a number of interrelated features of both plant and bacterium.

ACKNOWLEDGEMENTS

It is a pleasure to express my gratitude to Dr J W Mansfield for his continual advice and encouragement throughout this period of research and in the preparation of this thesis. Sincere thanks are also due to Dr R Sexton, Mr T Forrest, Mr A Richardson and Mr W Struthers for assistance in the electron microscopial aspects of the work, Mr L Taylor for assistance with photography, Mr I Brown and staff of the University greenhouses for preparation of plant material, Dr M Horne for advice on antibacterial assays. Finally, I thank the staff and postgraduates of the Biology Department, University of Stirling, who made this period of research enjoyable.

I am most grateful to Mrs P Brown for typing this thesis and to my wife for her patience throughout the duration of this research. The author acknowledges the support of a Science Research Council Research Studentship.

REFERENCES


ALLINGTON, W B & CHAMBERLAIN, D W (1949). Trends in the populations of pathogenic bacteria within leaf tissues of susceptible and immune plant sepcies. Phytopathology, 39, 656-660.

ANDERSON, A J & JASALAVICH, C (1979). Agglutination of pseudomonad cells by plant products. Physiological Plant Pathology, 15, 149-159.

ANON (1968). CMI Plant Pathologists Pocketbook. Commonwealth Agricultural Bureaux, London.

ARNESON, P A & DURBIN, R D (1967). Hydrolysis of tomatine by Septoria lycopersici: A detoxification mechanism. Phytopathology, 57, 1358-1360.

ATKINSON, M M, HUANG, J-S & VAN DYKE, C G (1981). Adsorption to tobacco cell walls and its significance to bacteria-host interactions Physiological Plant Pathology, 18, 1-5.

AVERRE, C W & KELMAN, A (1964). Severity of bacterial wilt as influenced by ratio of virulent to avirulent cells of Pseudomonas solanacearum in inoculum. Phytopathology, 54, 779-783.

AYERS, A R, AYERS, S B & GOODMAN, R N (1979). Extracellular polysaccharide of Erwinia amylovora: a correlation with virulence. Applied and Environmental Microbiology, 38, 659-666.

BAKER, E A & SMITH, I M (1977). Antifungal compounds in winter wheat resistant and susceptible to Septoria nodorum. Annals of Applied Biology, 87, 67-73.

BASHAN, Y, OKON, Y & HENIS, Y (1980). Ammonia causes necrosis in tomato leaves infected with Pseudomonas tomato (Okabe) Alstatt. Physiological Plant Pathology, 17, 111-119.

BATEMAN, D F & MILLAR, R L (1966). Pectic enzymes in tissue degradation. Annual Review of Phytopathology, 4, 119-146.

BLANQUET, P R (1976a). Ultrastructural study on the ruthenium red surface staining. I. Processes which give rise to electron-dense marker. Histochemistry 47, 63-78.

————. (1976b). Ultrastructural study on the ruthenium red surface staining. II. Nature and affinity of the electron-dense marker. Ibid. 47, 175-189.

BONATTI, P M, DARGENI, R & MAZZUCHI, U (1979). Ultrastructure of tobacco leaves protected against Pseudomonas aptata (Brown et Jamieson) Stevens. Phytopathologische Zeitschrift 96, 302-312.

BRADBURY, J F (1970). Isolation and preliminary study of bacteria from plants. Review of Plant Pathology, 49, 213-218.

BRUEGGER, B B & KEEN, N T (1979). Specific elicitors of glyceollin accumulation in the Pseudomonas glycinea - soybean host-parasite system. Physiological Plant Pathology, 15 , 43-51.

CALDWELL, R M, MULVEY, R R & COMPTON, L E (1946). Benton and Clinton, new disease resistant spring oats. Bulletin of the Indiana Agriculture Experiment Station, 514, 2-10.

CALLOW, J A (1975). Plant lectins. Current Advances in Plant Sciences, 18, 181-193.

———. (1977). Recognition, resistance and the role of plant lectins in host-parasite interactions. Advances in Botanical Research, 4 1-49.

CARLSON, R R, VIDAVER, A K, WYSONG, D S & RIESSELMAN, J H (1979). Pressure injection device for inoculation of maize with bacterial phytopathogens. Plant Disease Reporter, 63, 736-738.

CASON, E T, RICHARDSON, P E, ESSENBERG, M K, BRINKEROFF, L A, JOHNSON, W M & VENERE, R J (1978). Ultrastructural cell wall alterations in immune cotton leaves inoculated with Xanthomonas malvacearum. Phytopathology, 68, 1015-1021.

CHAND, J N & WALKER, J C (1964). Relation of age of leaf and varietal resistance to bacterial multiplication in cucumber inoculated with Psuedomonas lachrymans. Ibid. 54, 49-50.

CHENG, C-P & ROANE, C W (1968). Sources of resistance and inheritance of reaction to Pseudomonas coronafaciens in oats. Ibid. 58, 1402-1405.

COOK, A A (1975). Effect of low concentrations of Xanthomonas vesicatoria infiltrated into pepper leaves. Phytopathology, 65, 487-489.

——— & STALL, R W (1968). Effect of Xanthomonas vesicatoria on loss of electrolytes from leaves of Capsicum annum. Ibid. 58, 617-619.

——— & ———. (1977). Effects of watersoaking on response to Xanthomonas vesicatoria in pepper leaves. Ibid. 67, 1101-1103.

CORCUERA, L J, WOODWARD, M D, HEGELSON, J P, KELMAN, A & UPPER, C D (1978). 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one, an inhibitor from Zea mays with differential activity against soft-rotting Erwinia species. Plant Physiology, 61, 791-795.

CROSTHWAITE, L M & PATIL, S S (1978). Isolation of an endotoxin from Pseudomonas phaseolicola which induces cell collapse in bell pepper. Phytopathologische Zeitschrift, 91, 80-90.

——— & SHEEN, S J (1979). Inhibition of ribulose 1,5-biphosphate carboxylate by a toxin isolated from Pseudomonas tabaci. Phytopathology, 69, 376-379.

CUNFER, B M & SCHAAD, W (1976). Halo blight of rye. Plant Disease Reporter, 60, 61-64.

———, ——— & MOREY, D D (1978). Halo blight of rye: multiplicity of symptoms under field conditions. Phytopathology, 68, 1545-1548.

DAUB, M E & HAGEDORN, D J (1980). Growth kinetics and interactions of Psuedomonas syringae with susceptible and resistant bean tissues. Ibid. 70, 429-436.

DAVIES, D L G, NOBLE, M & HORMAN, T. (1955). Halo blight of oats. Plant Pathology, 4, 33.

DEFAGO, G (1977). Role des saponins dans la resistance des plantes aux maladies fongiques. Berichte-Schweizerische Botanische Gesellschaft, 87, 79-132.

DEVERALL, B J (1977). Defence mechanisms of plants. Cambridge monographs in experimental biology, 19. Cambridge University Press.

——— (1982). Introduction. In: Phytoalexins (Eds. J A Bailey and J W Mansfield) Blackie and Son. In press.

