RESISTANCE AND SUSCEPTIBILITY IN BEAN TO BACTERIAL PATHOGENS

By

Charles Ivor Keith Lallyett B.Sc. (Leeds)

A thesis submitted in part fulfillment of the requirements for the degree of Doctor of Philosophy of the University of London

Department of Botany and Plant Technology,
Imperial College of Science and Technology,
LONDON, S.W.7.

JUNE, 1977

ABSTRACT

Resistance and susceptibility of French bean (Phaseolus vulgaris) leaves to Pseudomonas spp. was investigated, using cultivars Red Mexican and Canadian Wonder. Attempts were made to alter the hypersensitive (resistant) (HR) and susceptible reactions (SR) using inhibitors, enzymes, systemic chemicals, sudden changes of temperature, and other bacteria. Prevention of HR and SR was obtained when certain inhibitors and certain bacteria were used. Results with inhibitors suggested that induction of HR was a function of de novo synthesis of bacterial protein.

Cell walls of Pseudomonas fluorescens, P. lachrymans, P. mors-prunorum and P. phaseolicola races 1 and 2 were examined by polyacrylamide gel electrophoresis and amino acid analysis for common (recognition) factors which would induce HR or SR. Some specificity was shown at the race and species levels.

Transmission electron microscopy of trifoliate leaves 14 days after inoculation of monofoliate leaves showed an accumulation of darkly staining material in the vacuole. Bacterial numbers in the inoculation site and in trifoliate leaves increased by 1000/leaf disk until c. 8 d and then declined. Chlorophyll levels in trifoliate leaves declined over 14 days at rates twice as high for virulent bacteria as for avirulent bacteria or uninoculated controls.

Scanning electron microscopy of stem and petiole segments from monofoliate and trifoliate leaves following

inoculation of monofoliate leaves with P. phaseolicola showed the bacteria mostly in the xylem.

Microautoradiography of tritium labelled bacteria produced photographs of bacteria with labelled nucleic acids.

CONTENTS

INTRODUCTION

This investigation studied the hypersensitive (resistant) and susceptible reactions between French bean cv. Canadian Wonder, Red Mexican and the bacteria *P. phaseolicola* races 1 and 2, and *P. mors-prunorum*.

In the hypersensitive reaction plant response is rapid *c.* 24 h after inoculation of monofoliate leaves with bacteria, secondary symptoms do not occur and bacteria are contained within the inoculation zone. In the susceptible reaction symptoms in monofoliate leaves develop 72 h after injection of bacteria, secondary symptoms develop and bacteria spread upwards to the developing trifoliate leaves.

The object of the investigation was to obtain further knowledge of the differing mechanism of plant response to virulent and avirulent bacteria, and if possible to alter this response advantageously. This was done using different bacteria with the same cultivar or the same bacterium with different cultivars. Red Mexican when challenged with *P. phaseolicola race 1* reacts hypersensitively, but Canadian Wonder reacts susceptibly. Resistance in Red Mexican is monogenic (Coyne *et al*, 1966). Thus a single gene difference at the reaction interface can determine HR or SR.

The HR in plants can be suppressed by high temperature (Klement and Nemeth, 1966); preinoculation with low concentrations of live bacteria cells (Turner and Novacky, 1974); heat killed cells (Lozano and Sequeira, 1970); calcium and other divalent cations (Cook and Stall, 1971);

bacterial sonicates (Sleesman et al. 1970); cytokinins (Novacky, 1972); plant extracts (Süle et al. 1973).

The SR can be suppressed by infiltration of heat killed cells prior to inoculation of live cells (Lovrekovich and Farkas, 1965).

In much of this investigation the same treatment was used simultaneously and under similar conditions for HR and SR. Responses were recorded on different levels - molecular, ultrastructural, morphological.

The advantages of a bacterial system are three fold - rapid growth, ease of numerical assessment in inocula and ease of uniform inoculation into a prescribed area of leaf tissue.

LITERATURE REVIEW

(1) Infection of plants by bacteria

A) Method of entry into plants

Bacteria infect plants after penetrating through natural openings such as the stomata, or hydathodes, or through wounds at the surface. A prerequisite for infection is very high relative humidity (R.H.). Shaw (1935) showed that 97% R.H. suppressed the growth of Erwinia amylovora. Between this value and 99% R.H. growth of the bacterium increased rapidly.

Stomata are the most important sites of entry by bacteria. The sub stomatal cavity has a R.H. approaching 100% with the pores on some plants up to 5 μ wide and 30 μ long. Most phytopathogenic bacteria are c. 1.0 μ wide and 1.5 μ long. The importance of stomatal entry was shown by Rolfs (1915) who inoculated 183 and 149 peach leaves on the lower and upper surfaces respectively with suspensions of Xanthomonas pruni. Peach leaves have no stomata on upper surfaces. Of the 183 leaves inoculated on the lower surface, 177 became infected but none became infected from inoculation of upper leaves.

Lewis and Goodman (1965) reported that a suspension of Erwinia amylovora (10^6 cells/ml) placed on lenticels of apple trees caused infection in young shoots above the inoculation site.

Hildebrand and MacDaniels (1935) first showed that nectaries and hydathodes are important sites of penetration in apple trees by E. amylovora.

Most bacteria also penetrate through wounds. Some, such as soft rot bacteria e.g. Erwinia caratovora, only penetrate through wounds. The crown gall bacterium Agrobacterium tumefaciens additionally requires for infection a substance produced by wounded cells.

Some bacteria penetrate through wounds caused by nematodes. Stewart and Schlinder (1956) reported that plants in soils infested with root nematodes and Pseudomonas caryophyllii become three to four times more seriously infected as plants in soil infected only with bacteria. Kalinenko (1936) reported that nematodes were vectors of Erwinia caratovora and Xanthomonas phaseoli infecting Taraxacum.

Insects are also vectors of bacteria. Waite (1896) showed that bees and wasps disseminated E. amylovora among blossoms. Gaümann (1950) reported that Pseudomonas savastanoi has Dacus oleae, the olive fly, as its obligate vector.

B) The inoculum threshold

The inoculum threshold is the number of bacteria required to start an infection. Many bacterial diseases only become predictable when inocula contain many more than one bacterium. Meynell and Stocker (1957) postulated the hypotheses of independent and co-operative action. In the former infection is caused by a single bacterium and in the latter by joint action of several bacteria which combine to overcome host defences.

Hildebrand (1937) studied this problem by infecting

pear and apple blossom with single cells of E. amylovora. Differences between the arrangement of pear and apple blossom obscured the results but it was concluded that a single cell could initiate an infection. A similar conclusion was reached by Hildebrand (1942) and Lipincott and Haberlein (1965) for infection by Agrobacterium tumefaciens. Corynebacterium michagenense and Erwinia caratovora have also been shown to act independently in infection of tomato plants and potato tuber discs respectively (Ercolani, 1967; Perombelon, 1971). Thyr (1968) reported that a single cell of C. michagenense can infect tomato.

Thus in compatible (virulent) combinations independent action of bacteria during infection seems usual.

In incompatible (avirulent) combinations evidence exists for co-operative action of bacteria during infection. Thus, Ercolani (1973) using six pathogenic pseudomonads in virulent and avirulent combinations with various hosts, reported that single cells of any of the six pseudomonads could not infect heterologous hosts (avirulent combination). This view, that symptoms develop in the avirulent combination only after use of large inocula is supported by Logan (1960); Klement and Goodman (1967); Hildebrand and Riddle (1971). The hypersensitive reaction (HR) is a common type of avirulent reaction. It is characterised by rapid host response c. 24 h after bacterial inoculation, compartmentation of bacteria within the lesion and absence of secondary symptoms. For review, Klement and Goodman (1967).

In HR, Ercolani postulates that co-operative action

of bacteria reflects the joint cumulative effect of bacterial components on the plant. Such components may occur in the surface layers of pathogenic bacteria (Sequeira and Ainslie, 1969; Ercolani, 1970; Sleesman, Perley and Hoitink, 1970; and Gardner and Kado, 1972).

In tobacco leaves c. 5.0×10^6/ml of P. syringae and in bean leaves c. 1.0×10^8 cells/ml of P. phaseolicola are needed to induce a confluent HR lesion. With inocula containing fewer bacteria than needed to evoke a confluent lesion, a HR still occurs but this time at a microscopic level (Klement, Farkas and Lovrekovich, 1964; Klement and Lovrekovich, 1962; Stall and Cook, 1966). Stall and Cook (1973) reported that in the tobacco/Pseudomonas cichorii system c. 100 bacterial cells per plant cell are required for a macroscopic lesion. Turner and Novacky (1974) working with tobacco / Pseudomonas pisi reported that a ratio 1 : 1 host cell to bacterium could cause HR at the microscopic level. This conclusion was however based on the specificity of a staining reaction.

In summary, the compatible (virulent) reaction appears to require no more than one bacterium to cause the host response (disease), whereas in the incompatible reaction it appears that more than one cell is needed.

C) Growth and interactions of plants and bacteria

Klement (1971) distinguished three models of interaction between plants and bacteria - compatibility, incompatibility and plants infected by saprophytes. Compatibility results when virulent bacteria are challenged with a susceptible host. Incompatibility may result from potentially virulent

bacteria in a resistant host, or pathogenic bacteria in a non-host plant. During compatible and incompatible reactions, bacterial multiplication takes place and visible necroses usually appear, though not always in the incompatible response. With saprophytes, the bacterium does not multiply in the plant but often survives for long periods.

Bacterial multiplication in vivo has been used to characterize susceptibility or resistance of a plant to infection. Once the bacterium has entered the plant multiplication occurs in the intercellular spaces. The pathogen now either establishes itself (usually by degrading the intercellular cell wall materials and spreading throughout the plant), or is suppressed (usually in localised areas) by the hosts defences.

a) Bacterial growth

Allington and Chamberlain (1949) were among the first to use bacterial multiplication in intercellular spaces of leaves as a characteristic of infection. Using bean (Phaseolus vulgaris) and soybean (Glycine max) and Pseudomonas glycinea and Xanthomonas phaseoli, they concluded that bacterial multiplication in the leaf was similar in resistant and susceptible varieties until the second day when bacterial numbers in the susceptible variety far exceeded that of resistant. This response was concomitant with macroscopic collapse of leaf tissue at about the third day. Diachum and Troutman (1954) working with four varieties of tobacco, (of different susceptibility), and Pseudomonas tabaci obtained similar results.

Klement and Lovrekovich (1961) studying the effects of

various pathogenic pseudomonad and xanthomonad bacteria on fruit of Capsicum annuum, found that reactions caused by pseudomonads were faster and more intense than those caused by xanthomonads, and that multiplication of bacteria in tissues had stopped by the time of visible symptom development. A non-pathogen P. aeroguinosa did not multiply.

Klement (1964) investigated infection by infiltrating leaves of tobacco with a virulent pathogen, avirulent pathogens and saprophytes. Symptoms appeared in three to five days with the virulent pathogen, in nine hours with the avirulent pathogens (hypersensitive reaction) and saprophytes did not cause any symptoms. Bacterial numbers of the pathogen (P. tabaci) continued to rise until symptoms developed (c. 3-5 d), whereas the avirulent pathogens stopped growth within 24 hours. Cessation of growth was associated with collapse of the infiltrated tissue. Saprophytes did not multiply.

Stall and Cook (1966), working with Xanthomonas vesicatoria and pepper reported that numbers of bacteria in resistant and susceptible tissues increased similarly until the third day when numbers in resistant leaves decreased whereas those in susceptible leaves increased for three days more. Cessation of growth again was associated with collapse of leaf tissue.

Omer and Wood (1969) using races 1 and 2 of P. phaseolicola and cv. Red Mexican found a similar pattern of growth as did Ercolani and Crosse (1966).

In summary, it appears that virulent and avirulent bacteria increase in numbers after inoculation until

collapse of host tissue. In the susceptible reaction this usually occurs two to three days after the resistant (hypersensitive) reaction and so the numbers of recoverable bacteria are much higher. Non bacterial pathogens do not multiply after inoculation.

b) Interactions of plants and bacteria

The microflora constitutes an important factor in the bacterial pathogenicity syndrome as interactions occur not only between the pathogen and the microflora but also between the various organisms of the microflora and the microflora and the plant.

In some cases it has been reported that plants infected by an avirulent micro-organism become protected against subsequent infection by virulent pathogens (Wood and Tveit, 1955; Farabee and Lockwood, 1958; Kassanis 1963). Averre and Kelman (1964) showed that avirulent strains of bacteria were able to protect against infection when in mixed inoculations with P. solanacearum.

Farabee and Lockwood (1958) isolated a yellow bacterium from fireblight cankers, of E. amylovora. The acid produced by the saprophyte was sufficient to inhibit growth of E. amylovora in vitro and when the bacterium was inoculated into apple trees at 5×10^8 cells/ml prior to E. amylovora inoculation it managed to protect a significant number of shoots against infection. This work supports that of Baldwin and Goodman (1963).

This ability of a saprophyte to protect against subsequent infection by a pathogen has also been observed by Leben (1963) for Xanthomonas vesicatoria and Crosse (1965)

for Pseudomonas mors-prunorum. Ercolani (1970) reported that tomato plants preinoculated with P. fluorescens or an auxotrophic mutant of P. syringae were protected against subsequent infection by C. michiganense. In bean and tomato leaves pre-inoculation with incompatible X. phaseoli, X. vesicatoria and X. campestris and P. fluorescens, decreased multiplication of compatible pathogens (Hsu and Dickey, 1972).

Protection can be effected by heat killed cells or with bacterial extracts. Lovrekovich and Farkas (1965) showed that pretreatment of tobacco leaves by heat killed cells of P. tabaci protected them against subsequent infection by P. tabaci. Apple and pear can be protected from E. amylovora by cell free sonicates of E. amylovora (McIntyre, Kuc and Williams 1973). Synergistic effects have been reported particularly when the virulent pathogen is included in the combination e.g. a combination of pseudomonads on bean including P. phaseolicola and a combination on carnation including P. caroyphylli. Braithwaite and Dickey (1970) suggested that the combination led to an increased release of nutrients from the host which favoured development of the incompatible bacteria. Enhancement of disease by non bacterial pathogens has also been reported. Maino Schroth and Vitanza (1974) reported that P. syringae and a species of Achromobacter enhanced infection by P. phaseolicola of bean, probably by contributing to a breakdown of cell walls. From the above reports it can be seen that live cells, heat killed cells and bacterial extracts may protect against disease. The role of components (implicated in HR induction) in the surface layers of bacteria has been reported, (Sequeira and Ainslie, 1969; Ercolani,

1970; Sleesman et al. 1970 and Gardner and Kado, 1972). As a mechanism for protection Sequeria et al. (1972) suggested that the bacteria released both the proteinaceous HR inducer and HR protector when inoculated into host tissue but that in most cases the effect of the second is masked. It may well be that the HR inducer produced by pathogens when inoculated into incompatible host tissue in combination with other live, heat killed or extracts of bacteria does itself become masked by surplus production of protector purely as a result of the combination.

D) Factors of pathogenicity and susceptibility

Ercolani (1973) defined pathogenicity factors as "factors which control general metabolic activities common to all pathogenic bacteria in plant tissue. Virulence factors govern the specific activities resulting in the induction of a progressive disease in the host plants." This definition suggests that the susceptible reaction is more specific than the resistant reaction.

Goodman et al. (1974) working with E. amylovora have shown that a host specific toxin is produced which causes wilting only in rosaceous plants. This has been suggested as a virulence factor. The toxin produced by P. phaseolicola is non-host specific since a susceptible reaction can be induced in resistant bean leaves by application of the toxin (Rudolph, 1972). However, the optimum temperature for toxin production and for disease are similar. Toxins involved in other bacterial diseases have been reported - Corynebacterium sp. (Strobel, 1974); X. campestris (Strobel, 1974); P. solanacearum (Keen and Kennedy, 1974).

Syringomycin the toxin of P. syringae appears to be host specific in that peach isolates of the pathogen do not produce the toxin in bean and vice versa (Backman and DeVay, 1974). However, a negative correlation between toxin production and pathogenicity suggested that the antibiotic properties of the toxin ecologically favoured weakly pathogenic stains of P. syringae.

The presence of a common antigen as a prerequisite of a compatible relationship has been suggested (DeVay, 1972). The inference being that less disruption occurs in the host system if common metabolites are present. Several common antigens have been demonstrated in plant-bacterial systems (DeVay, 1972). Capsular antigens of P. lachrymans and P. phaseolicola have been suggested as pathogenicity factors (Grogan et al., 1965). The specificity of rhizobia for their hosts has been attributed to a 'recognition fit' between bacterial wall polysaccharides and host lectins (Dixon, 1969). This supports a previous suggestion that during a compatible reaction, a virulence factor allows for attachment of bacteria on multiplication sites in the host (Ercolani, 1970). This has been shown for A. tumefaciens (Lippincott and Lippincott, 1972). In the incompatible reaction agglutination of bacteria on host cell walls has been reported (Sequeira et al, 1976) (Goodman, 1974) (Patil - personal communication). Sequeira and Donald (1976) have shown that a major difference between the compatible and incompatible strains of P. solanacearum is the ability of the latter to form polysaccharide slime. The polysaccharide is vital because of its ability to bind to host cell lectins, and hence block binding sites in the host. Thus the incompatible pathogen attaches to other sites on host cell walls and from there a product which

affects host cell membrane integrity is released.

The complexity of the interactions that determine the type of response by the plant is demonstrated by the following. Kennedy et al. (1971) reported that detached leaves or tissue cultures of soybean could be colonized by bacteria which previously could not infect whole plants. Young (1974) showed that plants at 100% relative humidity lose their susceptibility. Stall et al. (1973) reported that in pepper, inoculation with virulent X. vesicatoria decreased resistance to subsequent inoculation with avirulent bacteria. McIntyre et al. (1975) showed that uptake of DNA of E. amylovora by pear shoots protected against subsequent inoculation by virulent Erwinia amylovora. Additionally the breaking of resistance by a bacterial toxin (Rudolph, 1972) indicates that reactions of plants can be altered by bacteria. Ercolani's definition of pathogenicity suggests that susceptibility is more specific than the resistant response. Thus it would seem that any susceptible reaction produced by pathogenic bacteria would have to overcome the factors encoded in the plant for resistance.

(2) The susceptible reaction - 'halo blight'

A) General description

The causal organism of this disease was first isolated from Phaseolus vulgaris L. by Burkholder (1926) and named Phytomonas phaseolicola (Burk). The bacterium was later renamed Pseudomonas phaseolicola (Burk) Dows. by Dowson (1943).

The disease is characterised by a general dwarfing of

the plants, the appearance on foliage of translucent water soaked spots c. 2 mm diameter which may or may not have a chlorotic halo, and by the systemic interveinal chlorosis of developing trifoliate leaves. Halo blight is disseminated by infected seed which produce cotyledons bearing necrotic brown spots containing the bacteria. The bacteria then invade the stelar tissue and systemically colonize most tissues in the plant except the roots. The pathogen is spread in the field by wind and rain water 'splash' and 'spatter' droplets on to neighbouring plants.

B) Systemic nature of the bacterium

Zaumeyer (1932) using infected seed reported that bacteria rarely penetrate the host cells and were to be found in intercellular spaces and xylem elements of the plant. In severe infection, however, maceration of host cell walls becomes apparent and large cavities may develop within the plant. The bacteria moved mainly upwards as few were found in tissues of the hypocotyl below the cotyledonary nodes.

Waitz and Schwatz (1956) found a poor correlation between chlorotic symptoms and bacteria in the leaves situated above the infected lower leaves. The presence of the bacteria in chlorotic leaves was variable, and some bacteria were also found in healthy leaves. They concluded that the bacteria do not spread in large masses, but rather a few migrated in the transpiration stream and then multiplied locally. This systemic spread was usually upwards from the inoculation site.

Omer (1966) also found few bacteria below the inocula-

tion site and concluded that transport of the bacterium was in the transpiration stream. He also suggested that more migrate in susceptible than in resistant plants.

Hildebrand and Schroth (1971) suggested that failure to isolate bacteria from chlorotic leaves depended on the method used. By using a different technique of infiltration followed by centrifugation as opposed to infiltration and isolation in a pestle and mortar, they achieved recovery from 39 out of 40 leaves. Using the mortar technique recovery from only 16 out of 40 leaves was achieved.

Hale et al. (1972) investigating growth responses of bean to infection by P. phaseolicola reported that in 10 d old plants inoculation of both monofoliates suppressed trifoliate development. Inoculation of one monofoliate suppressed the expansion of that leaf, though its opposite expanded to a size significantly greater than in any of the controls. Inoculation of trifoliate leaves reduced growth of the plant by as much as 30%.

If bacterial infection in very young seedlings is severe then the apical meristem of the plant is killed. This and the general dwarfing of infected plants is attributed to an exotoxin, phaseotoxin, produced by Pseudomonas phaseolicola. The primary effects of this toxin appear to be on apical division and leaf cell expansion.

C) Phaseotoxin - its production, composition, mode of action and role in halo blight disease

a) Production

The halo around leaf spots of inoculated areas of leaves and the systemic nature of the interveinal chlorosis

of trifoliate leaves is attributed to phaseotoxin production by P. phaseolicola. Patel and Walker (1963) found that development of the halo and trifoliate chlorosis were temperature dependent. At 16-20°C large haloes and pronounced chlorosis were evident, at 24°C the effects were considerably less and at 28°C were rarely present. Skoog (1952) found that this temperature range correlated well with toxin production in vitro. After screening thirteen isolates of P. phaseolicola, Jensen and Livingstone (1944) reported that not all produced halo or chlorotic symptoms; those that did cause halo failed to do so at 28°C.

b) Composition and action

Hoitink et al. (1966) stated that culture filtrates caused haloes but not chlorosis. The toxin, from culture filtrates, was heat stable and dialysable. Rudolph (1969) concluded that the phytotoxin from culture filtrates was predominantly composed of hexose units and had a molecular weight of 2100. The toxin induced chlorosis and caused accumulation of ornithine in leaves as originally reported by Patel and Walker (1963) and Rudolph and Stahmann (1964). Patil (1970) reported that the phytotoxin inhibits ornithine carbamyl transferase. It was later reported that the toxin from P. phaseolicola and P. glycinea Coerp. was the same whether produced in culture or in infected plants (Hoitink and Sinden, 1970).

Patil (1970) also purified a phytotoxin from culture filtrates of P. phaseolicola and found it to be ninhydrin-positive with a molecular weight much smaller than that reported by Rudolph. In later work Patil (1972) has shown

the toxin to be composed of three known and three unknown amino acid and residues.

Chlorosis induced by the toxin is prevented by application of citrulline, the product inhibited in toxin treated tissues, Patil (1970).

c) Role

It has recently been suggested that phaseotoxin suppresses the hypersensitive reaction and phytoalexin accumulation in bean thus allowing continued growth of the pathogen in susceptible tissue (Patil and Gnanamanickam, 1976).

D) Ultrastructural changes in the susceptible reaction

Bajaj et al. (1969) reported that the granae of chloroplasts were disrupted in trifoliate leaves which had been naturally infected or toxin treated. Apart from the accumulation of large starch grains in the chloroplast other cellular organelles appeared normal. Leseman and Rudolph (1969) reported that the toxin induced a helix-like aggregation of ribosomes in plant cells though other organelles appeared normal. Siegge and Epton (1976) reported that in monofoliate bean leaves infection by P. phaseolicola caused breakdown of the chloroplast and an increase in cytoplasmic ribosomes.

It has been reported that in tobacco leaves treated with the toxin of Pseudomonas tabaci chloroplasts swelled, and cytoplasmic and chloroplastic ribosomal content decreased. There was no accumulation of starch grains (Goodman, 1971).

E) The host range of Pseudomonas phaseolicola in Phaseolus and related genera

In 1932 Burkholder and Zaleski reported that no differences in the susceptibility of a number of varieties could be found to two American and one European strain of P. phaseolicola. Later it was reported that the varieties Red Mexican and Schwart No. 27 were resistant (Jensen and Goss, 1942). The resistance of Red Mexican was confirmed by Zaumeyer and Thomas (1957) who also found that Michelite, Great Northern, Pinto and Pinks were also highly resistant.

In 1955 Ferguson et al. working in Canada reported that Red Mexican in that area was susceptible to P. phaseolicola. Patel and Walker (1964), then identified Races 1 and 2 of the pathogen, and Race 2 was found to be virulent to Red Mexican and a number of other varieties previously reported resistant. Patel and Walker (1965) screened large numbers of Phaseolus spp. for their reaction to P. phaseolicola Races 1 and 2. Reactions fell into the following groups: (1) Species susceptible to both races - P. radiatus, P. lathyroides, P. lunatus, P. coccineus, P. bracteatus, P. polystachus, P. polyanthis, P. acutifolius. (2) Species hypersensitive to Race 1 - P. calcarateus, P. mungo, P. acontifolius, P. aureus and P. augularis. No Phaseolus species was hypersensitive to Race 2. (3) Other genera tested for resistance to Races 1 and 2 - Vignia sinensis, V. sesquipedalis, Vicia faba, Pisum sativum and Trifolium pratense. (4) Other genera susceptible to both races - Pueraria hursuta and Glycine max.

Of species tested from different countries, 22% from Asia, 8% from America, 4% from Africa and 3% from Europe showed resistance or tolerance to P. phaseolicola. In 1965 Schuster et al. reported isolates of P. phaseolicola more virulent than Races 1 and 2 in that they had a wider host range and infected cultivars of P. vulgaris tolerant to Races 1 and 2. Coyne et al. (1966) confirmed that resistance in Red Mexican to P. phaseolicola was dominant and monogenic.

(3) The resistant reaction of plants to pathogenic bacteria

A) The hypersensitive response (General description)

The hypersensitive response (HR) has been a well known phenomenon in plants infected with fungal or viral pathogens for most of this century (Muller, 1959). In plants infected by bacteria the HR was first emphasized by Klement, Farkas and Lovrekovich (1964) who showed only incompatible avirulent bacteria caused HR. Klement (1971) distinguished three phases during the development of HR - induction period, latent period, and collapse of host cells. The induction period is the time necessary for bacterium to initiate HR. After this time bacteria may be killed by injection of antibiotics into the leaf and the reaction will still go to completion. The latent period is that period dependent on the plant but independent of the bacterium when the host is symptomless although the respiration rate has been reported to rise (Nemeth et al. 1969). The tissue collapse phase is characterised by large permeability changes in host cells. Goodman (1968) reported that

the host cell membranes become altered.

The characteristic features of HR following infiltration of incompatible bacteria into intercellular spaces are rapid changes in host cell permeability (evident by electrolyte loss) within 8-10 h accompanied by dramatic ultrastructural changes. In tobacco the plasma membrane, tonoplast and membranes of chloroplasts and mitochondria become deranged (Goodman and Plurad, 1971). Within 18-24 h collapse of host cell tissue is complete and a well demarcated border separates the inoculated zone from the rest of the leaf tissue. The main differences between an incompatible reaction (HR) and the compatible reaction (SR) are the rapidity of the response, the systemic nature of the bacteria and the bacterial multiplication. In the HR symptoms are apparent 6-12 h after introduction of bacteria into the leaf, whereas in the SR symptoms are not normally apparent until 36 h or later. In HR bacterial numbers increase at first then drop in level while in SR multiplication occurs at a linear rate. In the HR the bacteria do not move to other parts of plants and remain confined within the necrotic lesion while in SR considerable spread of the bacteria to other parts of the plant occurs.

The concentration of incompatible bacteria in the inoculum does not influence the formation of HR but merely affects the development of visible necroses, as below the inoculum threshold microscopical lesions are produced (Klement, 1964). In tobacco leaves injected with Pseudomonas syringae an inoculum of 5.0×10^6 cells/ml as required to induce a confluent HR (Klement, 1967), but in bean leaves injected with Pseudomonas mors-prunorum an inoculum

of 6.8×10^7 cells/ml is required.

From the work of Klement and Goodman (1967) it has been further shown that HR in tobacco leaves could not be reproduced by plant pathogenic bacteria that had been killed, sonicated, treated with antibiotics or by culture filtrates. In beans 'Mickle' treated bacterial cells, bacterial cell walls, culture filtrates or bacterial cytoplasm of Pseudomonas mors-prunorum also failed to produce the HR in the leaves (O'Brien, 1973). More specifically, when pathogen DNA was injected into bean leaves no HR resulted. Thus it appears that only living incompatible bacteria are able to produce HR. The role of living bacteria in HR centres around the induction period, which is independent of the host but dependent on the metabolic activity of the bacterium.

The induction period is measured by infiltrating antibiotics into the intercellular spaces of leaves at time intervals after bacterial injection. In tobacco leaves injected with Pseudomonas syringae the induction period is 25 min. (Klement and Goodman, 1967), but in bean leaves injected with Pseudomonas mors-prunorum it is 150 min. In tobacco leaves inoculated with avirulent P. solanacearum at c. 10^8 cells/ml the induction period was determined using rifampicin and streptomycin to be c. 3 h (Sequeira, 1976). The fact that the induction period is so short diminishes the possibility of bacterial multiplication being an important factor in HR. Also as only living cells induce HR a specific metabolite has to be attributed to the bacterium.

B) Specificity in hypersensitive reaction

Several attempts have been made to implicate different bacterial substances in HR induction. Lovrekovich et al. (1969, 1970) implicated ammonia evolved by bacteria in leaf tissues. This was later disproved by Goodman (1971, 1972) and Stall et al. (1972) when it was shown that ammonia reached toxic concentrations 16 h after inoculation whereas cell membrane damage was detected at 6 h. Additionally it was suggested that most of the ammonia was derived from leaf cell metabolism and not the bacteria. Sequeira and Ainslie (1969) reported that a HR inducing fraction from bacteria could induce HR in tobacco when the cell free preparation was purified using chromatographic procedures. Gardner and Kado (1972) reported that a proteinaceous high molecular weight substance derived from the osmotic shock fluid of Erwinia rubrifaciens induced HR in tobacco leaves which was accompanied by electrolyte leakage.

"Shock protein" preparations from the non pathogen E. herbicola failed to induce HR. This osmotic shock procedure has previously been used to release proteins from surface layers of enterobacteria.

Sleesman et al. (1970) reported that a proteinaceous heat labile fraction from sonicated cells of P. glycinea could induce HR. Sequeira et al. (1972) further managed to obtain a proteinaceous fraction from cells of P. solanacearum which would protect against the HR: this protection factor did not involve the bacterial cell wall or the extracellular polysaccarides or nucleic acids of P. solanacearum and was non specific since it reduced both number and size of TMV lesions on tobacco leaves. Lobenstein and

Lovrekovich (1966) reported a similar protective effect with heat killed cells of P. syringae, but additionally showed that the protective effect was inhibited by actinomycin-D thus implicating RNA synthesis.

Sequeira et al. concluded that incompatible bacteria in tobacco leaves release both the HR inducing factor and the factor that prevents the HR, but that the response to the first was so rapid that the effect of the second is masked. Both factors appear to be proteinaceous (Gardner and Kado, 1972; Sequeira and Ainslie, 1969; Sequeira et al. 1970).

However, in the cases mentioned the fractions obtained tended to produce non specific effects and all were of unknown nature.