————, WONG, P T W & McLEOD, S (1979). Failure to implicate antifungal substances in cross-protection of wheat against take-all. Transactions of the British Mycological Society, 72, 233-236.

DIACHUN, S & TROUTMAN, J (1954). Multiplication of Pseudomonas tabaci in leaves of Burley tobacco, Nicotiana longiflora and hybrids. Phytopathology, 44, 186-187.

DINSDALE, D, MORRIS, E J, & BACON, J S D (1978). Electron microscopy of the microbial populations present and their modes of attack on various cellulosic substrates undergoing digestion in the sheep rumen. Applied and Environmental Microbiology 36, 160-168.

DURBIN, R D & SINDEN, S L (1967). The effect of light on the symptomatology of oat halo blight. Phytopathology 57, 1000-1001.

———, UCHYTIL, T F, STEELE, J A & RIBEIRO, R (1978). Tabtoxinine-$\beta$-lactam from Pseudomonas tabaci. Phytochemistry 17, 147.

EL-BANOBY, F E & RUDOLPH, K (1979a). A polysaccharide from liquid cultures of Pseudomonas phaseolicola which specifically induces water-soaking in bean leaves (Phaseolus vulgaris L.). Phytopathologische Zeitschrift 95, 38-50.

———— & ———— (1979b). Induction of water-soaking in plant leaves by extracellular polysaccharides from phytopathogenic pseudomonads and xanthomonads.

Physiological Plant Pathology 15, 341-349.

———— & ———— (1980a). Purification of extracellular polysaccharides from Pseudomonas phaseolicola which induce water-soaking in bean leaves. Ibid. 16, 425-437.

———— & ———— (1980b). Agglutination of Pseudomonas phaseolicola by bean leaf extracts. Phytopathologische zeitschrift 98, 91-95.

———— , ———— & HUTTERMAN, A (1980). Biological and physical properties of an extracellular polysaccharide from Pseudomonas phaseolicola. Physiological Plant Pathology 17, 291-301.

————, ———— & MENDGEN, K (1981). The fate of extracellular polysaccharide from Pseudomonas phaseolicola in leaves and leaf extracts from halo blight susceptible and resistant bean plants. Ibid. 18, 91-98.

ELLIOTT, C (1920). Halo blight of oats. Journal of Agricultural Research XIX, 139-172.

———— (1927). Bacterial stripe blight of oats. Ibid 35, 811-824.

———— (1951). Manual of bacterial plant pathogens (second edition). Chronica Botanica Company.

ERCOLANI, G L & CROSSE, J E (1966). The growth of Pseudomonas phaseolicola and related pathogens in vivo. Journal of General Microbiology 45, 429-439.

ESSENBERG, M, CASON, E T, HAMILTON, B, BRINKERHOFF, L A, GHOLSON, R K & RICHARDSON, P E (1979). Single cell colonies of Xanthomonas malvacearum in susceptible and immune cotton leaves and the local resistant response to colonies in immune leaves, Physiological Plant Pathology 15, 53-68.

————————, HAMILTON, B, CASON, J T, BRINKERHOFF, L A, GHOLSON, R K & RICHARDSON, P E (1979). Localized bacteriostasis indicated by water dispersal of colonies of Xanthomonas malvacearum within immune cotton leaves. IBid. 15, 69-78.

FERGUSON, A R & JOHNSTON, D S (1980). Phaseolotoxin: chlorosis, arnithine accumulation and inhibition of ornithine carbomoyltransferase in different plants. Ibid. 16, 269-275.

FETT, W F & SEQUEIRA, L (1980). A new bacterial agglutinin from soybean. I. Isolation, partial purification and characterization. Plant Physiology 66, 847-852.

———— & ———— (1980). A new bacterial agglutinin from soybean. II. Evidence against a role in determining pathogen specificity. Ibid. 66, 853-858.

FLETCHER, M & FLOODGATE, G D (1973). An electron microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. Journal of General Microbiology 74, 325-334.

———— & ———— (1976). The adhesion of bacteria to solid surfaces. In: Microbial Ultrasctructure for use of the Electron Microscope (eds. Fuller, R and Lovelock, D W) Academic Press, London. pp 101-107.

FOX, R T V (1972). The ulstrastructure of potato tubers infected by the soft rot organism Erwinia carotovora var. atroseptica. Proceedings of the Third International Conference on Plant Pathogenic Bacteria, Wageningen. pp. 95-120.

GABORJANYI, R, O'BRIEN, F & KLEMENT, Z (1974). Inhibition of the hypersensitive response of tobacco leaves to bacteria by foreign proteins. Acta Phytopathologia Academiae Scientiarum Hungaricae 9, 31-33.

GARIBALDI, A (1972). Pectolytic and cellulolytic enzymes produced by Xanthomonas perlargonii. Proceedings of the Third International Conference on Plant Pathogenic Bacteria, Wageningen. pp. 351-356.

GIDDIX, L R, LUKEZIC, F L & PELL, E J (1981). Effect of light on bacteria-induced hypersensitivity in soybean. Phytopathology 71, 111-115.

GLAUERT, A (1965). Section staining, cytology, autoradiography and immunochemistry for biological specimens. In: Techniques for Electron Microscopy (ed. D. Kay). Blackwell Scientific Publications, pp. 254-310.

GNANAMANICKAM, S S & PATIL, S S (1976). Bacterial growth, toxin production, and levels of ornithine carbomoyltransferase in resistant and susceptible cultivars of bean inoculated with Pseudomonas phaseolicola. Phytopathology 66, 290-294.

——— & ———(1977a). Accumulation of antibacterial isoflavonoids in hypersensitively responding bean leaf tissues inoculated with Pseudomonas phaseolicola. Physiological Plant Pathology 10, 159-168.

——— & ——— (1977b). Phaseotoxia suppresses bacterially induced hypersensitive reaction and phytoalexin synthesis in bean cultivars. Ibid. 10, 169-179.

———— & MANSFIELD, J W (1981). Selective toxicity of wyerone and other phytoalexins to Gram-positive bacteria. Phytochemistry, 20, 997-1000.

———— & SMITH, D A (1980). Selective toxicity of isoflavonoid phytoalexins to Gram-positive bacteria. Phytopathology 70, 894-896.

GOODMAN, R N (1967). A possible biochemical basis for the hypersensitive reaction induced in plants by pathogenic bacteria. Phytopathology 57, 812-813.

———— (1968). The hypersensitive reaction in tobacco. A reflection of changes in host cell permeability. Ibid. 58, 872-873.

———— (1972). Electrolyte leakage and membrane damage in relation to bacterial population, pH, and ammonia production in tobacco leaf tissue inoculated with Pseudomonas pisi. Ibid. 62, 1327-1331.

———— (1976). Physiological and cytological aspects of the bacterial infection process. Encyclopedia of Plant Physiology, New Series 4, 172-196.

———— & BURKOWICZ, A (1970). Ultrastructural changes in apple leaves inoculated with a virulent or an avirulent strain of Erwinia amylovora. Phytopathologische Zeitschrift 68, 258-268.

———— , HUANG, P Y & WHITE, J A (1976). Ultrastructural evidence for immobilization of an incompatible bacterium, Pseudomonas pisi, in tobacco leaf tissue. Phytopathology 66, 754-764

———— & PLURAD, S B (1971). Ultrastructural changes in tobacco undergoing the hypersensitive reaction cuased by plant pathogenic bacteria. Physiological Plant Pathology 1, 11-15.

————, POLITIS, D J & WHITE, J A (1977). Ultrastructural evidence of an 'active' immobilization process of incompatible bacteria in tobacco leaf tissue: a resistant reaction. In: Cell Wall Biochemistry (eds. B Solheim and J Raa). Scandinavian University Books. pp. 423-438.

GOTO, M, TAKEMURA, I & YAMANAKA, K (1979). Leakage of electrolytes and amino acids from susceptible and resistant citrus leaf tissues infected by Xanthomonas citri. Annals of the Phytopathological Society of Japan 45, 625-636.

GRAHAM, T L & SEQUEIRA, L (1977). Interaction between plant lectins and cell wall components of Pseudomonas solanacearum: role in pathogenicity and induced disease resistance. In: Cell Wall Biochemistry (eds. B Solheim and J Raa). Scandinavian University Books. pp. 417-422.

————, ———— & HUANG, T R (1977). Bacterial lipopolysaccharides as inducers of disease resistance in tobacco. Applied and Environmental Microbiology 34, 424-432.

GRIFFITHS, D J & PEREGRINE, W T H (1956). Halo blight of oats. Plant Pathology 5, 95-97.

———— & ———— (1960). Control of halo blight in oats. Ibid. 9, 10-14.

———— & ———— (1964). The inheritance of resistance to halo blight of oats. Ibid. 13, 56-59.

GROSS, D C & DE VAY, J E (1977). Population dynamics and pathogenesis of Pseudomonas syringae in maize and cowpea in relation to the in vitro production of

syringomycin. Phytopathology 67, 475-483.

GULYAS, A, BARNA, B, KLEMENT, Z & FARKAS, G L (1979). Effect of plasmolytica on the hypersensitive reactions induced by bacteria in tobacco: a comparison with the virus-induced hypersensitive reaction. Ibid. 69, 121-124.

HAGBORG, W A F (1970). A device for injecting solutions and suspensions into thin leaves of plants. Canadian Journal of Botany 48, 1135-1136.

———— (1974). Notes on bacterial diseases of cereals and some other crop plants. Canadian Plant Disease Survey 54, 129-151.

HAMMERSCHMIDT, R & KUC, J (1979). Isolation and identification of phytuberin from Nicotiana tabacum previously infiltrated with an incompatible bacterium. Phytochemistry 18, 874-875.

HANCHEY, P, PASTALKA, T & NOVACKY, A (1974). Ultrastructure of tobacco protected against the hypersensitive response. Proceedings of the American Phytopathological Society 1, 74.

HARDER, D E & HARRIS, D C (1973). Halo blight of oats in Kenya. East African Agricultural and Forestry Journal 38, 241-245.

HARTMAN, J R, KELMAN, A & UPPER, C D (1975). Differential inhibitory activity of a corn extract to Erwinia spp. causing soft rot. Phytopathology 65, 1082-1088.

HEARON, S S, SHERALD, J L & KOSTKA S J (1980). Association of xylem limited bacteria with elm, sycamore and oak leaf scorch. Canadian Journal of Botany 58, 1986-1993.

HIETALA, P K & WAHLROOS, O (1956). The synthesis of 6-methoxy-2(3)-benzoxazolinone. Acta Chemica Scandinavica 10, 1196-1197.

HICKEY, E L & COFFEY, M D (1978). A cytochemical investigation of the host-parasite interface in Pisum sativum infected by the downy mildew fungus Peronospora pisi. Protoplasma 97, 201-220.

HILDEBRAND, D C (1972). Pectolytic enzymes of Pseudomonas. Proceedings of the Third International Conference on Plant Pathogenic Bacteria, Wageningen. pp. 331-343.

————, ALOSI, M C & SCHROTH, M N (1980). Physical entrapment of pseudomonads in bean leaves by films formed at air-water interfaces. Phytopathology 70, 98-109.

HOLDEN, J (1980). Relationship between pre-formed inhibitors in oats and infection by Gaeumannomyces graminis and Phialophora radicicola. Transactions of the British Mycological Society 75, 97-105.

HOLLIDAY, M J, KEEN, N T & LONG, M (1981). Cell death patterns and the accumulation of fluorescent material in the hypersensitive response of soybean leaves to Pseudomonas syringae pv. glycinea. Physiological Plant Pathology 18, 279-287.

HSU, S T & DICKEY, R S (1972). Comparative growth of Xanthomonas phaseoli and Xanthomonas vesicatoria and development of symptoms in bean and tomato leaves. Phytopathology 62, 329-332.

HUANG, J S & VAN DYKE, C G (1978). Interaction of tobacco callus tissue with Pseudomonas tabaci, P. pisi and P. fluorescens. Physiological Plant Pathology 13, 65-72.

HUANG, P Y, HUANG, J S & GOODMAN, R N (1975). Resistance mechanisms of apple shoots to an avirulent strain of Erwinia amylovora. Ibid. 6, 283-287.

INGHAM, J L (1973). Disease resistance in higher plants. The concept of pre-infectional and post-infectional resistance. Phytopathologische Zeitschrift, 78, 314-335.

JOHNSON, J (1937). Relation of water-soaked tissues to infection by Bacterium angulatum and Bacterium tabacum and other organisms. Journal of Agricultural Research 55, 599-618.

JONES, L R, JOHNSON, A G & REDDY, C S (1917). Bacterial blight of barley. Ibid. 11, 625-643.

JUTTE, S M & DURBIN, R D (1979). Ultrastructural effects in Zinnia leaves of a chlorosis-inducing toxin from Pseudomonas tagetis. Phytopathology 69, 839-842.

KAWAMOTO, S O & LORBEER, J W (1972). Multiplication of Pseudomonas cepacia in onion leaves. Ibid. 62, 1263-1265.

KEEN, N T (1978). Phytoalexins: efficient extraction from leaves by a facilitated diffusion technique. Ibid. 68, 1237-1239.

———— & KENNEDY, B W (1974). Hydroxyphaseollin and related isoflavonoids in the hypersensitive reaction of soybeans to Pseudomonas glycinea. Physiological Plant Pathology 4, 137-185.

————, ERSEK, T, LONG, M, BRUEGGER, B B & HOLLIDAY, M J (1981). Inhibition of hypersensitive reaction of soybean leaves to incompatible Pseudomonas spp. by blasticidin S, streptomycin or elevated temperature. Ibid. 18, 325-337.

KELMAN, A & SEQUEIRA, L (1972). Resistance in plants to bacteria. Proceedings of the Royal Society of London B 181, 247-266.

KENDRICK, J B (1926). Holcus bacterial spot of Zea mays and Holcus species. Iowa Agriculture Experiment Station Research Bulletin 100, 303-334

KINGSOLVER, C H (1944). Pathogenicity on Avena and growth response of Pseudomonas coronafaciens (Elliott) Stapp. Iowa State College Journal of Science 19, 29-31.