These facts suggest three points:-

(a) the extraction procedures from bacteria in vitro are not stringent enough to allow for the lability of the pathogenic metabolite,

(b) and/or the pathogenic inducer may need a complementary metabolite supplied by the plant before it becomes active. This may take the form of a receptor site in the plant leaf or a specific plant metabolite,

(c) or the pathogen may not produce the HR factor in vitro.

When bacterial free intercellular fluid was removed at 10 min intervals from tobacco leaves inoculated with either Pseudomonas syringae or Erwinia amylovora it failed to induce HR (Klement, 1965). Similarly when bacterial free

intercellular fluid was sequentially removed from bean leaves inoculated with Pseudomonas mors-prunorum no HR resulted (O'Brien, 1973).

Additionally, all attempts to extract the HR inducer in vivo have failed with one exception. Howes et al. (1973) working with wheat cv. Chinese Spring and Puccinia graminis tritici race 56 (containing P6 gene), showed that an RNAase sensitive extract from the leaves of wheat containing the Sr6 gene (R line) could elicit the HR. This response was measured by the significant increase in necrotic lesions when the RNAase fraction was reinjected back into plants (R line) infected with a virulent race (p6). Further the RNAase sensitive extract was shown to be produced by the fungus but only in an avirulent situation. Specificity of the extract was shown as extracts from Sr6/P6 combination showed no activity in an Sr5 bioassay. These RNA active extracts could only be formed by utilising the temperature sensitivity (threshold) of the Sr6 gene. To enable sufficient avirulent fungus to grow so that a fraction containing high concentration of RNA could be extracted the temperature was raised to 26^{o}C for 72 h from inoculation. This enabled the leaves to be extensively colonised and then the temperature was dropped to 20^{o}C for 30 h prior to extraction.

The significance of this work is paramount, as it is consistent with the gene for gene hypothesis (Flor, 1956) and has shown that a gene specific RNA is responsible for initiating the resistant reaction in wheat against stem rust. This work also clearly shows that the interaction between genes involves the transfer of substances across

the host/parasite interface.

The specificity of the host parasite interaction in HR can be demonstrated using other examples. In the incompatible system Pseudomonas phaseolicola and tobacco the induction period is 3 - 4 h while the macroscopic appearance of HR in the leaves occurs after 9 h (Klement, 1971). In bean leaves cv. Red Mexican and P. phaseolicola the induction period is $2\frac{1}{2}$ h and the appearance of HR is 26 h. Both systems were incubated at 25^{o}C. In the tobacco/P. syringae system the induction period is 25 min (Klement, 1967) while in bean (cv. Red Mexican)/P. mors-prunorum system the appearance of HR is 18 h at 25^{o}C.

It can thus be seen that both the pathogen and the host are intimately involved in pathogenicity. The type of reaction (HR/SR) and the speed of the reaction is not directly a function of the external environment in which the plant is placed but is a direct function of the factors contained by the host and pathogen at the reaction interface. This can be further substantiated by the presence of resistant gene thresholds. In wheat (R line) and in tobacco the temperature thresholds are 26^{o}C and 36^{o}C respectively. Above these temperatures resistant reactions become susceptible thus strongly implicating a change in metabolic production in the form of recognition factor(s). The undoubted presence and importance of these recognition factors can be further illustrated. When Pseudomonas phaseolicola race 1 is injected into bean cv. Red Mexican a resistant (HR) reaction results. When the same bacterium at the same inoculum concentration, incubation temperature and light regimes is injected into bean cv. Canadian Wonder

a susceptible reaction results. Resistance in Red Mexican is monogenic (Coyne et al. 1966). Thus in this pathogenicity syndrome a single gene difference in the plant can confer resistance or susceptibility.

Phytopathogenic bacteria, once introduced into the plant, are to be found in the vascular system, the intercellular spaces or resting on the host cell walls. The fluid in the intercellular space contains an abundance of nutrients necessary for bacterial growth (Klement, 1965). The bacteria rarely penetrate the cell and then only in times of extreme infection (Zaumeyer, 1932). Thus in HR induction metabolites have to pass out of the bacterial cell across the intercellular space (unless the bacteria are resting on the host cell wall) and into the host cell or vice-versa. Ercolani (1970) proposed that the phytopathogenic bacteria become attached to multiplication sites on the cell surface and depending on the factors contained by the bacterium a HR or SR is produced. It has more recently been reported that the avirulent bacteria agglutimate on the host cell wall (Sequeira, 1976; Goodman, 1975; Patil, S. S. (personal communication)).

As a mechanism it was proposed by Klement and Goodman (1967) that the HR inducer alters the permeability of cells by denaturing the s-s bonds in the protein lamellae of host cell membranes with a sulphydryl compound.

C) Suppression of hypersensitive reaction

The HR in plants can be suppressed by high temperature. No HR develops in tobacco when the plants are kept at 37^{o}C (Klement and Nemeth, 1966). In wheat possessing the Sr6

gene a similar situation arises at 26°C to the pathogen Puccinia graminis var. tritici. Kassanis (1957) reported a similar effect in virus diseases. It is suggested that a thermo sensitive period exists in host tissue following the induction period (Klement, 1972).

Turner and Novacky (1974) reported that preinoculation with low concentrations of bacteria inhibit HR if the plants are subsequently inoculated with concentrations sufficient to induce HR. This contrasts with the work of Cook (1975). Infiltration of calcium into plant leaves prior to bacterial inoculation also inhibits HR (Cook and Stall, 1971). Other methods for inhibition include the use of precipitates from bacterial sonicates (Sleesman et al. 1970), cytokinins (Novacky, 1972), plant extracts (Süle et al. 1973) and heat killed bacterial cells (Lozano and Sequeira, 1970; Stall et al. 1974). Lovrekovich and Farkas (1965) also managed to inhibit the compatible (SR) in tobacco leaves by infiltrating heat killed cells of P. tabaci, P. syringae and C. flaccumfaciens prior to inoculation with live cells of P. tabaci.

Sequeira et al. (1972) and Wacek (1974) have shown that suppression of HR by heat killed cells of P. solanacearum is associated with glycoproteins found in the periplasmic space of the bacterial cell wall. Extracellular polysaccharide DNA or peptidoglycan failed to give a protective response (Wacek and Sequeira, 1973). Treatment with trypsin and pronase destroyed activity of the protection fraction (Sequeira et al. 1972).

Protective glycoproteins from the periplasmic space have also been obtained using osmotic shock of bacterial

cells (Wacek, 1974). The method is similar to that of Gardner and Kado (1972) who obtained a HR inducing fraction by osmotic shock of cells of E. rubrifaciens. Both the HR inducer and HR protectant appear to be proteinaceous since proteolytic enzymes destroy their activities. Sequeira et al. (1972) postulated that the bacteria release small amounts of both these proteins but during HR induction the protectant is masked by the inducer.

Evidence suggests that the HR protecting fraction (derived from heat killed cells or otherwise) causes moderate injury or irritation to the plant which in turn produces its own fraction to resist necrotization of tissue caused by the incompatible bacteria (Ryan, 1974). Intercellular fluid extracted from plants which have been protected against HR contained bacterial growth inhibitors. The populations of compatible, incompatible and saprophytic bacteria decline rapidly in protected leaf tissue (Sequeira and Hill, 1974). It further appears that protection in tobacco leaves at least is light dependent since bacterial growth was inhibited to a greater extent from fluid of plants kept in the light than those in the dark (Rathmell and Sequeira, 1975). A wound hormone which systemically migrates through the plant is thought to induce production of the bacterial inhibitors (Ryan, 1974). This systemic protection is similar to that found in cucumber leaves infected with Colletotrichum lagenarium (Kuc et al. 1975).

In summary it appears that protection is dependent on (a) the time between the treatment and challenge (b) the relative concentrations of heat killed cells to live cells (c) the temperature (d) light regime.

D) Resistance mechanisms other than hypersensitive reaction

It has in the past been generally regarded that HR is a chief mechanism of resistance of plants to bacteria though evidence from a fungal system Phytophthora infestans on potato tuber suggests that HR is a consequence rather than a cause of plant resistance (Ersek et al. 1973). Kelman and Sequeira (1972) divided bacterial resistance into (a) preformed and (b) induced mechanisms.

The HR in plant tissue can be inhibited by using heat killed cells (Lozano and Sequeira, 1970), low concentrations of HR inducing bacteria (Turner and Novacky, 1974), precipitates from bacterial sonicates (Sleesman et al. 1972), cytokinins (Novacky, 1972), plant extracts (Sule et al. 1973), calcium and other salts (Cook and Stall, 1971). In all the above cases, bacterial multiplication was still prevented and a susceptible reaction did not develop.

a) Role of nutrients

Bacteria when introduced into host tissue mostly multiply within the intercellular space. The availability of these nutrients within the intercellular space must be regarded as a vital factor to any syndrome. In both HR and SR an increase in the host cell permeability occurs, but it is only in the compatible system that the bacteria manage to regulate this nutrient flow into the intercellular space. In bean tissue inoculated with P. phaseolicola, the bacterial toxin decreases the host cell permeability and so acts as a regulator (Zeller and Rudolph, 1972). Rudolph (1972) suggests this is how the toxin protects against HR and elicits SR when P. phaseolicola is injected

into bean leaves. Direct evidence of the qualitative influence of nutrients *in vitro* was shown when *P. phaseolicola* only produced its toxin on one of several artificial media (Rudolph, 1969).

Quantitatively it has been shown that bacterial infection sites accumulate organic and inorganic substances (Shaw and Samborski, 1956).

b) Chemical involvement in bacterial resistance

Walker and Stahmann (1955) reported that many compounds serve in resistance to fungal diseases but not in bacterial diseases. The majority of these inhibitory compounds have been classified as phytoalexins. These are compounds which accumulate in plants following infection and stress and constitute a type of induced resistance (Kelman and Sequeira, 1972). Phaseollin has been shown to be induced in bean tissue following bacterial infection but subsequent tests showed that the phytoalexin did not inhibit growth of the pathogens *in vitro* (Cruickshank and Perrin, 1971; Stholasuta *et al.* 1971). However induction of hydroxyphaseollin in the HR of soybean to *P. glycinea* and induction of coumestrol in HR of bean to *P. phaseolicola* and *P. mors-prunorum* have been shown to inhibit bacterial growth *in vitro* (Keen and Kennedy, 1974; O'Brien and Wood, 1973). Similarly 6-methoxybenzoxoline extracted from sweet corn inhibited *in vitro* growth of *X. stewartii* (Whitney and Mortimore, 1961). The phenolics have been reported to be important in fungal disease resistance but little evidence exists for bacterial disease resistance (Farkas and Kiraly, 1962; Cruickshank and Perrin, 1964; Rohringer and Samborski, 1967). The resistance of

pathogenic pseudomonads to phenolics has been attributed to their ability to reduce quinones to phenols and their inability to produce hydrogen peroxide which affects the oxidation of the reverse reaction. Non pathogens were inhibited (Moustaffa and Whittenby, 1970).

Extracts of pepper leaves following inoculation by X. vesicatoria inhibited bacterial multiplication in vitro (Stall and Cook, 1968). Similarly extracts from tobacco leaves inhibited P. solanacearum (Lozano and Sequeira, 1970) and bacteriostatic compounds were detected in extracts of bean leaves inoculated with water (Rudolph and Cinar, 1971) and from potato (Zalewski and Sequeira, 1973). Some preformed or constituative mechanisms of resistance to bacteria have been reported (Kelman, 1972).

Arbutin, a glycoside of pear was shown to be involved in resistance to bacterial pathogens (Hildebrand and Schroth, 1964; Hildebrand, 1970). Arbutin is hydrolysed by a glycosidase to yield the aglucone, hydroquinone. This is then oxidised to yield semiquinone which is toxic to E. amylovora causing a resistant reaction. Phloridzin in apple trees has a similar function. In the resistant response phloridzin is hydrolysed to glucose and the phenolic phloretin (Goodman, 1967). In susceptible varieties phloridzin and arbutin are not hydrolysed. Antagonism of E. herbicola towards E. amylovora has been related to hydroquinone formation from arbutin by E. herbicola (Chatterjee et al. 1969).

Kelman (1972) working with maize and E. chrysanthemi extracted a differentially inhibitory fraction from the

host which was more toxic to soft rot Erwinia sp. than to the pathogen E. chrysanthemi. Other pathogenic and saprophytic bacteria were also inhibited by the fraction. Other maize pathogens tested proved insensitive.

In addition to the above preformed inhibitors a large number of antibiotics and saponins have been isolated from higher plants but their role in resistance is unknown (Nickell, 1959; Tschesche and Wulff, 1965).

c) Other mechanisms

Apart from the effect of nutrients and production of inhibiting compounds by the host, whether preformed or induced, other mechanisms by which a plant could confer resistance exist. Bacteria have a large affinity for moisture, and a characteristic of bacterial disease is watersoaked lesions. Keen and Williams (1971) isolated a lipomucopolysaccharide from P. lachrymans which induced water soaking in cucumber leaves. In the resistant reaction water soaking rarely occurs, so failure to induce a water soaked lesion will favour a resistant reaction. Cessation of bacterial growth is concomitant with desiccation and collapse of host tissue (see section of bacterial growth). In the incompatible system P. pisi/and tobacco the bacterial population increased during an experiment when plants were kept in a humidity chamber (Goodman, 1972). In the incompatible systems bean/P. lachrymans and bean/P. syringae the bacteria multiplied at an equal rate to that of the compatible system bean/P. phaseolicola when the leaves were saturated with water after infection. Even non pathogenic bacteria increased in numbers under these conditions (Young, 1974).

Finally Erikson (1945) and Fox *et al.* (1972) have proposed that phellogen and a mechanical barrier limited the spread of *P. syringae* in plum tree cankers and *E. caratovora* in potato tissue respectively.

MATERIALS AND METHODS

(1) Biological material

A) Higher plants

a) Cultivars

Phaseolus vulgaris L. cv. Red Mexican U.I.34, were grown at Imperial College Field Station, Silwood Park (original stocks from Honey Seed Co., Twin Falls, Idaho, U.S.A.) cv. Canadian Wonder were supplied by Sutton and Sons Ltd., Reading.

b) Plant culture

Plants were grown from seed which was germinated in plastic boxes lined with moist tissue paper at 25°C. After 48 h germinated seedlings were removed and placed individually into seed trays (35 x 22 x 5 cm) containing John Innes No. 2 potting compost where they remained for a further 5 d until the monofoliate leaves emerged. The trays were then put in a growth cabinet at 23° ± 1°C supplemented with fluorescent lighting for a 16h/day at an intensity of 4,200 lx at the level of the leaves. The compost was soaked every 2 d with tap water pH 7.2.

c) Plant inoculation

The bacteria were forced into expanding monofoliate bean leaves of 10-13 d old plants by injection through main veins into intercellular spaces of interveinal areas of the leaf. This was done using a sterile hypodermic syringe fitted with a No. 30 needle until water soaking appeared. The most usual point for inoculation was at the junction of the lamina and petiole.

Immediately after inoculation leaves were washed in a

stream of sterile water. The plants were then replaced in the growth cabinet. The water soaked leaves appeared normal about 30 min after inoculation.

B) Bacteria

a) Cultures

Pseudomonas fluorescens isolate No. 1964; Pseudomonas lachrymans isolate No. 277; Pseudomonas phaseolicola race 1 isolate No. 605; Pseudomonas phaseolicola race 2 isolate No. 1321; Pseudomonas solanacearum isolate No. 325; Pseudomonas tabaci isolate No. 1408; Xanthomonas phaseoli isolate No. 1420 were all obtained from the National Collection of Plant Pathogenic Bacteria, (M.A.A.F., Hatching Green, Herpenden). An isolate (D5) of Pseudomonas mors-prunorum was kindly supplied by Dr. J. E. Crosse, East Malling Research Station, Maidstone, Kent. Erwinia atroseptica and Erwinia caratovora were obtained from the Plant Pathology Culture Collection, Imperial College. Xanthomonas cassava isolate 1120 CIAT and Xanthomonas manihotis 1059 CIAT were obtained from Dr. T. Ikotun, Imperial College, now at University of Ibadan, Nigeria.

b) Growth of bacteria

Stock cultures were kept on storage nutrient agar slopes pH 7.2, of the following composition: 13 g Oxoid Nutrient Broth No. 1 (CMI), 15 g Davis Standard Agar, 1 litre distilled water. The cultures were stored at 4^{o}C and at - 20^{o}C.

For inoculation, bacteria were grown on plates of nutrient agar growth medium, pH 7.3, of the following composition: 13 g Oxoid Nutrient Broth No. 2 (CM67), 15 g Davis Standard Agar, 1 litre distilled water.

For studies into bacterial cell walls, bacteria were grown in liquid culture in conical flasks containing 200 mls Nutrient Broth No. 2 (CM1) (10g/l) for 48 h rotated on an orbital shaker.

For growth under minimal nutrient conditions a salts medium was used of the following composition: 4.6 g Casamino acids, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 10 ml trace element solution, distilled water to 1 litre. The final trace element constituents were:

Trace element	Source	Concentration p.p.m. of trace element in final culture solution
Iron	$Fe.SO_4.7H_2O$	0.5
Zinc	$ZnSO_4.7H_2O$	0.1
Molybdenum	$Na_2MoO_4.2H_2O$	0.2
Copper	$CuSO_4.5H_2O$	0.1
Manganese	$MnCl_2.4H_2O$	0.1

c) Standardization of bacterial inoculum

Bacteria were grown for 18-22 h at 27°C on plates of nutrient agar. About 10 ml sterile distilled water was added to each plate and the cells suspended by scraping the surface of each plate with a glass rod. The cells were centrifuged (10 min x 6,000 g) and washed once by resuspension and centrifugation.

Washed bacteria were resuspended in sterile distilled water and the suspension adjusted until the required reading of 35 on a nephetometer (Eel "Unigalvo") was obtained. This reading corresponded to a viable count 2.6×10^8 cells/ml, the concentration used for inoculation.

C) Grading of host reaction

The following system was adopted for macroscopic observations of host response.

Grade 0 - No visible response.

a) Resistant reaction grades

(1) Inoculation area appears shiny.

(2) Area of collapse within inoculation area is shown by pale discoloration.

(3) Collapse of inoculated area, brown/white in colour.

(3*) Incomplete collapse of inoculated area.

(4) Bronze/white lesion becomes light brown and desiccated.

(5) Light brown lesion becomes dark brown in colour.

b) Susceptible reaction grades

(1) - (5) As in resistant reactions.

(6) Trifoliate leaves show early signs of chlorosis usually at leaf margins near the petiole, and in the lateral before terminal leaflets. Primary leaves sometimes with a watersoaked zone around inoculation area.

(7) Pronounced interveinal chlorosis in all trifoliates. Halo develops around the advancing lesion.

(8) Primary leaf now very chlorotic and sometimes necrotic. Successive trifoliates becoming chlorotic in interveinal regions. Plant exhibits

stunted growth.

(9) Plant stops growth and dies.

(2) Chemicals

A) General chemicals

All chemicals routinely used were of Analar grade and were obtained from British Drug Houses (BDH) Ltd., Poole, Dorset; Hopkin and Williams Ltd., London E.C.1.; Koch-Light Ltd., Colnbrook, Bucks; Oxoid Ltd., London, S.E.1.

B) Special chemicals

a) Inhibitors

Actinomycin D, chloramphenicol, cyclohexamide, 5-fluorouracil, erythromycin, puromycin, rifampicin, were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey. Spectinomycin was obtained from Upjohn Ltd., Fleming Way, Crawley, Sussex.

b) Enzymes

Deoxyribonuclease, ribonuclease, trypsin were obtained from Sigma.

c) Systemic compounds

Abscisic acid, gibberellic acid, indole-3-acetic acid, 6-methyl amino purine, were all supplied by Sigma.

Alar (wettable powder, 85% active ingredient (WP85)); Alsol (emulsifiable concentrate, 40% active ingredient (EC 400)); gibberellin (WP50); CCC (EC400); Ethrel (EC480); Lasso (EC500); Off Shoot "T" (EC630) were kindly supplied by Ciba-Geigy Ltd., CH-4002, Basel, Switzerland. In addition to the above commercial chemicals the following experimental chemicals were also supplied by Ciba-Geigy: CGA 17'048

(WP50); CGA 22'640 (WP25); CGA 37'407 (WP50); CGA 45'072 (WP50); CGA 45'434 (WP50).

d) Nucleic acids

Deoxyribonucleic acid from calf thymus (D-1501); deoxyribonucleic acid from E. coli strain B (D-2001) were supplied by Sigma.

e) Chemicals for electrophoresis

Acrylamide (electrophoresis purity); bis (electrophoresis purity); temed (electrophoresis purity) and coomaisse brilliant blue were supplied by BioRad Laboratories Ltd., Honesdale Road, Bromley, Kent.

f) Chemicals for autoradiography

Tritiated uridine ((5,6-^{3}H) Uridine 5' triphosphate, ammonium salt, specific activity 40,000-60,000 m Ci/m mol) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Photographic emulsion (K5) was obtained from Ilford Nuclear Research Laboratory, Ilford, Essex.

(3) Measurement of bacterial growth

A) In vitro

1 ml samples were removed at intervals from bacterial liquid shake cultures on minimal medium and serial 10,100-fold dilutions were made. At the end of the dilution sequence 1 ml was transferred to petri dishes (8.5 cm diam) and 10 mls of nutrient agar at 43^{o}C quickly poured in. The bacteria and agar was mixed, then cooled and incubated at 27^{o}C for 4 d. Three replicates each of the last two dilutions were made.

From numbers of bacterial colonies per plate the concentration of bacteria in the original suspension was calculated.

B) In vivo

At various time intervals inoculated leaves were removed, washed thoroughly in a stream of sterile water to remove most surface bacteria, and 4 disks (4.5 mm diam) were punched out of inoculated areas using a sterile cork borer. Disks were ground up with a mortar (6.5 cm diam) and pestle in 1 ml sterile distilled water. The homogenate was then diluted and plated as described above. Numbers of bacteria/leaf disk were calculated.

(4) Measurement of electrolyte loss from leaves

Ten disks (5.5 mm diam) were removed from inoculated areas of leaves after various time intervals after leaves had been washed in a stream of sterile water. The disks were put into vials containing 10 ml of deionized water and the conductivity measured in a "Chandos" conductivity bridge (A21) immediately and after 1 h. The difference was taken as a measure of electrolyte loss from the disks at 25^{o}C.

(5) Methods used in attempts to alter the hypersensitive and susceptible reactions

A) Use of injected chemicals

The plants used for these experiments remained firmly rooted in the trays of potting compost in which they were grown. In some cases the bacteria and the appropriate quantity of chemical, drawn from two stock solutions, were

mixed in the syringe prior to injection into the leaves. Otherwise either the bacterium or the chemical was injected first. In all cases the visible bacterial concentration remained constant.

B) Use of systemic chemicals

Plants in these experiments were carefully unearthed from the John Innes potting compost in which they were grown, so as to avoid root damage. The roots were washed under running tap water, rinsed in bowls of tap water so as to remove as much soil as possible, and then immersed in a solution of the appropriate systemic chemical contained in 50 ml flasks. The plants were returned to the growth cabinet and remained in the solution for 24 h at 23°C ± 1°C before bacteria were inoculated into the leaves. In a few experiments hypocotyls were excised at soil level and placed in the solution of the systemic chemical before bacteria were inoculated into the leaves.

C) Use of alteration of temperature to induce shock

In these experiments whole plants were carefully unearthed from John Innes potting compost and their roots were put into 50 ml flasks of tap water pH 7.3 for 24 h in the growth cabinet. The plants were then subjected to a rapid change in air temperature for periods of between 5-15 min. Leaves were then injected with bacteria and the plants were replaced in the growth cabinet.

D) Use of cross protection

Plants remained in the trays of potting compost in which they were grown. Different bacteria were injected simultaneously or sequentially into the same area of a leaf.

After inoculation the plants were kept at $23^{o} \pm 1^{o}C$ in the growth cabinet. Live and heat killed cells (see later) were used.

(6) Extraction of deoxyribonucleic acid from Pseudomonad species

A modification of the method of Marmur (1961) was used.

A) Harvesting and lysis of bacterial cells

5 g of wet packed bacterial cells were harvested from nutrient broth cultures (Nutrient Broth No. 2 at 10 g/l) during the exponential growth phase (18-20 h) by centrifugation at 10,000 g x 10 min. The cells were then washed once in saline-EDTA (0.15 M NaCl and 0.1 M Acetate EDTA pH 8). After collection by centrifugation (10,000 g x 10 min) cells were resuspended in 25-30 mls saline-EDTA and lysed by addition of an equal volume of 25% sodium lauryl sulphate. The mixture was placed in a water bath at $60^{o}C$ for 15 min, then cooled to room temperature.

This treatment results in a rapid increase in viscosity associated by the release of nucleic acids and other cytoplasmic components.

B) Nucleic acid isolation

Sodium perchlorate (5 M) was added to the lysed suspension to a final concentration of 1 M and the mixture was shaken with an equal volume of chloroform in a ground glass stoppered flask for 30 min on a reciprocal shaker. The resulting emulsion was separated into three layers by centrifugation (10,000 g x 10 min). The upper aqueous phase containing the nucleic acids was pipetted off. The middle layer (denatured protein and occluded nucleic acids) was

collected by decanting and an equal volume of dilute saline citrate (0.015 M NaCl and 0.0015 M trisodium citrate) was added and the mixture shaken for 10-15 min. The mixture was centrifuged (10,000 g x 10 min) and the supernatant pipetted off and added to the aqueous phase. The nucleic acids were then precipitated by gently layering 2 vol ethyl alcohol (95%) on to the aqueous phase evenly distributed in test tubes. The layers were mixed by using a thin stirring rod which was swirled back and forth, thus allowing the nucleic acids to 'spool' as threadlike precipitates. The nucleic acids were removed on the rod, excess alcohol drained by pressing the rod against the tube, and then the nucleic acids were redissolved in 5 ml dilute saline citrate. It was essential at this stage to keep the concentration of DNA at c. 0.5 mg/ml. Too low a concentration results in degradation and loss of biological activity (Hershey and Burgi, 1960). Once all the precipitated nucleic acids were redissolved, the concentration of the saline citrate was raised by adding an equal volume of concentrated saline citrate (1.5 M NaCl and 0.15 M trisodium citrate).

Dissolved nucleic acids were shaken as before with an equal volume of chloroform for 15 min, centrifuged (10,000 g x 10 min) and the supernatant and the nucleic acids precipitated again. This deproteinization was continued until very little protein was seen at the interface.

C) Separation of deoxyribonucleic acid from ribonucleic acid

To the nucleic acids dissolved in saline citrate, bovine ribonuclease was added to a final concentration of 50 µg/ml and the mixture incubated at 37°C for 30 min.

Digestion of RNA released into solution ribonucleoprotein which had earlier resisted the chloroform deproteinization.

The solution was subjected to a final chloroform deproteinisation by shaking for 15 min, centrifuging (10,000 g x 10 min), the supernatant precipitated out and redissolved in dilute saline citrate.

0.5 volume Acetate EDTA (3.0 M sodium acetate and .001 M EDTA pH 7.0) was added to the solution of nucleic acids which was stirred rapidly. Isopropyl alcohol was then added dropwise into the vortex. This precipitated the DNA in a fibrous form. From 1 g wet packed cells c. 1 mg of DNA was obtained. The precipitated DNA was redissolved in dilute saline citrate and the purity checked spectrophotometrically by measuring the ratio of absorbance at 260 : 230 : 280 mμ which should be close to 1.0 : 450 : 515.

D) Note on procedure

The stirring rod was made from a glass rod 2-3 mm diameter and 150 mm length. The rod was bent to form an upturned 'L' shape with the slightly upturned base c. 4-5 mm in length.

(7) Disintegration of bacterial cells

A) Mickle shaker disintegration

For bacterial cell wall preparations 3-5 g wet packed cells were harvested by centrifugation (10,000 g x 10 min) from nutrient broth cultures grown as previously described (1B(b)).

Cells were washed three times by centrifugation

(10,000 g x 10 min at 10°C) in cooled phosphate buffered saline (0.05 M NaCl and 0.01 M sodium phosphate pH 7.3). Cells not ready for use at this stage were frozen at -20°C.

The concentration of the cell suspension was adjusted with buffered saline phosphate such that c. 1 g wet packed cells went into each of the two flat bottomed glass cups (60 mm x 40 mm). An equal volume of glass Ballotini beads (No. 12) was added to each cup which were then closed with rubber stoppers. Cups were cooled in an ice bath for 10 min, then screwed into the disintegrator arms and shaken at maximum amplitude for 15 min. The cups were then cooled in the ice bath again and the process repeated to give a run of 45-50 min until disruption was complete as determined by viable counts (c. 95.0% breakage), and microscopic examination.

The final preparation was centrifuged (2,000 g x 5 min) to separate unbroken cells and ballotini beads from the supernatant, which was further centrifuged (10,000 g x 10 min) to obtain a cell wall pellet.

B) Heat treatment

This was applied to bacterial cells which were to be used in a cross protection experiment.

Suspensions of 3.0×10^8 and 6.0×10^8 cells/ml were kept in a water bath at 100°C for 30 min. Cells were recovered by centrifugation (10,000 g x 10 min) and resuspended in sterile distilled water.

(8) Dried bacterial cell wall preparation

Bacterial cells were disintegrated in a Mickle shaker

by the method given above. Cells were resuspended in distilled water, washed by centrifugation (13,000 g x 10 min at 10°C) and then resuspended in phosphate buffered saline (0.05 M NaCl in 0.01 M sodium phosphate pH 7.3) and washed again by centrifugation (10,000 g x 10 min at 10°C). To the preparation (resuspended in 0.01 M phosphate buffer pH 7.4) 0.2 mg/ml of deoxyribonuclease, ribonuclease and trypsin were added and this was incubated in a water bath at 35°C for 8 h. The preparation was then centrifuged (2,000 g x 10 min) to remove debris, and the resulting supernatant pipetted off and re-centrifuged (10,000 g x 10 min) to give a cell wall pellet. This was washed a further three times by centrifugation: (a) phosphate buffered saline (10,000 g x 10 min) (b) sterile distilled water x 2 (13,000 g x 10 min). The pellet was transferred to a vial (1.5 cm x 3.0 cm), placed in a vacuum desiccator which was evacuated by a 'Speedivac' high vacuum pump (.005 mm mercury) and dried under vacuum over P_2O_5. Dried cell wall was then ground up to a powder in a glass mortar (6.5 cm diam).

(9) Polyacrylamide gel electrophoresis of bacterial cell walls

A) Gel preparation and equipment

Acrylamide gels are prepared by polymerisation of the monomer, acrylamide, with the cross linking agent, bis acrylamide (N, N' methylene-bis acrylamide), in aqueous solution. On the addition of a catalyst, such as 'Temed' (N, N, N' N' - tetraethylmethylethylene diamine), and ammonium persulphate, polymerisation follows with the formation of

a homogenous gel of defined pore size. Gels between 2.4 and 30% may be prepared. Gels between 4 and 10% are used for protein separation, while soft, low concentration gels (2.4 - 4%) are used for RNA separations (Loening, 1967).

The equipment used was a Shandon basic outfit for disk electrophoresis: a disk electrophoresis apparatus, Vokam constant voltage/constant current D.C. power supply with output 50-300 v at 40 mA max. as a constant voltage supply; 0-40 mA at 300 v max. as a constant current supply.