KLARMAN, W L & SANFORD, J B (1968). Isolation and purification of an antifungal principle from infected soybeans. Life Sciences 7, 1095-1103.

KLEMENT, Z (1963). Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199, 299-300.

——— (1965). Method of obtaining intercellular fluid from the intercellular spaces of foliage and fluids merit as substrate for phytobacterial pathogens. Phytopathology 55, 1033-1034.

——— (1971). The hypersensitive reaction of plants to bacterial infections. Acta Phytopathologia Academiae Scientiarum Hungaricae 6, 115-118.

——— (1972). Development of the hypersensitivity reaction induced by plant pathogenic bacteria. Proceedings of the Third International Conference on Plant Pathogenic Bacteria, Wageningen. pp. 157-164.

——— (1977). Cell contact recognition versus toxin action in induction of bacterial hypersensitive reaction. Acta Phytopathologia Academiae Scientiarum Hungaricae 12, 257-261.

———, FARKAS, G L & LOVREKOVICH, L (1964). Hypersensitive reaction induced by phytopathogenic bacteria

in the tobacco leaf. Phytopathology 54, 474-477.

——— & GOODMAN, R N (1967a). The role of the living cell and induction time in the hypersensitive reaction of the tobacco plant. Ibid. 57, 322-323.

——— & ——— (1967b). The hypersensitive reaction to infection by bacterial plant pathogens. Annual Review of Phytopathology 5, 17-44.

———, HEVESI, M & SASSER, M (1978). Mechanism of the development of wildfire disease in susceptible tobacco plant. Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria, Angers. pp. 679-685.

——— & LOVREKOVICH, L (1961). Defence reactions induced by phytopathogenic bacteria in bean pods. Phytopathologische Zeitschrift 41, 217-227.

LACY, G H, HIRANO, S S, VICTORIA, J L, KELMAN, A & UPPER, C D (1979). Inhibition of soft-rotting Erwinia spp. strains by 2,4-dihydroxy-7-methoxy-2H-1,4-benzoazin-3 (4H)-one in relation to their pathogenicity on Zea mays. Phytopathology 69, 757-763.

LARGE, E C (1954). Growth stages in the cereals: illustration of the Feekes scale. Plant Pathology 3, 128-129.

LELLIOTT, R A, BILLING, E & HAYWARD, A C (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. Journal of Applied Bacteriology 29, 470-489.

LOEBENSTEIN, G. & LOVREKOVICH, L (1966). Interference with tobacco mosaic virus local lesion formation in tobacco by injecting heat-killed cells of Pseudomonas syringae. Virology 30, 587-591.

LOVREKOVICH, L & FARKAS, G L (1965). Induced protection against wildfire disease in tobacco leaves treated with heat-killed bacteria. Nature 205, 823-824.

———— & LOVREKOVICH, H (1970). Tissue necrosis in tobacco caused by a saprophytic bacterium. Phytopathology 60, 1279-1280.

————, ———— & GOODMAN, R N (1970). Ammonia as a necrotoxin in the hypersensitive reaction caused by bacteria in tobacco leaves. Canadian Journal of Botany 48, 167-171.

LOZANO, J C & SEQUEIRA, L (1970). Prevention of the hypersensitive reaction in tobacco leaves by heat-killed bacterial cells. Phytopathology 60, 875-879.

LUFT, J H (1971). Ruthenium red and violet I. Chemistry, purification, methods for use for electron microscopy and mechanism of action. Anatomical Record 171, 347-368.

LUND, B M & LYON, G D (1975). Detection of inhibitors of Erwinia carotovora and E. herbicola on thin-layer chromatograms. Journal of Chromatography 110, 193-196.

LUNING, H U & SCHLOSSER, E (1975). Role of saponins in antifungal resistance. V. Enzymic activation of avenacosides. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 82, 699-703.

———— & ———— (1976). Saponine in Avena sativa. Angewandte Botanik 50, 49-60.

LYON, G D (1978). Attenuation by divalent cations of the effect of the phytoalexin rishitin on Erwinia carotovora var. atroseptica. Journal of General Microbiology 109, 5-10.

———— & BAYLISS, C E (1975). The effect of rishitin on Erwinia carotovora var. atroseptica and other bacteria. Physiological Plant Pathology 6, 177-186.

LYON, F M & WOOD, R K S (1975). Production of phaseollin, coumestrol and related compounds in bean leaves inoculated with Pseudomonas spp. Ibid. 6, 117-124.

———— & ———— (1976). The hypersensitive reaction and other responses of bean leaves to bacteria. Annals of Botany 40, 479-491.

MANSFIELD, J W (1982). The role of Phytoalexins in disease resistance. In: Phytoalexins (eds. J A Bailey and J W Mansfield). Blackie and Son. In press.

MAZZUCHI, U & PUPILLO, P (1976). Prevention of confluent hypersensitive necrosis in tobacco leaves by a bacterial protein-lipopolysaccharide complex. Physiological Plant Pathology 9, 101-112.

————, BAZZI, C & PUPILLO, P (1979). The inhibition of susceptible and hypersensitive reactions by protein-lipopolysaccharide complexes from phytopathogenic pseudomonads: relationship to polysaccharide antigenic determinants. Ibid. 14, 19-30.

MITCHELL, R E (1978). Halo blight of beans: toxin production by several Pseudomonas phaseolicola isolates. Ibid. 13, 37-49.

MOHANAKUMARAN, N, GILBERT, J C & BUDDENHAGEN, I W (1969). Relationship between tomatin and bacterial wilt resistance in tomato. Phytopathology 59, 14.

MULLER, H J (1964). Studies on the leaf spot diseases of oats. III. Bacterial leaf spot pathogens of oats

(P. coronafaciens and P. striafaciens). Phytopathologische Zeitschrift 49, 325-338.

MULLER, K O (1956). Einige einfache versuche zum nachweis von phytoalexin. Ibid. 27, 237-254.

———— & BORGER, H (1941). Experimentelle untersuchungen uber die Phytophthora - Resistenz der kartoffel. Zugleich ein beitrag zum problem der erworbenen resistenz in pflanzenreich. Arb.Biol.Anst.(Reichanst.) Berlin 23, 189-231. (Abstract: Review of Applied Mycology 29, 226, 1950).

NEMETH, J & KLEMENT Z (1967). Changes in respiration rate of tobacco leaves infected with bacteria in relation to the hypersensitive reaction. Acta Phytopathologia Academiae Scientiarum Hungaricae 2, 303-308.

————, ———— & FARKAS, G L (1969). An enzymological study of the hypersensitive reaction induced by Pseudomonas syringae in tobacco leaf tissues. Phytopathologische Zeitschrift 65, 267-278.

NOBLE, M (1949). Halo blight of oats. Scottish Agriculture 29, 162-165.

NOVACKY, A (1972). Suppression of the bacterially induced hypersensitive reaction by cytokinins. Physiological Plant Pathology 2, 101-104.

————, ACEDO, G & GOODMAN, R N (1973). Prevention of bacterially induced hypersensitive reaction by living bacteria. Ibid. 3, 133-136.

———— & HANCHEY, P (1976). Effect of internal leaf injury on bacterial hypersensitivity. Acta Phytopath 2, 217-222.