The gels contained between 7.5 - 8.0% (w/v) acrylamide, 25-35% (v/v) acetic acid and 6 M urea. The electrophoresis buffer was 10% (v/v) acetic acid. For a 7.5% gel pH 2.3 containing 25% acetic acid and 6 M urea the stock solutions were as follows.

Buffer: urea 36.0 g, 10N KOH 4.8 ml, glacial acetic acid 52.2 ml, temed 1.15 ml, H_2O to 100 ml (4 parts).

Acrylamide: urea 36.0 g, acrylamide 30.0 g, bis 0.8 g H_2O to 100 ml (2 parts).

Initiator: ammonium persulphate 0.7 g, urea 9.0 g H_2O to 25 ml (2 parts).

The shelf life of the stock solutions was one month except for the initiator which had to be freshly prepared.

The prepared mixture was degassed under vacuum to remove the oxygen which inhibited polymerization. Aliquots (2.5 - 3.0 ml) were pipetted into glass tubes (75 mm x 7 mm) sealed at their bases with 'suba-seal' serum caps. Each gel was then carefully overlayed with 3-4 mm of distilled water from a hypodermic syringe plugged with a thin

strand of cotton wool. (The water excluded oxygen and provided a flat gel surface).

After polymerisation (15-20 min), the water was decanted, the stoppers removed, and the tubes were inserted into the upper electrode buffer reservoir and bottom electrode assembly. Electrode buffer 250 ml (10% v/v acetic acid) was poured into the lower and upper reservoirs. The protein sample was then layered on to the gel below the electrode buffer using an attenuated pasteur pipette. To do this the sample was made more dense by dissolving sucrose in it. The lower electrode served as the cathode and electrophoresis was carried out for 3.5 h at a constant current of 5 ma per tube.

B) Estimation of protein content in bacterial cell walls

The amount of protein in cell wall samples was determined according to Lowry et al. (1951). The reagents were as follows.

Alkaline solution: 4% (w/v) Na_2CO_3 (100 vol), 2% (w/v) $CuSO_4$ (1 vol), 4% (w/v) Na K tartrate 1 vol. (It was sometimes necessary to filter the Na_2CO_3 before adding the Na K tartrate and $CuSO_4$).

Folin-Ciocalteau Reagent: 1 volume stock diluted with 2 volumes of distilled water.

The method used was as follows:

0.75 ml of protein sample was put into a test tube and 7.5 ml of alkaline solution was added and the two solutions were mixed and warmed for 15 min in a water bath at $40^{o}C$.

Folin reagent 0.75 ml was added and the mixture was left for 30 min. The O.D. was measured in an EEL portable calorimeter using a red filter and the results are expressed as μg/ml of tyrosine equivalents from a calibration curve previously obtained.

C) Solubilisation of bacterial cell walls

Once the amount of total protein in each dried bacterial cell wall sample was determined (from method above), then equal amounts of protein from each sample were weighed into different test tubes (the amount weighed into each tube was calculated such that in 100 μl sample of the solubilised protein there was between 150-200 μg protein). The protein of the cell wall material was solubilised according to the method of Takayama et al. (1966) utilizing phenol : acetic acid : water (2 : 1 : 0.5, w/v/v). The acetic acid and urea helped to transform the hydrophobic proteins to their monomeric form and prevented the reaggregation of the monomers during migration in the gels. To each of the solubilised samples sucrose was added to a final concentration 5% (w/v) and 100 μl sample was pipetted on to the gel in the method given above.

D) Staining and destaining the gels

Once electrophoresis was completed, the tubes were removed from the apparatus and gels removed from the tubes by 'rimming'. A 10 ml hypodermic syringe was filled with distilled water and the needle gently inserted between the gel and the wall of the tube. By slowly rotating the tube while discharging the syringe, the gel was separated from the tube wall by a film of liquid. By applying a rubber teat to the end of the tube the gel was easily expelled

under a slight pressure.

Gels were stained in a solution of 2% (w/v) Coomaisse Brilliant Blue in 7% (v/v) acetic acid for 20 min and destained by extensive washing in 7% acetic acid. This process was sometimes hastened by including a small quantity of Amberlite IRA 45, ion-exchange resin, which bound to the eluted stain. Gels were stored in tubes (80 mm long x 10 mm wide) in 7% acetic acid.

E) Recording of results

The positions of the bands in the gels were recorded in a two fold manner (a) by photography (b) by scanning in a Chromoscan.

For photography the gels were supported in glass specimen tubes (80 mm x 10 mm) filled with 7% acetic acid and photographed against a white screen illuminated with fluorescent light.

For scanning, a Gilford spectrophotomer 240 with linear transport attachment was used. The gels were placed in quartz glass cuvette (90 mm x 10 mm) in 7% acetic acid, and scanned at 550 nm at a scan rate of 1 cm/min. The results were recorded on paper at a chart rate of 1 cm/min such that the length of gel corresponded exactly to distance of the base line on paper for a particular gel i.e. 1 cm on the gel was represented by 1 cm on the chart paper. Each band in the gel thus gave rise to a peak or inflection on paper. Using this method the positions of the bands in the gels in the various samples were accurately compared, by measuring the distance from the top of the gel to the various bands down the gel.

Those bands which appeared at the same distance from the top were similar proteins. Using this method any similarity or dissimilarity in the protein make-up of the bacterial walls of different species or sub species became clear.

(10) Hydrolysis and amino acid analysis of bacterial cell walls

A) Hydrolysis of bacterial cell walls

10 mg of dried bacterial cell wall preparation (see (8) above) were weighed into 10 ml freeze drying ampoules (FBG Trident, Charlton, London. (No. 3/B/A/19/002/01/A)). To this 2 µm solution of nor-leucine was added. The ampoules were then 'necked' in a methane-oxygen burner so that the internal diameter of the neck of the ampoule was c. 3 mm. To the ampoules 10 ml of 6N HCl was added using attenuated Pasteur pipettes. Ampoules were then frozen in a bath of liquid nitrogen while the content were rotated so as to ensure a thin frozen layer around the periphery of the inside of the ampoule wall. Ampoules were then sealed in vacuo using a high vacuum pump (.005 mm mercury) and a 'Calor Gaz' blow torch.

Ampoules were then placed in an oven at 105°C for 30 h after which time any residue remaining was centrifuged down (13,000 g x 15 min). The supernatants were evaporated down at room temperature in glass vials (50 mm x 20 mm) in a vacuum desiccator over concentrated sulphuric acid and potassium hydroxide pellets.

B) Amino acid analysis of bacterial cell walls

Hydrolysates of dried bacterial cell walls were pre-

pared in the method above. To the evaporated hydrolysate 2 ml of 0.1 N HCl was added in the glass vial. From this solution a 10 λ sample was removed, put into a sample cup and analysed on an amino acid analyser which used the ninhydrin reaction. Results were measured calorimetrically on two charts at 550 and 420 nm, and were expressed as amounts of amino acid in gram %. Absorbance peaks which occur on the charts at positions equivalent to that of the standard, (to which the analyser had previously been programmed for eighteen amino acids) were regarded as similar amino acids. The second chart (420 nm) was required to measure proline since at 550 nm the absorbance of proline is poor.

(11) Extraction of phenolics

Total phenolics were extracted by homogenising 1.0 - 1.5 g fresh wt trifoliate leaves in 20 ml of 80% (v/v) ETOH for 10 min. The homogenate was filtered and the residue washed with 10 ml ETOH. The filtrate was shaken with petroleium ether (2 vol) for 10 min and partitioned by allowing to stand for 10 min. The alcohol phase was removed and shaken three times more with petroleum ether. The final straw coloured solution was evaporated in a Buchi rotary evaporator and the residue dissolved in 5 ml water. Total phenolics were estimated by the Folin method and results expressed as catechol equivalents in μg/g fresh wt leaf.

(12) Extraction of chlorophyll

Chlorophyll from trifoliate leaves was extracted in 80% (v/v) acetone by the method of Mackinney (1941). Results

are expressed as mg/litre chlorophyll/g fresh wt leaf.

(13) Microscopy

The following microscopes were used: a Reichert Zetopan with Binolux twin lamp unit, equipped with phase contrast, interference (Nomarski) contrast systems; a Bausch and Lorib zoom dissecting microscope; AEI 6B transmission electron microscope, Cambridge Stereoscan Mk IIA scanning electron microscope.

A) Light microscopy

Trifoliate leaves were examined by two methods. In the first, sections were cut free hand using a razor blade to give sections c. 25 μ thick. These were stained in the manner to be described below for tannins, phenols or lipids. Leaf material in the second case, was fixed in glutaraldehyde, dehydrated and embedded (as described below) and 5 μ sections taken. These were also examined for tannins, phenols or lipids.

a) Staining for phenols

Ortho-dihydroxyphenols were located by a modified method of Reeve(1960). Single drops of 10% acetic acid and 10% sodium nitrite solution were mixed on a slide to which the sections were transferred. After 3 min, the nitrous acid formed by this reaction was neutralised by an equal volume of 2N sodium hydroxide. A positive reaction was indicated by a scarlet coloration.

b) Staining for tannins

A few drops of the test solution (2% $FeCl_3$ in 95% alcohol) were placed on the sections for 3 min. After this time a positive reaction was indicated by a greyish blue

coloration.

c) Staining for lipids

Lipids were located by the use of Sudan IV reagent (Jensen, 1962). Sections were placed in 50% alcohol for 2-3 min and then transferred to a saturated, filtered solution of Sudan IV in 70% alcohol. A positive reaction was indicated by a bright red coloration.

B) Transmission of electron microscopy

a) Fixation

Samples were cut from leaves by use of a razor blade and fine scissors under fixative in an embryo cup to c. 2 mm^3. The fixative was 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.2. The leaf material was left in fixative for 2 h. This was removed and replaced by three washes in buffer. After the last wash material was left in buffer for 30 min. Post fixation was carried out by replacing buffer with 1% solution of osmium tetroxide in buffered phosphate (0.1 M) for 1 h.

b) Dehydration

The osmium tetroxide was replaced by 20% ethanol and dehydration completed by serial passage through 20%, 30%, 40% and 50% for 10 min each and at 75%, 95%, 100% for 20 min. The absolute alcohol was changed twice and the material left overnight in covered embryo cups.

c) Embedding

The absolute alcohol was initially replaced by epoxypropane in three changes at 10 min intervals. Some embedding resin, Epon 1a : 1b (Luft, 1961) was poured in after one third of the epoxy propane had been withdrawn. A glass cover-

slip was placed over the embryo cup and the resin was left for 1 h to allow diffusion into the leaf material. The coverslip was then left slightly open to allow the epoxy propane to evaporate overnight.

After 24 h the specimens were transferred to fresh resin in a different embryo cup and left for a further 24 h. Specimens were then transferred to polythene embedding cups (3.5 cm diam) in some residual resin and equidistantly separated around the periphery, and fresh resin poured in. The cups were kept at 60°C for 72 h to allow the resin to polymerise.

d) Sectioning

Leaf material was cut from the embedding resin using a flexible hand saw and mounted on 1.5 cm cylindrical blocks of perspex using 'Durafix' adhesive and set at 60°C for 30 min. The resin around the leaf material was trimmed under a dissecting microscope using a sharp razor blade to give finally a trapezoidal pyramid. The specimen block was then sectioned with a diamond knife on a Reichert ultra-microtome.

The sections grey-silver (550 - 800Å) were transferred to 100 mesh copper grids which had previously been filmed with formvar.

e) Staining

Sections were stained with uranyl acetate and lead citrate. Each grid was immersed in a drop of 2% uranyl acetate in a groove on a polythene sheet, which itself was placed in a covered Petri dish for 30 min at 60°C.

The grids were washed in a stream of glass distilled water and then immersed in water for 5 min, dried and then immersed in a solution of lead citrate for 20 min as before for uranyl acetate. The polythene sheet in this case was placed on filter paper soaked in concentrated sodium hydroxide solution to avoid precipitation in the lead citrate stain. The grids were washed, dried and placed in a covered Petri dish on clean filter paper ready for viewing in the microscope. The grids were viewed in an AEI EM6B transmission electron microscope at 60 kV.

C) Scanning electron microscopy

Longitudinal and transverse sections of material to be examined were first dehydrated in a serial gradation of alcohol 50%, 60%, 80% for 10 min then 100% for 20 min. A further change in absolute before two changes in acetone was then undertaken, and finally the material was immersed in liquid carbon dioxide. The material was transferred to a critical point drier (Polaron E3000) and dried at 31.5°C at a pressure of 1100 p.s.i.

Material was then mounted on aluminium rivets using 'Durafix' adhesive and coated with gold in a Polaron E5000 for 2 min at an accelerating voltage of 1.2 kV and current 40 m amps. This gave a covering film of $\leq$. 480Å.

Mounted material was viewed in a Cambridge Stereoscan Mk 2A. Photographs were taken using an Exa 1A camera loaded with Ilford Pan F film.

(14) Autoradiography

This technique was used to monitor by photographs the passage of metabolites from one position in the plant to

another, or from a bacterium to the plant. RNA in bacteria was labelled with tritiated uridine as a preliminary experiment to perfect a method which it was hoped could be used at a later date.

A) Labelling the bacteria

Pseudomonas mors-prunorum grown on plates of nutrient agar was harvested during exponential growth (18 h), washed by centrifugation (10,000 g x 10 min) and resuspended in sterile distilled water in the manner described previously. The concentration was then adjusted turbidmetrically to 9.0 x 10^8 cells/ml.

A bacterial growth medium of nutrient broth 2 (CM1 30 g/l) was also prepared.

The radioactive nucleotide (5,6-^{3}H) uridine 5' triphosphate ammonium salt was supplied as 1 m Ci/ml samples with a specific activity of 52 m Ci/mM in a vial.

For the pulse phase (labelling) the following constituents were added to an incubation vial (50 mm x 20 mm):-

1.5 ml P. mors-prunorum (9.0 x 10^8 cells/ml)
1.5 ml nutrient broth (30 g/l)
1.5 ml ^{3}H-uridine (125 μ Ci)
4.5 ml Total

This then gave the incubation medium final concentrations of:-

P. mors-prunorum	3.0 x 10^8 cells/ml
Nutrient broth	10 g/l
^{3}H-uridine	41 μ Ci

The incubation vial was shaken periodically every 5 min

at a temperature of 25°C, for 150 min, the optimum period for labelling. This was determined by using serial labelling times of 30, 80, 100, 130, 150, 180 min. After 150 min the amount of radioactivity incorporated by the bacteria appeared to drop slightly (Table 1).

After labelling the bacteria, the contents of the vial were centrifuged (10,000 g x 10 min), the supernatant was pipetted off, and the bacteria resuspended in 3.0 ml of distilled water and washed a further 6 times by centrifugation (10,000 g x 10 min). Using liquid scintillation (see below) this was shown to be the optimum number of washings required to reduce the label in the supernatant and on the bacterial surface to an acceptable level above the background emission (Table 2).

Table 1. Determination of incubation period for bacterial labelling

Incubation time (min) at 25°C	Radioactivity (c.p.m.) emitted by bacteria after 6 washes
30	542
80	1,665
100	2,110
130	2,883
150	3,240
180	3,120

Table 2. Determination of number of bacterial washings needed to reduce supernatant label to an acceptable level

No. of washes in distilled water	Radioactivity (c.p.m.) of supernatant
1	6,714.0
2	175.0
3	132.0
4	95.0
5	84.0
6	79.0
7	78.0
Background radiation	66.0

B) Liquid scintillation counting and calculation of quenching

a) Counting

This is a technique whereby low emission isotopes i.e. tritium can be detected by the fluorescence of materials placed in the solvent system with the radionucleotide. The radioactive particles excite the solvent molecules, which transmit their energy of excitation to the fluorescent material, which in turn respond by emitting a flash of light. The individual flashes (each corresponding to a single radioactive disintegration) were detected by sensitive photomultiplier tubes and counted on a scaler. However, quenching reduced the counting efficiency and was checked from sample type to sample type.

Samples (0.1 ml) were counted in 15 ml of solvent in stoppered glass vials and were prepared away from u v or bright sunlight to avoid prdliminary fluorescence. The scintillation solvent was: Toluene (scintillation grade) containing 4 g/l PPO (2,5-Diphenyloxazole) and 0.05 g/l POPOP (1,4-Di-2-(5-Phenyloxazolyl)-benzene. The toluene also contains 20% (v/v) ethanol). To 15 ml of scintillation fluid 0.1 ml of aqueous radioactive sample was added and the sample counted in a Nuclear Chicago Scintillation System.

b) Quenching (Efficiency)

Toluene is a good solvent since it has a low energy absorption (quenching) but as the aqueous radioactive sample approaches the maximal amount permitted (0.15 ml) then quenching becomes prevalent since water causes severe quenching.

Calculation:-

Given that 1 μ Ci = 3.7×10^4 d.p.s. (decays sec^{-1})

= 2.22×10^6 d.p.m. (decays min^{-1})

From results:

.1 ml 37 μ Ci sample which is $\frac{37}{10 \times 15}$ = .25 μ Ci/ml

.25 μ Ci/ml sample = 2.60×10^5 c.p.m. (counts min^{-1})

Equilibriate to 1 μ Ci $2.60 \times 10^5 \times 4$ c.p.m.

= 1.04×10^6 c.p.m.

Efficiency $\frac{1.04 \times 10^6}{2.22 \times 10^6} \times 100$

= 46%

This figure suggests that 3,240 c.p.m. after 150 min pulse (see Table 1) should read c. 4840 c.p.m. for the

bacterial labelling. However, introducing .25 μ Ci into the scintillation vial tends to overload the photomultiplier so that c.p.m. are last, thus making the efficiency considerably lower than it should be.

C) Application of labelled bacteria

Labelled bacteria (Pseudomonas mors-prunorum) were adjusted to 2.0 x 10^8 cells/ml turbidmetrically and injected into monofoliate leaves of Red Mexican. The resulting hypersensitive response indicated that the incorporation of tritiated uridine of a high specific activity had not reduced the bacterial viability to an unacceptable level.

Labelled bacteria were also used in a further experiment involving a whole cell autoradiography and thin section autoradiography.

a) Whole cell autoradiography

To 0.5 ml of labelled bacteria (8.0 x 10^8 cells/ml) an equal volume of 4% glutaraldehyde was added. The suspension was fixed for 2 h, rinsed in 0.1 M phosphate buffer pH 7.1 and post-fixed 1 h in 2% OsO_4. After fixation, suspension was washed by centrifugation (10,000 g x 10 min) in buffer. The cells were then pipetted on to 300 mesh copper grids which had previously been coated with formvar. The grids were dried in an oven at 60°C for 5 min. Grids were then attached to strips of double-backed sellotape on clean glass slides and coated with K5 photographic emulsion and left for 10 d in fridge at 3°C and finally developed in chemical (D19B) and physical developers.

b) Thin section autoradiography

To 1.0 ml of labelled bacterial suspension (1×10^9 cells/ml) an equal vol of 4% glutaraldehyde was added for 4 h. The suspension was rinsed in phosphate buffer by centrifugation (10,000 g x 10 min) and post-fixed in 1% OsO_4 for 150 min. Cells were rinsed again in buffer, then resuspended to 1.0 ml in distilled water and added to 1.0 ml 4% Davis standard agar set at 50°C and cooled. Agar containing the bacteria was dehydrated through serial alcohols cut into 2 m m^3 and embedded in Epon 1a : 1b as in the method for electron microscopy. Blocks were trimmed and sectioned as previously described and 800-1000 Å sections were put on formvar coated 300 mesh copper grids. The grids were attached to thin strips of 'double backed' sellotape on clean glass slides and then coated with K5 photographic emulsion, and left for 2 months in a light proof container at 3°C.

D) Photographic emulsion application

All procedures were carried out in a dark room fitted with an Ilford Safelight 902S. A Mettler P162 balance used during the procedure was carefully blacked out.

11.5 g of K5 emulsion was put into a 250 ml beaker and 20 ml distilled water added. The beaker was then put into a water bath set at 45°C for 10 min and the emulsion melted while being stirred thoroughly using a glass rod.

The emulsion was then filtered through a double layer of muslin into a 300 ml beaker which was then put back into the water bath for 15 min and stirred.

The beaker was then transferred to an ice bath for

exactly 3 min and then left to stand at room temperature (22°C) for a further 3 min. A wire loop (3.5 cm diam) was dipped into the emulsion and withdrawn slowly. The film of emulsion was placed over the grids on the slide by gently touching the loop to the slide. The film (from preliminary experiments) following this procedure should contain a monolayer of silver halide grains as required for high resolution microautoradiography. The workable time of the gel in the loop phase is c. 20 sec after which time the emulsion becomes too viscous and no film appears. If the emulsion is insufficiently cooled then the distribution of silver halide grains in the film in the loop becomes uneven.

Once the grids on the slides were coated with emulsion they were placed in slide boxes (9.0 x 7.0 cm) which were wrapped into black 'light proof' paper and finally put into another box and kept at 3°C for 2 months.

E) Development

A physical developer and chemical developer were used. All procedures were carried out in a dark room.

a) Physical developer

Constituents were 1.0 M sodium sulphite and
0.1 M p-phenylenediamine.

Preparation:

Dissolve sodium sulphite in distilled water at 50°C. Add the p-phenylenediamine to give final concentration of 0.1 M. Cool the solution and filter.

b) Chemical developer (D19B)

Constituents were

2.2 g	4 methylamino-phenosulphate
72.0 g	anhydrous sodium sulphite
8.8 g	crystalline hydroquinone
48.0 g	anhydrous sodium carbonate
4.0 g	potassium bromide.

Preparation:

Dissolve chemicals in the order given in 750 ml distilled water at 40°C and then adjust to 1000 ml. Care should be taken to ensure that each chemical is fully dissolved before the next is added.

For development in both the chemical and physical developer the following procedure was adopted. The slide with the grids still attached were put into developer (2 min for chemical; 1 min for physical), then dipped in a stop bath 10 sec (5% acetic acid) and then fixed for 5 min. Finally the grids were washed in running water for 5 min and then put into distilled water for 1 min. During all these procedures the slides were put into coplin jars containing the appropriate chemical.

The grids were then removed from the slides, dried and viewed in an AEI 6B transmission electron microscope.

EXPERIMENTAL RESULTS

(1) Interactions between bean leaves and bacteria

Preliminary experiments were made to determine the response of bean leaves to a variety of bacterial species from a number of genera. In section 3 this information was used to determine if host response, following various treatments, to these bacteria could be altered.

A) Responses of bean leaves to bacteria

Four Red Mexican and four Canadian Wonder plants were each injected with suspensions (3.0×10^8 cells/ml) of washed cells of the following species of bacteria; *Pseudomonas fluorescens*, *P. lachrymans*, *P. mors-prunorum*, *P. phaseolicola* races 1 and 2, *P. solanacearum*, *P. tabaci*, *Erwinia atroseptica*, *E. caratovora*, *Xanthomonas cassava*, *X. manihotis*, *X. phaseoli*. Plants were incubated after inoculation at $23^{\circ}C$ for 14 d. Results are given in Tables 3 and 4.

The most rapid responses occurred with *P. lachrymans*, *P. mors-prunorum*, *P. tabaci* (Table 4). In these cases the inoculated area had collapsed by 18-20 h and was desiccated and brown in colour by 4 d. There was no development of a chlorotic halo around the inoculation area, or of interveinal chlorosis in the trifoliate leaves. The inoculation area did not have a watersoaked margin and an abrupt demarcation separated healthy and necrotic tissue (Plate 1). These bacteria, therefore, caused a typical hypersensitive reaction (HR).

The HR of Red Mexican to *P. phaseolicola* race 1 developed more slowly (Table 4). The inoculation area did

Plate 1. A 3 day old hypersensitive reaction (HR) in Red Mexican (RM) caused by injecting a suspension of P. mors-prunorum (3.0×10^8 cells/ml) into the main veins of the leaf

Table 3. Responses of bean leaves to injection with suspensions of bacteria

Bacterium	Cultivar	
	Red Mexican	Canadian Wonder
Pseudomonas fluorescens	NIL	NIL
Pseudomonas lachrymans	HR	HR
Pseudomonas mors-prunorum	HR	HR
Pseudomonas phaseolicola race 1	HR	SR
Pseudomonas phaseolicola race 2	SR	SR
Pseudomonas solanacearum	NIL	NIL
Pseudomonas tabaci	HR	HR
Erwinia amylovora	NIL	NIL
Erwinia caratovora	NIL	NIL
Xanthomonas cassava	NIL	NIL
Xanthomonas manihotis	NIL	NIL
Xanthomonas phaseoli	SR	SR

Nil - No response
HR - Hypersensitive response
SR - Susceptible response

Table 4. Reaction grades of responses of bean leaves to *Pseudomonas* spp.

Cultivar	Time (d) after inoculation	Reaction Grades				
		Bacterium				
		P. lac	P. m-p	P. phas 1	P. phas 2	P. tab
Red Mexican	1	3	3	2	NIL	3
	2	4	4	3	1	4
	3	4	4	4	3	4
	5	5	5	4	4	5
	7	5	5	5	5	5
	10	5	5	5	6	5
	14	5	5	5	7	5
	16	5	5	5	8	5
Canadian Wonder	1	3	3	NIL	NIL	3
	2	4	4	1	1	4
	3	5	4	3	3	4
	5	5	5	4	4	5
	7	5	5	5	5	5
	10	5	5	6	6	5
	14	5	5	7	7	5
	16	5	5	8	8	5

Table 5. Inoculum thresholds for hypersensitive reactions of bean leaves to Pseudomonas species

Bacterium	Inocula (bacterial cells/ml) 3.0 x 10^8		2.0 x 10^8		1.5 x 10^8		1.0 x 10^8		7.5 x 10^7		6.0 x 10^7		5.0 x 10^7	
	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW
Pseudomonas lachrymans	3	3	3	3	3	3	3	3	3	3	3*	3*	3*	3*
Pseudomonas mors-prunorum	3	3	3	3	3	3	3	3	3	3	3*	3*	3*	3*
Pseudomonas phaseolicola 1	3	–	3	–	3	–	3*	–	3*	–	NIL	–	NIL	–
Pseudomonas tabaci	3	3	3	3	3	3	3	3	3	3	3*	3*	3*	3*

RM Red Mexican
CW Canadian Wonder
3 Hypersensitive response
3* Hypersensitive response but incomplete collapse of inoculation area.

not collapse until after 30 h and desiccation was not complete until 5 d. There was no development of chlorotic haloes or of interveinal chlorosis in the trifoliate leaves.

In Canadian Wonder, P. phaseolicola race 1 caused a susceptible reaction (SR) in which the inoculation area did not collapse until 3 d and desiccation occurred at 6 d. By 7 d a watersoaked margin usually developed around the inoculation area which was itself surrounded after 8-9 d by a chlorotic halo. By 10 d mild interveinal chlorosis of trifoliate leaves had occurred. This usually developed first at leaf margins near the petiole, and in lateral leaflets before terminal leaflets. By 14 d there was pronounced chlorosis of trifoliate leaves. The plant was dwarfed and successive trifoliates were retarded in development. A similar response to P. phaseolicola race 2 was obtained in Red Mexican (RM) and Canadian Wonder (CW) plants.

There was no response in CW or RM to the saprophyte P. fluorescens or to the pathogens P. solanacearum, E. atroseptica, E. caratovora. X. cassava and X. manihotis caused a mild chlorotic discoloration in the inoculation zone.

X. phaseoli caused a rather similar SR in CW and RM as did P. phaseolicola. The inoculation area collapsed 4 d after injection and chlorotic haloes were visible 4 d later. There was some brownish discoloration of the stem and mild chlorosis of trifoliate leaves by 14 d.

B) Inoculum thresholds for resistant reactions of bean leaves to Pseudomonas spp.

The inoculum threshold is the concentration of bacteria

required to give a confluent necrotic lesion within the inoculation area. Thresholds were determined using inocula of decreasing concentrations of bacteria. Four plants each of CW and RM were injected with P. lachrymans, P. morsprunorum, P. phaseolicola race 1, P. tabaci at each inoculum. Plants were incubated at 23°C and results (Table 5) taken 30 h after inoculation show that all HR had an inoculum threshold of c. 6.8×10^7 cells/ml except that caused by P. phaseolicola race 1, which was c. 1.3×10^8 cells/ml.

To ensure a well developed HR a concentration of 2.6×10^8 cells/ml was used for all subsequent bacterial injections unless stated otherwise.

(2) Effects of inhibitors on the hypersensitive and susceptible responses

A) Effects of inhibitors on the hypersensitive response

Klement (1971) divided the hypersensitive response (HR) into three periods - induction, latent and collapse period. The induction period is defined as being dependent on the bacterium but independent of the host and has been shown to vary from between 25 min (Klement and Goodman, 1967) to 3 h (Sequeira, 1976). The latent period is dependent on the host but independent of the bacterium and the collapse period is the time when permeability of host cells increases sharply. Use of specific inhibitors of particular steps in enzyme synthesis is a standard method for determining whether enhanced activities of enzymes is due either to de novo synthesis or to formation from pre-existing protein.

De novo synthesis of metabolites follows the pathway - DNA —— RNA —— PROTEIN since the production of the new metabolite has to be derived from transcription of a gene previously 'dormant'. By blocking HR at one of the points in this pathway with inhibitors used at concentrations not sufficient to reduce bacterial numbers below the inoculum threshold, we may infer that the reaction is dependent on de novo synthesis.

In these experiments, the cultivar Red Mexican was used exclusively in conjunction with P. mors-prunorum and P. phaseolicola race 1. Both bacteria cause a HR in Red Mexican (RM).

Responses in RM were noted 24 h after injection of P. mors prunorum and 30 h after injection of P. phaseolicola at which time in HR collapse of the tissue in the inoculation zone is complete. Reaction grade 3 represents this collapse. The inoculum thresholds are 6.8×10^7 and 1.4×10^8 cells/ml respectively for P. mors-prunorum and P. phaseolicola. The inoculum concentration used for both bacteria was 2.6×10^8 cells/ml.

a) Effects of inhibitors on the induction period

The induction period was determined in the following manner. Six RM plants were used in conjunction with P. mors-prunorum and P. phaseolicola, and with rifampicin and chloramphenicol 15 µg/ml. Rifampicin specifically inhibits RNA transcription in bacteria by binding to the DNA dependent RNA polymerase. Chloramphenicol specifically inhibits protein synthesis in bacteria by binding to the

50S ribosomal subunit. Both antibiotics at these concentrations are highly specific for procaryotic systems.

Bacterial suspensions were injected into monofoliate leaves at the junction of the petiole and lamina followed 60, 90, 120, 150, 180, 240 min later by solutions of an inhibitor into the same areas. Plants were then incubated in a controlled environment at $23^{o} \pm 1^{o}C$. Results (Table 6) show that association between host cells and bacteria for 150 min or longer led to the development of HR irrespective of addition of inhibitor.

Table 6. Determination of the induction period of the hypersensitive response in Red Mexican to Pseudomonas mors-prunorum and Pseudomonas phaseolicola race 1

Time (min) between injection of bacteria and inhibitor	Response[a]	
	Rifampicin (15 µg/ml)	Chloramphenicol (15 µg/ml)
60	NIL	NIL
90	NIL	NIL
120	NIL	NIL
150	3*	3*
180	3	3
210	3	3
240	3	3

a. NIL - No response

3 - HR

3* - Incomplete collapse of inoculation area.