OBUKOWICZ, M & KENNEDY, G S (1981). Phenolic ultracytochemistry of tobacco cells undergoing the hypersensitive reaction to Pseudomonas solanacearum. Physiological Plant Pathology 18, 339-344.

O'BRIEN, F & WOOD, R K S (1973a). Role of ammonia in infection of Phaseolus vulgaris by Pseudomonas spp. Ibid. 3, 315-325.

———— & ————, (1973b). Antibacterial substances in hypersensitive responses induced by bacteria. Nature 242, 532-533.

O'NEILL, T M (1981). Narcissus smoulder: cause, epidemiology and host resistance. PhD Thesis, University of Stirling.

OMER, M E H & WOOD, R K S (1969). Growth of Pseudomonas phaseolicola in susceptible and in resistant bean plants. Annals of Applied Biology 63, 103-116.

PASSMOOR, M & EPTON, H A S (1980). The relationship between toxin release and the formation of vesicles and blebs by Pseudomonas phaseolicola. Phytopathologische Zeitschrift 98, 289-295.

PATE, J L & ORDAL, E J (1967). The fine structure of Chondrococcus columnaris. III. The surface layers of Chondrococcus columnaris. Journal of Cell Biology 35, 37-51.

PATIL, S S (1974). Toxins produced by phytopathogenic bacteria. Annual Review of Phytopathology 12, 259-279.

———— & GNANAMANICKAM, S S (1976). Suppression of bacterially-induced hypersensitive reaction and phytoalexin accumulation in bean by phaseotoxin. Nature 259, 486-487.

PEROMBELON, M C M & KELMAN, A (1980). Ecology of the soft rot Erwinias. Annual Review of Phytopathology 18, 361-387.

PINKAS, Y & NOVACKY, A (1971). The differentiation between bacterial hypersensitive reaction and pathogenesis by the use of cycloheximide. Phytopathology 61, 906-907.

POLITIS, D J & GOODMAN, R N (1978). Localized cell wall appositions: incompatibility response of tobacco leaf cells to Pseudomonas pisi. Ibid. 68, 309-316.

——— & ——— (1980). Fine structure of extracellular polysaccharide of Erwinia amylovora. Applied and Environmental Microbiology 40, 596-607.

RATHMELL, W G & SEQUEIRA, L (1975). Induced resistance in tobacco leaves: the role of inhibitors of bacterial growth in the intercellular fluid. Physiological Plant Pathology 5, 65-73.

REDDY, C S & GODKIN, J (1923). A bacterial disease of brome-grass. Phytopathology 13, 75-89.

REYNOLDS, E S (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology 17, 208-212.

RIBEIRO, R, DURBIN, R D, ARNY, D C & UCHYTIL, T F (1977). Characterization of the bacterium inciting chocolate spot of corn. Phytopathology 67, 1427-1431.

ROANE, C W & KURIGER, W E (1976). The Pseudomonas coronafaciens syndrome of oats. Proceedings of the American Phytopathological Society 3, 291.

ROEBUCK, P, SEXTON, R & MANSFIELD, J W (1978). Ultrastructural observations on the development of the hypersensitive reaction in leaves of Phaseolus vulgaris cv. Red Mexican inoculated with Pseudomonas phaseolicola (Race 1). Physiological Plant Pathology 12, 151-157.

ROLAND, J C (1978). General preparation and staining of thin sections. In: Electron Microscopy and Cytochemistry of Plant Cells (ed. J L Hall). Elsevier/North Holland Biomedical Press. pp. 1-62.

RUDOLPH, K (1978). A host specific principle from Pseudomonas phaseolicola (Burkh) Dowson, inducing watersoaking in bean leaves. Phytopathologische Zeitschrift 93, 218-226.

———— (1980). Multiplication of bacteria in leaf tissue. Angewandte Botanik 54, 1-9.

SABATINI, D D, BENSCH, K & BARRNETT, R J (1963). Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymic activity by aldehyde fixation. Journal of Cell Biology 17, 19-58.

SCANNERINI, S & BONFANTE-FASOLO, P (1979). Ultrastructural cytochemical demonstration of polysaccharides and proteins within the host-arbuscle interfacial matrix in an endmycorrhiza. New Phytologist 83, 87-94.

SCHAREN, A L (1959). Comparative population trends of Xanthomonas phaseoli in susceptible, field tolerant and resistant hosts. Phytopathology 49, 425-428.

SELLAM, M A & WILCOXSON, R D (1976). Bacterial leaf blight of wheat in Minnesota. Plant Disease Reporter 60, 242-245.

SEQUEIRA, L (1976). Induction and suppression of the hypersensitive reaction caused by phytopathogenic bacteria: specific and non-specific components. In: Specificity in Plant Diseases (eds. R K S Wood & A Graniti). Plenum Press, New York and London. pp. 289-306.

———————— (1978). Lectins and their role in host-pathogen specificity. Annual Review of Phytopathology 16, 453-481.

———————— (1979). Recognition between plant hosts and parasites. In: Host Parasite Interfaces (ed. B B Nickol). Academic Press, London. pp. 71-84.

————————, AIST, S & AINSLIE, V (1972). Prevention of the hypersensitive reaction in tobacco by proteinaceous constituents of Pseudomonas solanacearum. Phytopathology 62, 536-541.

————————, GAARD, G & DE ZOETEN, G A (1977). Interaction of bacteria and host cell walls: its relation to mechanisms of induced resistance. Physiological Plant Pathology 10, 43-50.

———————— & GRAHAM, T L (1977). Agglutination of avirulent strains of Pseudomonas solanacearum by potato lectin. Ibid. 11, 43-54.

———————— & HILL L M (1974). Induced resistance in tobacco leaves: the growth of Pseudomonas solanacearum in protected tissues. Ibid. 4, 447-455.

SHERWOOD, R T, OLAH, A F, OLESON, W H & JONES, E E (1970). Effect of disease and injury on accumulation of a flavonoid estrogen, coumestrol in alfalfa. Phytopathology 60, 684-688.

SIGEE, D C & EPTON, H A S (1975). Ultrastructure of Pseudomonas phaseolicola in resistanct and susceptible leaves of French bean. Physiological Plant Pathology 6, 29-34.

———————— & ———————— (1976). Ulstrastructural changes in resistant and susceptible varieties of Phaseolus vulgaris

following artificial inoculation with Pseudomonas phaseolicola. Ibid. 9, 1-8.

SINDEN, S L, DURBIN, R D & UCHYTIL, T F (1969). The production of convulsions by an exotoxin from Pseudomonas tabaci. Toxicology and Applied Pharmacology 14, 82-88.

———— & ———— (1970). A comparison of the chlorosis inducing toxin from Pseudomonas coronafaciens with wildfire toxin from Ps. tabaci. Phytopathology 60, 360-364.

SING, V & SCHROTH, M N (1977). Bacteria-plant cell surface interactions: active immobilization of saprophytic bacteria in plant cells. Science 197, 759-761.

SLEESMAN, H C, PERLEY, J E & HOITINK, A J (1970). Susceptible and hypersensitive reactions in tobacco and their prevention by cell-free extracts of Pseudomonas tabaci and Pseudomonas glycinea. Phytopathology 60, 1314.

SLUSARENKO, A J & WOOD, R K S (1981). Differential agglutination of races 1 and 2 of Pseudomonas phaseolicola by a fraction from cotyledons of Phaseolus vulgaris cv. Red Mexican. Physiological Plant Pathology 18, 187-193.

SMITH, E F (1920). An introduction to bacterial diseases of plants. W B Saunders Company.

SRIDHAR, R, MOHANTY, S K & REDDY, P R (1979). Physiology of bacterial leaf streak of rice: relationship between leaf senescence and post-infectional changes in detached rice leaves. Acta Phytopathologia Academiae Scientiarum Hungaricae 14, 95-101.

STALL, R E & COOK, A A (1966). Multiplication of Xanthomonas vesicatoria and lesion development in resistant and susceptible pepper. Phytopathology 56, 1152-1154.

———— & ———— (1968). Inhibition of Xanthomonas vesicatoria in extracts from hypersensitive and susceptible pepper leaves. Ibid. 58, 1584-1587.

———— & ———— (1979). Evidence that bacterial contact with the plant cell is necessary for the hypersensitive reaction but not the susceptible reaction. Physiological Plant Pathology 14, 77-84.

STHOLASUTA, P, BAILEY, J A, SEVERIN, V & DEVERALL, B J (1971). Effect of bacterial inoculation of bean and pea leaves on the accumulation of phaseollin and pisatin. Ibid. 1, 177-183.

TAYLOR, P A, SCHNOES, H K & DURBIN, R D (1972). Characterization of chlorosis-inducing toxins from a plant pathogenic Pseudomonas sp. Biochimica et Biophysica Acta 286, 107-117.

———— & DURBIN, R D (1973). The production and properties of chlorosis-inducing toxins from a Pseudomonad attacking Timothy. Physiological Plant Pathology 3, 9-17.

TESSI, J L (1953). Estudio comparativo de dos bacterios patógenos en avena y determinación de una toxina que origina sus differencias. Revista de Investigaciones Agricolas 7, 131-145.

TOMINAGA, T (1968). Halo blight of ryegrasses, brome grasses and fescues. Annals of the Phytopathological Society of Japan 34, 242-249.

TRABULSI, I Y (1975). Studies on halo-blight of oats and other diseases caused by toxigenic species of Pseudomonas. PhD Thesis, University College of North Wales, Bangor.

TURNER, E M (1953). The nature of resistance of oats to the take-all fungus. Journal of Experimental Botany 4, 264-271.

———— (1956). The nature of resistance of oats to the take-all fungus II. Inhibition of growth and respiration of Ophiobolus graminis Sacc. and other fungi by a constituent of oat sap. Ibid 7, 80-92.

———— (1961). An enzymic basis for pathogenic specificity in Ophiobolus graminis. Ibid. 12, 169-175.

TURNER, J G & NOVACKY, A (1974). The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopathology 64, 885-890.

VANHOOF, L, VANDENBERGHE, D A & VLIETINCK, A J (1980). Screening of poplar trees for antibacterial, antifungal and antiviral activity. Biologia Plantarum 22, 265-273.

WACEK, T J & SEQUEIRA, L (1973). The peptiodoglycan of Pseudomonas solanacearum: chemical composition and biological activity in relation to the hypersensitive reaction in tobacco. Physiological Plant Pathology 3, 363-369.

WAHLROOS, O & VIRTANEN, A I (1959). The pre-cursors of 6-methoxy-benzoxazoline in maize and wheat plants, their isolation and some of their properties. Acta Chemica Scandinavica 13, 1906-1908.

WALLIS, F M, RIJKENBERG, F H J, JOUBERT, J J & MARTIN, M M (1973). Ultrastructural histopathology of cabbage leaves infected with Xanthomonas campestris. Physiological Plant Pathology 3, 371-378.

WEBSTER, D M & SEQUEIRA, L (1977). Expression of resistance in bean pods to an incompatible isolate of Pseudomonas syringae. Canadian Journal of Botany 55, 2043-2052.

WELLER, D M & SAETTLER, A W (1980). Colonization and distribution of Xanthomonas phaseoli and Xanthomonas phaseoli var. fuscans in field-grown navy beans. Phytopathology 70, 500-506.

WHATLEY, M H, HUNTER, N, CANTRELL, M A, HENDRICK, C, KEEGSTRA, K & SEQUEIRA, L (1980). Lipopolysaccharide composition of the wilt-pathogen, Pseudomonas solanacearum-correlation with the hypersensitive response in tobacco. Plant Physiology 65, 557-559.

WHITNEY, N J & MORTIMORE, C G (1961). Effect of 6-methoxybenzoxazoline on the growth of Xanthomonas stewartii (Erw. Smith) Dowson and its presence in sweet corn (Zea mays var. saccharata Bailey). Nature 189, 596-597.

WYMAN, J G & VAN ETTEN, H D (1978). Antibacterial activity of selected isoflavonoids. Phytopathology 68, 583-589.

YOUNG, J M (1974a). Development of bacterial populations in vivo in relation to plant pathogenicity. New Zealand Journal of Agricultural Research 17, 105-113.

———— (1974b). Effect of water on bacterial multiplication in plant tissue. Ibid. 17, 115-120.

WALLIS, F M, RIJKENBERG, F H J, JOUBERT, J J & MARTIN, M M (1973). Ultrastructural histopathology of cabbage leaves infected with Xanthomonas campestris. Physiological Plant Pathology 3, 371-378.

WEBSTER, D M & SEQUEIRA, L (1977). Expression of resistance in bean pods to an incompatible isolate of Pseudomonas syringae. Canadian Journal of Botany 55, 2043-2052.

WELLER, D M & SAETTLER, A W (1980). Colonization and distribution of Xanthomonas phaseoli and Xanthomonas phaseoli var. fuscans in field-grown navy beans. Phytopathology 70, 500-506.

WHATLEY, M H, HUNTER, N, CANTRELL, M A, HENDRICK, C, KEEGSTRA, K & SEQUEIRA, L (1980). Lipopolysaccharide composition of the wilt-pathogen, Pseudomonas solanacearum-correlation with the hypersensitive response in tobacco. Plant Physiology 65, 557-559.

WHITNEY, N J & MORTIMORE, C G (1961). Effect of 6-methoxybenzoxazoline on the growth of Xanthomonas stewartii (Erw. Smith) Dowson and its presence in sweet corn (Zea mays var. saccharata Bailey). Nature 189, 596-597.

WYMAN, J G & VAN ETTEN, H D (1978). Antibacterial activity of selected isoflavonoids. Phytopathology 68, 583-589.

YOUNG, J M (1974a). Development of bacterial populations in vivo in relation to plant pathogenicity. New Zealand Journal of Agricultural Research 17, 105-113.

———— (1974b). Effect of water on bacterial multiplication in plant tissue. Ibid. 17, 115-120.