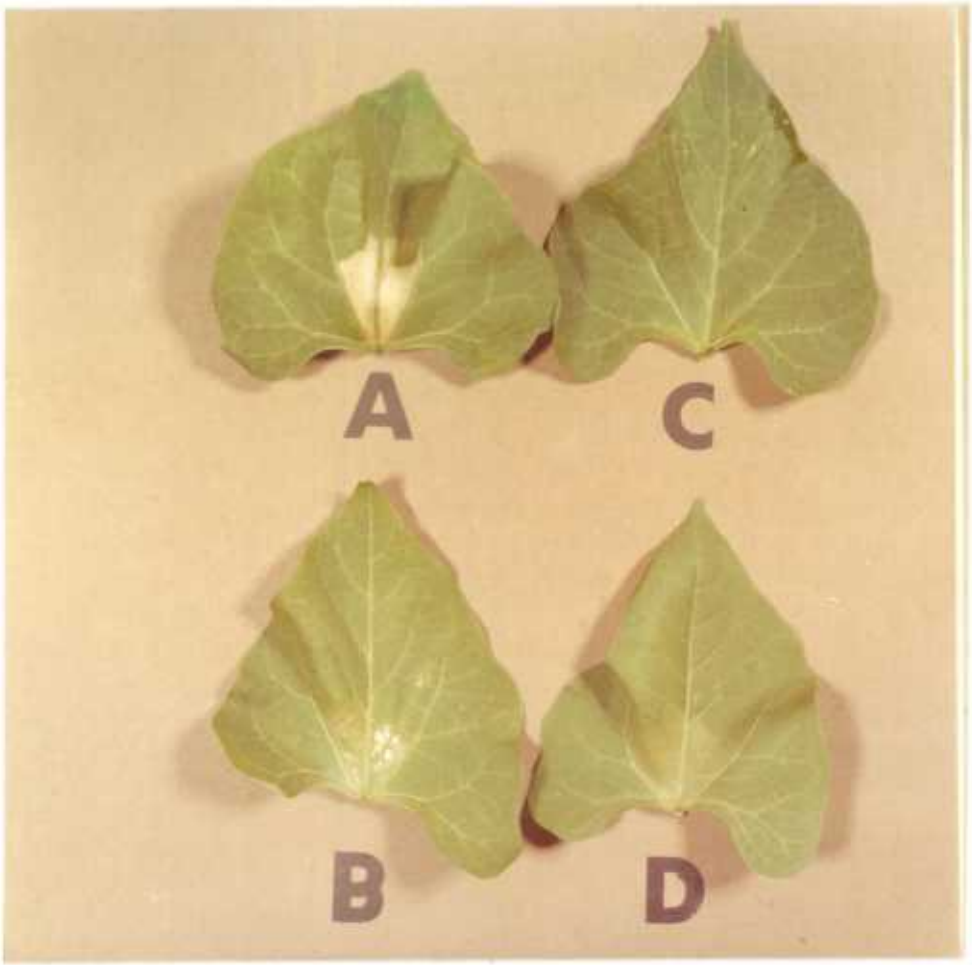


Plate 2. Effect of rifampicin on HR

A. A RM leaf showing confluent necrosis (HR) of the inoculation area caused by injection of P. mors-prunorum (2.6×10^8 cells/ml)

B. A RM leaf showing partial necrosis of the inoculation area caused by injection of P. mors-prunorum (5.0×10^7 cells/ml)

C. A RM leaf following injection of water

D. A RM leaf following simultaneous injection of P. mors-prunorum (2.6×10^8 cells/ml) and rifampicin (15 µg/ml)

Table 7. Mode and site of action of various inhibitors

Ampicillin	Inhibits the synthesis of the peptidoglycan component of bacterial cell walls by blocking the incorporation of UDP-acetylmuramyl-pentapeptide. Affects only growing bacterial cells. No effect on plants.
Actinomycin D	Inhibits transcription in eucaryotic and procaryotic systems. Acts by binding to G-C bases on DNA template. Tends to be more specific against m-RNA than r-RNA.
Chloramphenicol	Inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit. Specific to 70S systems of procaryotes i.e. does not inhibit protein synthesis on 80S ribosomes in plants.
Cycloheximide	Inhibits protein synthesis in plants, fungi, mammals but not in bacteria, and works by inhibiting the transfer reaction in peptide bond formation.
D-Cycloserine	Inhibits the synthesis of the peptidoglycan component of bacterial cell walls by blocking the incorporation of the dipeptide D-alanyl-D-alanine. No effect on plants.

Table 7 cont'd.

Erythromycin	Mode of action similar to chloramphenicol, i.e. inhibits at the 50S ribosomal subunit. Specific to 70S systems of procaryotes.
5-Fluorouracil	Enters m-RNA during transcription and acts by producing new proteins which are altered with respect to amino acid composition. Acts on procaryotic and eucaryotic systems.
Puromycin	Inhibits protein synthesis in eucaryotic and procaryotic systems. Acts by inducing release of peptides from ribosomes, by competing with the incoming t-RNA. Competes with chloramphenicol and erythromycin for site of action.
Rifampicin	Inhibits RNA transcription in procaryotic systems by binding to the DNA dependent RNA polymerase.
Spectinomycin	Inhibits protein synthesis in procaryotic systems. Site of action, 30S ribosomal subunit. Effects can be reversed by washing the cells.

Table 8. Effect of inhibitors of RNA and protein synthesis on the development of the hypersensitive response in Red Mexican to Pseudomonas mors-prunorum

Inhibitor	Molarity at 125 µg/ml	Reaction Grade[a] Concentration inhibitor (µg/ml)					
		125	60	30	15	5	1
Actinomycin D	9.0×10^{-5}	3[b]	3	3	3	3	3
Chloramphenicol	4.0×10^{-4}	NIL	NIL	NIL	NIL	3	3
Cycloheximide	4.5×10^{-4}	3	3	3	3	3	3
Erythromycin	1.7×10^{-4}	Effective at 800 µg/ml. Primarily a Gram + ve bacterial inhibitor.					
5-Fluorouracil	9.5×10^{-4}	NIL	NIL	3*	3	3	3
Puromycin	2.6×10^{-4}	NIL	3*	3	3	3	3
Rifampicin	1.7×10^{-4}	NIL	NIL	NIL	NIL	3	3
Spectinomycin	3.7×10^{-4}	NIL	NIL	NIL	3*	3	3

[a] - Mean of six plants

[b] Nil No response

3 - HR

3* - Incomplete collapse of inoculated area

Table 9. Effect of inhibitors of RNA and protein synthesis on the development of the hypersensitive response in Red Mexican to Pseudomonas phaseolicola race 1

Inhibitor	Molarity at 125 µg/ml	Reaction Grade[a] Concentration inhibitor (µg/ml)					
		125	60	30	15	5	1
Actinomycin D	9.0×10^{-5}	3[b]	3	3	3	3	3
Chloramphenicol	4.0×10^{-4}	NIL	NIL	NIL	NIL	3*	3
Cycloheximide	4.5×10^{-4}	3	3	3	3	3	3
Erythromycin	1.7×10^{-4}	Effective at 800 µg/ml. Primarily a Gram + ve bacterial inhibitor.					
5-Fluorouracil	9.5×10^{-4}	NIL	NIL	NIL	3*	3	3
Puromycin	2.6×10^{-4}	NIL	3*	3	3	3	3
Rifampicin	1.7×10^{-4}	NIL	NIL	NIL	NIL	3*	3
Spectinomycin	3.7×10^{-4}	NIL	NIL	NIL	3*	3	3

a - Mean of 6 plants/12 leaves

b NIL - No response

3 - HR

3* - incomplete collapse of inoculated area.

Results (Table 6) show that the induction period is therefore c. 3 h. After this time, host response as HR becomes independent of the bacterium (Klement, 1971).

In Plate 2 leaves are shown in which inhibition of HR is compared with a normal HR, an HR at threshold level and with water injection.

Two of the most specific inhibitors of RNA and protein synthesis used in this study were rifampicin and chloramphenicol. Both prevented development of HR. The mode of action of other inhibitors, of host and bacterial systems, used in this experiment is given in Table 7. Tables 8, 9 and 14 summarise the results obtained when they were injected into monofoliate leaves of RM simultaneously with the bacteria. Results were recorded as reaction grades and taken 24 and 30 h after injection of P. mors-prunorum and P. phaseolicola respectively.

The results in Tables 8 and 9 show that each of the bacterially specific inhibitors of RNA and protein synthesis prevented development of HR. Actinomycin D and cycloheximide were the only two inhibitors of RNA and protein synthesis which failed to inhibit HR. It can also be seen that concentrations of inhibitor needed to inhibit HR are almost identical for P. mors-prunorum and P. phaseolicola. To prove that de novo synthesis was implicated in preventing the HR it was necessary to show that concentrations of bacteria in the leaf had not been reduced to below the level required to give confluent collapse of the inoculated area (inoculum threshold). This was done by counting the numbers of viable bacteria in the areas of leaves in which HR

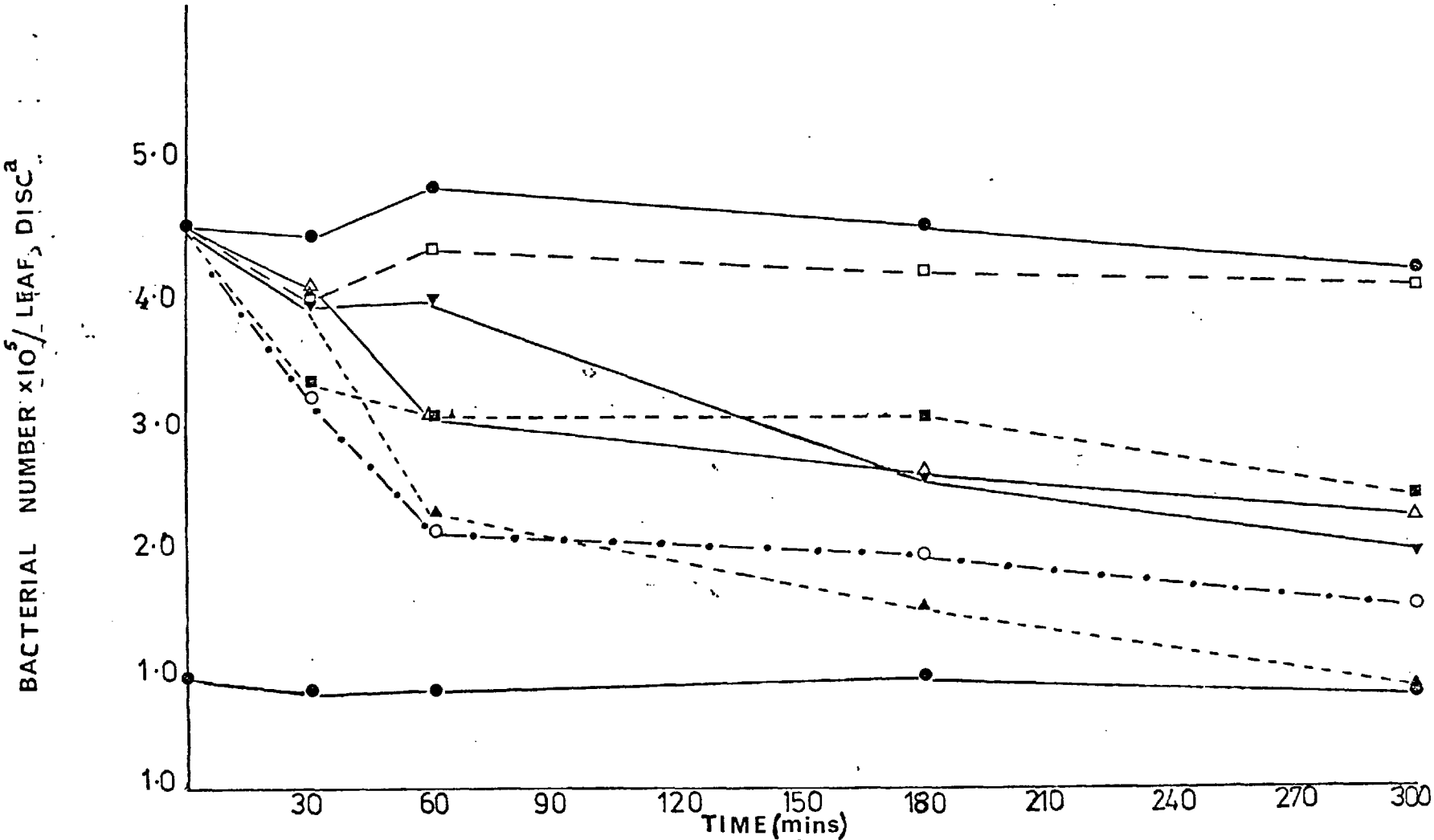


Figure 1. Effect of inhibitors of RNA and protein synthesis on numbers of P. mors-prunorum in leaves during the induction period. (All S.E.M. at 180 min 0.1). [a] Leaf disk 4.5 mm diam. (●——●) Control 1. Standard Inoculum (SI) 2.6 x 10^8 cells/ml P. mors-prunorum; (●——●) Control 2. Threshold Inoculum required to cause HR 6.8 x 10^7 cells/ml; (□- - -□) Chloramphenicol 15 µg/ml & SI; (■- - -■) Erythromycin 1000 µg/ml & SI; (△——△) 5-Fluorouracil 60 µg/ml & SI; (▲- - -▲) Puromycin 125 µg/ml & SI; (○—·—○) Rifampicin 15 µg/ml & SI; (▼——▼) Spectinomycin 30 µg/ml & SI.

had been prevented by the inhibitors (Materials and Methods 3). The results are expressed in Figure 1 as numbers/leaf disk and cover the first 5 h after injecting leaves with P. mors-prunorum. The induction period was previously shown to be c. 3 h (Table 6). The results in Figure 1 show that each of the inhibitors at concentrations which prevent the HR, do not reduce the bacterial numbers below levels needed to give a confluent necrotic lesion in the absence of inhibitors.

The effect of a wide range of concentrations of chloramphenicol and rifampicin on growth of P. phaseolicola race 1 over 24 h in vitro and in vivo was also determined. For in vitro studies, P. phaseolicola race 1 (3.0×10^8 cells/ml) was incubated with varying concentrations of inhibitors in a minimal medium on a reciprocal shaker. At 3, 12, 24 h periods later, 1 ml samples were removed and numbers of bacteria determined (Materials and Methods 3).

Results are given in Table 10 as bacterial numbers/ml. For in vivo studies, P. phaseolicola race 1 (3.0×10^8 cells/ml) was injected with varying concentrations of inhibitors into leaves of RM. At various time intervals leaf disks (4.5 mm diam) were removed from the inoculation area and numbers of viable bacteria determined (Materials and Methods 3). Results are given in Table 11 as bacterial numbers/leaf disk.

From results in Tables 10 and 11, it can be seen that of the two inhibitors rifampicin is the more bacteriocidal. From in vitro studies, bacterial numbers with the highest concentration of rifampicin (125 µg/ml) have fallen by c.