APPENDIX 1

LIGHT MICROSCOPY

Prior to ultrathin sectioning, bacteria were located by light microscopy using 2µm thick sections stained in toluidine blue. Bacteria were stained blue and in susceptible interactions were often seen close to vascular bundles (Plate A.1a). When tissues were fixed in glutaraldehyde containing ruthenium red, omission of toluidine blue staining did not affect visualization of compatible bacteria as pink staining masses were evident on sections (Plate A.1 b-d). The pink colouration was caused by staining of the fibrillar extracellular polysaccharide (EPS).

PLATE A.1 Light micrographs of leaves fixed in glutaraldehyde containing ruthenium red 24h after inoculation with pathogenic pseudomonads.

(a) Oat cv. Milford injected with *P. coronafaciens* stained in toluidine blue (Mag x 400).

(b) As for (a) but toluidine blue omitted, ruthenium red staining only (Mag x 400).

(c) Tobacco cv. Burley infiltrated with *P. tabaci* stained in toluidine blue (Mag x 160).

Photographs taken using Zeiss large Universal Research Microscope.

(a)

PLATE A.1 Light micrographs of leaves fixed in glutaraldehyde containing ruthenium red 24h after inoculation with pathogenic pseudomonads.

(a) Oat cv. Milford injected with P. coronafaciens stained in toluidine blue (Mag x 400).

(b) As for (a) but toluidine blue omitted, ruthenium red staining only (Mag x 400).

(b)

(c) Tobacco cv. Burley infiltrated with P. tabaci stained in toluidine blue (Mag x 160).

Photographs taken using Zeiss large Universal Research Microscope.

PLATE A

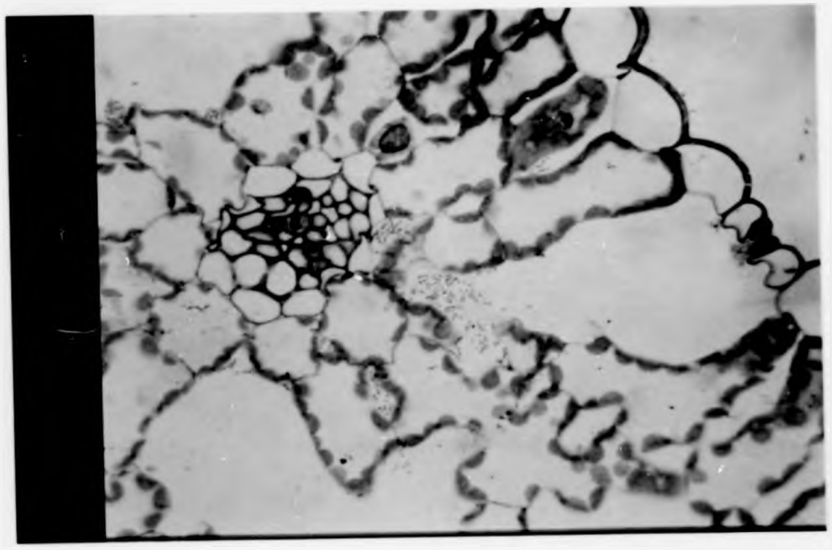
xed in
enium red 24h
nic pseudo-

with
luidine blue

blue omitted,
lag x 400).
trated with
blue

large Universal

(a)



(b)

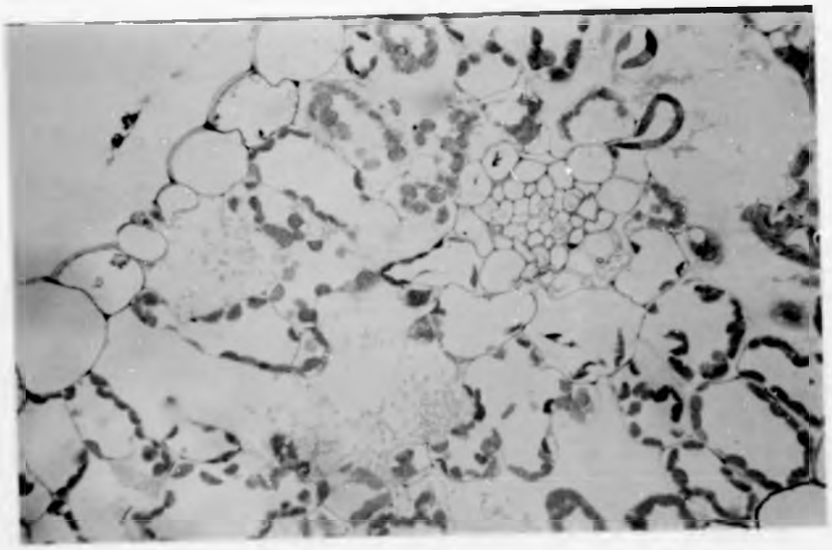


PLATE A.1

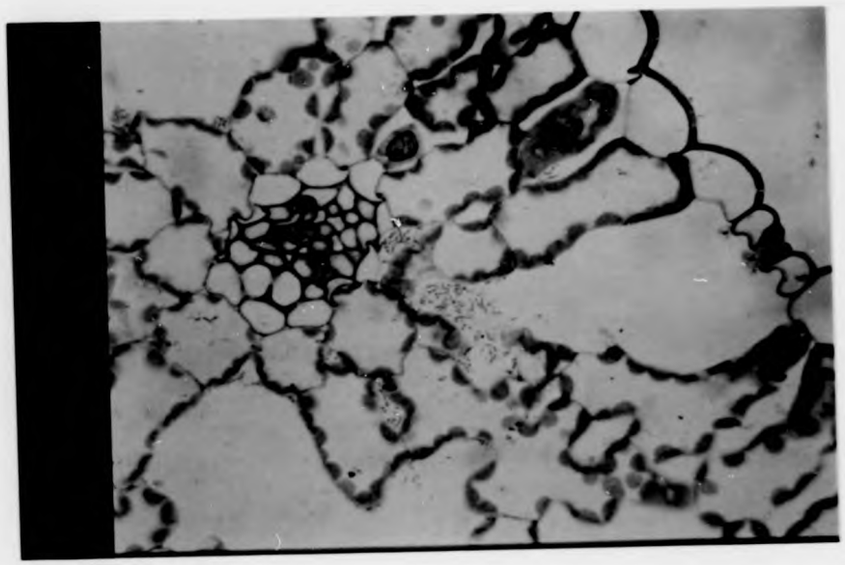
xed in
enium red 24h
nic pseudo-

with
luidine blue

blue omitted,
ag x 400).
rated with
blue

large Universal

(a)



(b)

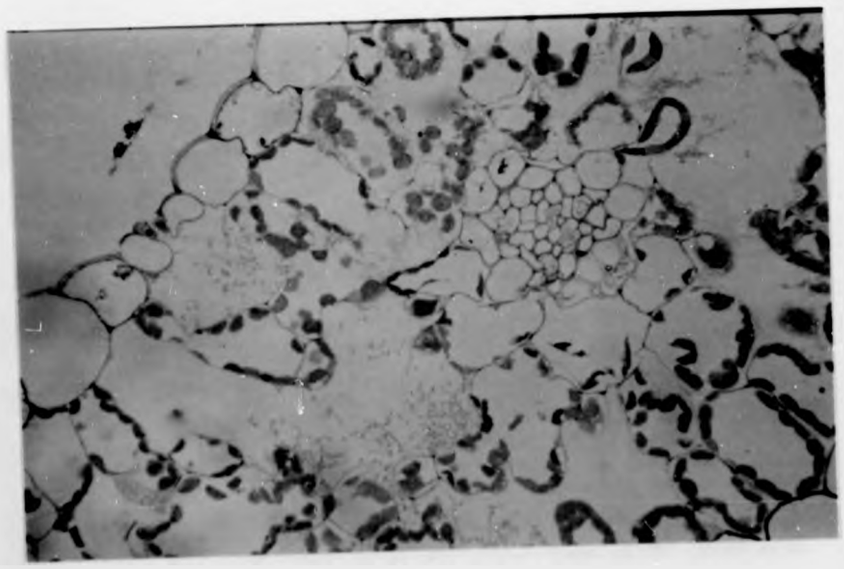
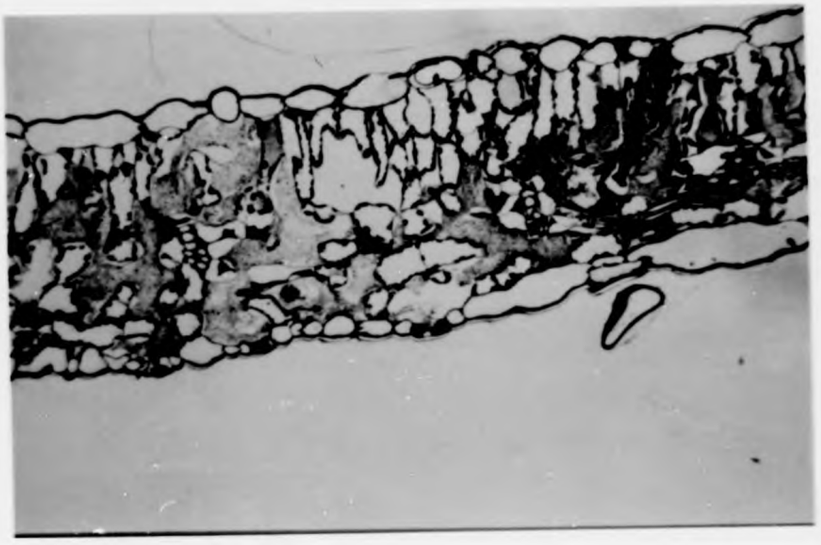


PLATE A.1

(c)



(d)

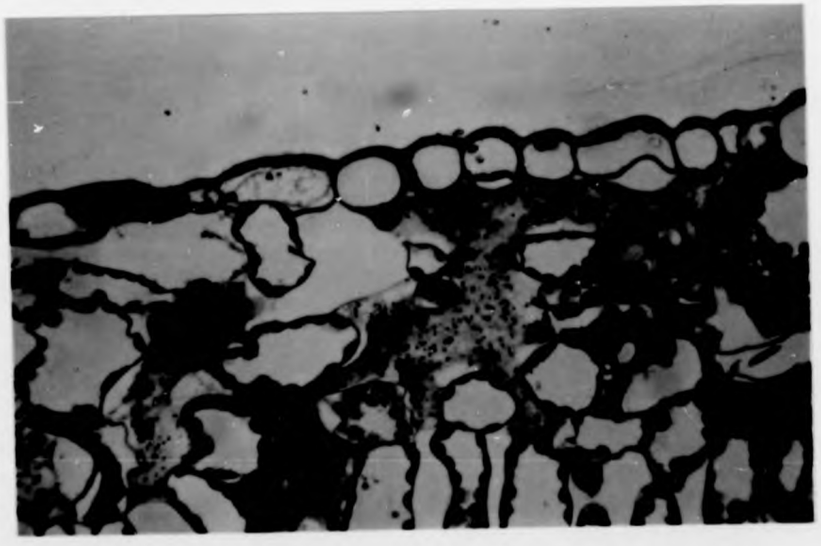
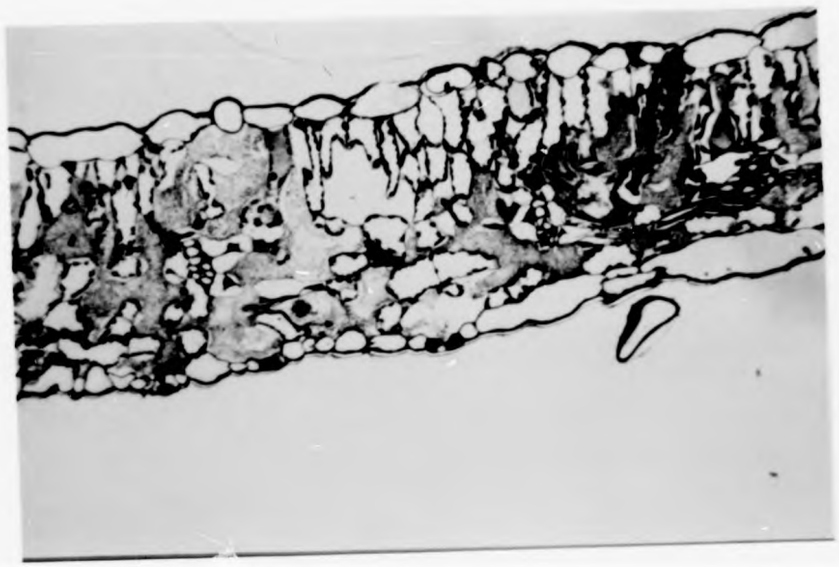


PLATE A.1

(c)



(d)

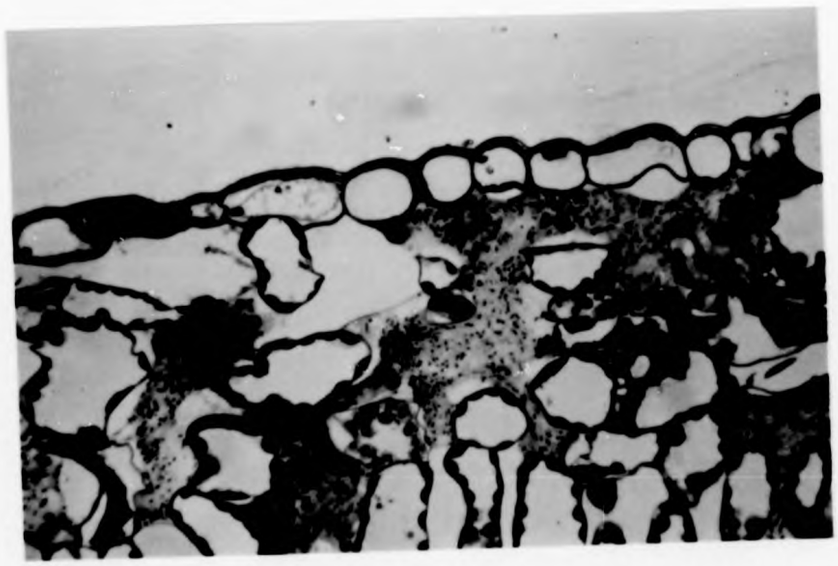


PLATE A.1

II