Table 10. Effect of chloramphenicol and rifampicin on the growth of P. phaseolicola race 1 in minimal medium

Time (h)	Inhibitor	Bacterial Number/ml					
		Concentration of inhibitor (μg/ml)					
		0	5	15	30	60	125
3	Chloramphenicol	a & b 6.6×10^8	5.5×10^8	4.9×10^8	3.6×10^8	3.5×10^8	3.2×10^8
	Rifampicin		7.4×10^8	1.1×10^8	6.0×10^7	5.7×10^6	1.2×10^6
12	Chloramphenicol	2.6×10^9	2.6×10^8	2.9×10^8	2.8×10^8	2.0×10^8	1.8×10^8
	Rifampicin		1.7×10^9	8.9×10^7	2.0×10^7	2.7×10^5	2.2×10^5
24	Chloramphenicol	3.2×10^9	2.8×10^8	3.1×10^8	2.0×10^8	1.2×10^8	4.4×10^7
	Rifampicin		2.5×10^9	8.2×10^7	2.3×10^7	4.5×10^4	4.3×10^4

a Bacterial numbers/ml at time 0 was 3.0×10^8 cells/ml

b Mean of six replicates

Table 11. Effect of chloramphenicol and rifampicin on the growth of P. phaseolicola race 1 leaves of Red Mexican

Time (h)	Inhibitor	Bacterial numbers/leaf disk[a]					
		Concentration of inhibitor (μg/ml)					
		0	5	15	30	60	125
0	Chloramphenicol (C)	6.0×10^5[b]	5.2×10^5	5.2×10^5	5.5×10^5	5.0×10^5	4.7×10^5
	Rifampicin (R)		3.5×10^5	3.2×10^5	3.0×10^5	2.0×10^5	1.5×10^5
1	C	5.0×10^5	5.2×10^5	5.2×10^5	5.0×10^5	4.0×10^5	4.2×10^5
	R		3.0×10^5	1.9×10^5	1.7×10^5	1.2×10^5	1.1×10^5
3	C	5.2×10^5	5.0×10^5	6.0×10^5	5.6×10^5	4.0×10^5	2.5×10^5
	R		3.0×10^5	1.8×10^5	9.2×10^4	1.0×10^5	6.2×10^4
5	C	5.0×10^5	4.7×10^5	5.0×10^5	5.2×10^5	5.5×10^5	2.5×10^5
	R		1.6×10^5	1.0×10^5	7.5×10^4	7.0×10^4	3.5×10^4
8	C	5.2×10^5	4.0×10^5	4.1×10^5	4.1×10^5	4.4×10^5	2.4×10^5
	R		1.1×10^5	5.0×10^4	5.0×10^4	3.0×10^4	2.2×10^4

Table 11 cont'd.

Time (h)	Inhibitor	Bacterial numbers/leaf disk[a]					
		Concentration of inhibitor (μg/ml)					
		0	5	15	30	60	125
12	C	5.7×10^5	4.2×10^5	4.4×10^5	4.1×10^5	3.6×10^5	2.2×10^5
	R		9.2×10^4	6.0×10^4	4.7×10^4	3.0×10^4	2.5×10^4
16	C	6.2×10^5	5.0×10^5	4.4×10^5	4.2×10^5	3.5×10^5	2.2×10^5
	R		7.0×10^4	7.0×10^4	5.2×10^4	2.4×10^4	2.4×10^4
20	C	6.0×10^5	4.2×10^5	4.1×10^5	3.7×10^5	2.2×10^5	2.3×10^5
	R		7.5×10^4	4.5×10^4	3.2×10^4	2.3×10^4	1.7×10^4
24	C	7.2×10^5	5.5×10^5	4.0×10^5	3.5×10^5	1.9×10^5	1.5×10^5
	R		5.2×10^4	4.8×10^4	2.4×10^4	2.1×10^4	6.5×10^3

a Leaf disk (4.5 mm diam)

b Mean 6 replicates

10,000 x after 24 h while with chloramphenicol at its highest concentration bacterial numbers have fallen by only c. 100 x. A similar trend is shown in vivo. At the highest concentration of rifampicin after 24 h numbers have fallen by c. 100 x while with chloramphenicol it is only c. 5 x.

With chloramphenicol and rifampicin at 15 μg/ml (the concentration required to inhibit HR) bacterial numbers in vitro and in vivo fall more slowly. In vitro after 24 h, numbers have fallen by c. 10 x and c. 15 x for chloramphenicol and rifampicin respectively but over the first 3 h (time required for HR induction) numbers have fallen by c. 0.5 x and c. 6 x for chloramphenicol and rifampicin respectively. In vivo numbers have fallen c. 1 x and c. 15 x over 24 h but over 3 h there is no fall with chloramphenicol and only c. 3 x fall with rifampicin. This pattern of reduction in bacterial numbers in vivo is also shown in Figure 1.

Two of the inhibitors used in this work, 5-fluorouracil (5-FU) and puromycin, tend to be non bacterially specific in that they affect both eucaryotic and procaryotic systems. Both inhibitors prevented HR from occurring. It thus becomes necessary to show that the inhibitors were preventing HR by working on the bacterial and not the plant system.

Puromycin causes nascent release of peptides from - RNA bound to ribosomes. This reaction can itself be inhibited by addition of erythromycin or chloramphenicol (Cundliffe and McQuillen, 1967; Traut and Monro, 1964).

Table 12. Determination of the site of action of puromycin in Red Mexican to P. mors-prunorum[c]

Treatment	Response [a,b]
Chloramphenicol 4 µg/ml and puromycin 60 µg/ml and bacteria [c].	NIL
Erythromycin 500 µg/ml and puromycin 60 µg/ml and bacteria.	NIL
Chloramphenicol 4 µg/ml and bacteria.	3
Erythromycin 500 µg/ml and bacteria.	3
Puromycin 60 µg/ml and bacteria.	3*

a Mean of 4 replicates

b NIL - No response
3 - HR
3* - Incomplete collapse of inoculation area

c P. mors-prunorum (2.6 x 10^8 cells/ml)

Table 13. Inhibitor activity in relation to a time sequence in Red Mexican to P. mors-prunorum[c]

	Reaction Grade [a]				
Inhibitor	Inhib. 24 h then bacteria	Inhib. 4 h then bacteria	Inhib. and bacteria	Bacteria 1 h then inhib.	Bacteria 4 h then inhib.
5 FU (100 µg/ml)	3[b]	3*	NIL	NIL	3
Puromycin (250 µg/ml)	3	3*	NIL	NIL	3

a Mean of 4 replicates

b NIL - No response
3 - HR
3* - Incomplete collapse of inoculation area

c P. mors-prunorum (2.6 x 10^8 cells/ml)

The inference is that as erythromycin and chloramphenicol both inhibit protein synthesis at the 50S ribosomal sub-unit then the aminoacyl site becomes occluded, hence preventing access to the site from which puromycin interacts with the nascent peptide chain. Thus by injecting erythromycin or chloramphenicol with puromycin at levels below which they inhibit HR together with the bacterium (2.6×10^8 cells/ml) it should be possible to determine whether puromycin is acting at the 70S or 80S ribosomal level, since both erythromycin and chloramphenicol are specific for 70S ribosomal inhibition. The results are expressed in Table 12.

From Table 12 it can be seen that inhibition of HR occurs when chloramphenicol and puromycin or erythromycin and puromycin are injected together at individual sub inhibitory levels with the P. mors-prunorum. From Table 8 the concentration of chloramphenicol, erythromycin and puromycin needed to inhibit HR was 15, 800 and 125 µg/ml respectively. Therefore inhibition of HR in Table 12 could only be achieved when the inhibitors are complementing each other and since erythromycin and chloramphenicol are specific to 70S protein inhibition then puromycin must also be inhibiting HR at the 70S ribosome i.e. working on the bacterial system.

The experiment can be done differently as illustrated in Table 13 for both puromycin and 5-FU when a time sequence is adopted between 1st and 2nd injections. In this case the inhibitor at concentrations which inhibits HR (Table 8) was injected into the leaf. After a set time

interval P. mors-prunorum was injected into the same area of the leaf. In two combinations the bacterial injection preceded that of inhibitor and in one case bacteria and inhibitor were injected simultaneously. Results from Table 13 show that HR inhibition occurs only during the period of bacterial induction; that is c. $2\frac{1}{2}$ h after the bacteria are injected into the leaf. If 5-FU or puromycin are injected 4 h after the bacterium then no inhibition of HR occurs. Failure of 5-FU and puromycin to inhibit HR when injected 24 h before the bacterium is thought to be due to a mobilisation of the inhibitor from the intercellular space at the site of injection.

Thus from Tables 12 and 13 it can be seen that puromycin and 5-FU inhibit HR by acting on the bacterium and not the host.

The effects of bacterially specific inhibitors with sites of action other than RNA and protein synthesis were investigated. Ampicillin and D-cycloserine both inhibit the synthesis of peptidoglycan, a component of the bacterial cell wall, and only act on growing cells. The effects of these inhibitors when injected simultaneously with P. mors-prunorum (2.6×10^8 cells/ml) into leaves of RM is given in Table 14. Results from Table 14 show that neither inhibitor at concentration of 1000 µg/ml inhibited the HR. To show that these inhibitors could gain access to the bacterial cell, the effect of ampicillin and D-cycloserine on the growth of P. mors-prunorum in vitro was determined (Figure 2). This was done by incubating the bacterium (2.6×10^8 cells/ml) and inhibitor (1000 µg/ml) in a minimal medium on a reciprocal shaker.

Table 14. Effect of inhibitors of bacterial cell wall synthesis on the development of the hypersensitive response in Red Mexican to P. mors-prunorum

Inhibitor	Molarity at 125 µg/ml	Reaction Grade [a] Concentration of inhibitor (µg/ml) 1000	500	250	125	60	30
Ampicillin	3.1×10^{-4}	3 [b]	3	3	3	3	3
D-Cycloserine	1.2×10^{-3}	3	3	3	3	3	3

a Mean of six replicates

b 3 - HR

Table 15. Effect of ribonuclease and trypsin on the development of the hypersensitive response in Red Mexican to P. mors-prunorum

Enzyme	Reaction Grade [a] Enz.[c] 24 h then bacteria	Enz. 4 h then bacteria	Enz. and bacteria	Bacteria 1 h then enz.	Bacteria 4 h then enz.
Bovine RNAase (1000 µg/ml)	3 [b]	3	3*	3	3
Bovine trypsin (1000 µg/ml)	3	3*	3*	3*	3

a Mean of six replicates

b 3 - HR
3* - incomplete collapse of inoculated area

c Enz - enzyme

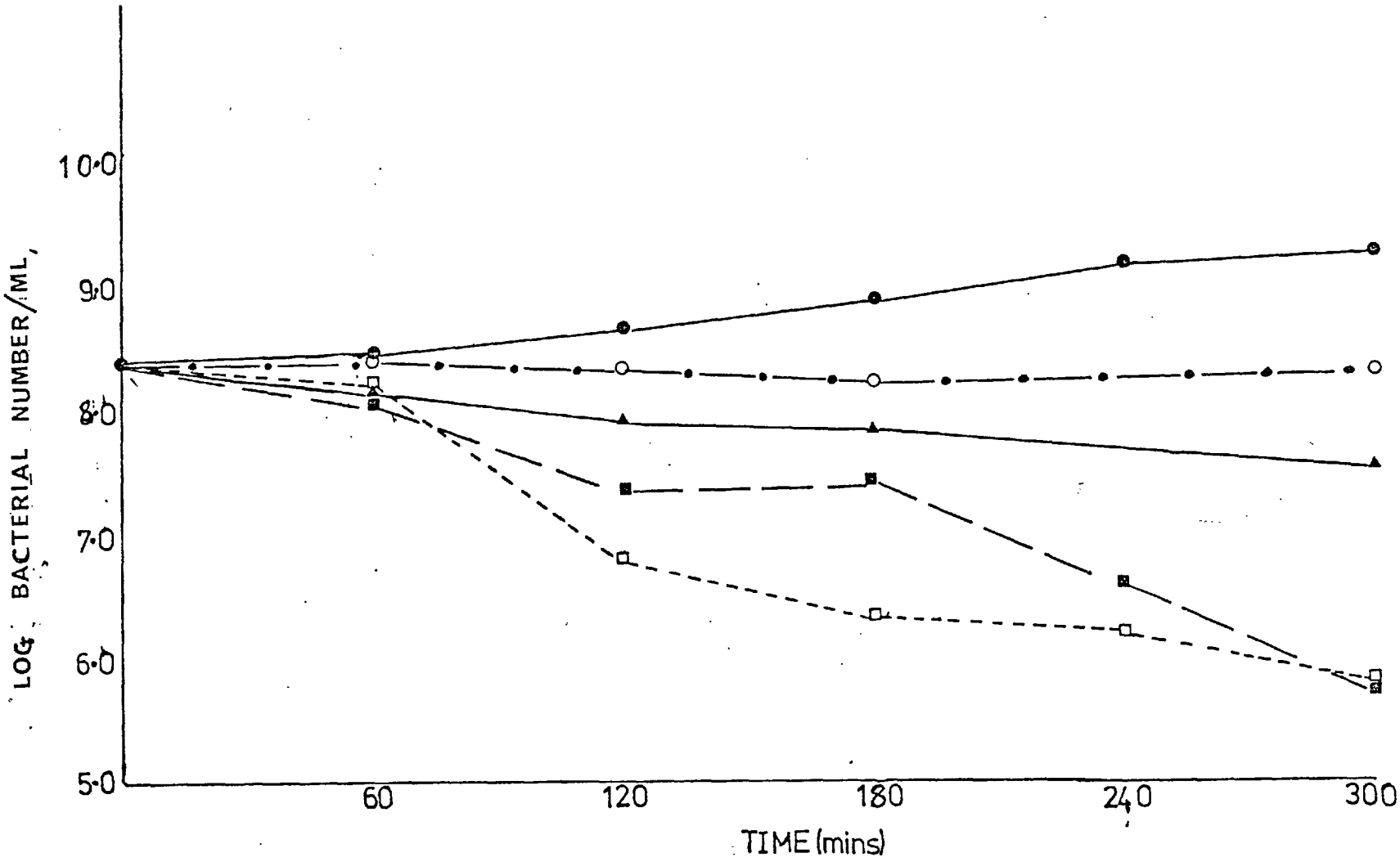


Figure 2. Effect of inhibitors of RNA, protein and cell wall synthesis on numbers of P. mors-prunorum in minimal medium during the induction period. (●——●) Control, Standard Inoculum (SI) 2.6 x 10^8 cells/ml P. mors-prunorum; (○—·—○) Chloramphenicol 15 µg/ml & SI; (▲——▲) Rifampicin 15 µg/ml & SI; (■ ■) D-Cycloserine 1000 µg/ml & SI; (□----□) Ampicillin 1000 µg/ml & SI.

At periods 1, 2, 3, 4 and 5 h later, 1 ml samples were withdrawn and bacterial viability assessed using dilution plating (Materials and Methods 3). For comparative purposes bacterial viability with HR inhibitory concentrations of chloramphenicol and rifampicin (15 μg/ml) were also determined. Results are given as bacterial numbers/ml.

Figure 2 shows that ampicillin and D-cycloserine after 5 h, had reduced bacterial numbers at least 25% more quickly than did concentrations of rifampicin and chloramphenicol which inhibited HR. Thus the inability of ampicillin and D-cycloserine to inhibit HR was not due to lack of penetration of the inhibitor into the bacterial cell.

If de novo synthesis of a bacterial metabolite is implicated in induction of HR then a nuclease or protease might inhibit the development of HR. Assuming de novo synthesised protein is involved in HR induction then as it passes from the bacteria into plant cells it might be susceptible to proteolytic enzyme attack in the intercellular spaces. This possibility was investigated. Solutions of bovine RNAase and trypsin were made up in phosphate buffer pH 7.3. The pH for optimum activity is pH 7.6 for trypsin and pH 7.0 for RNAase. The enzymes were used on a time sequence as for Table 13, and results are given in Table 15. Neither enzyme was phytotoxic to leaves of RM at 1000 μg/ml in the absence of the bacterium. Results from Table 15 show that neither enzyme inhibited completely the development of HR, though of the two protease had the greater effect.

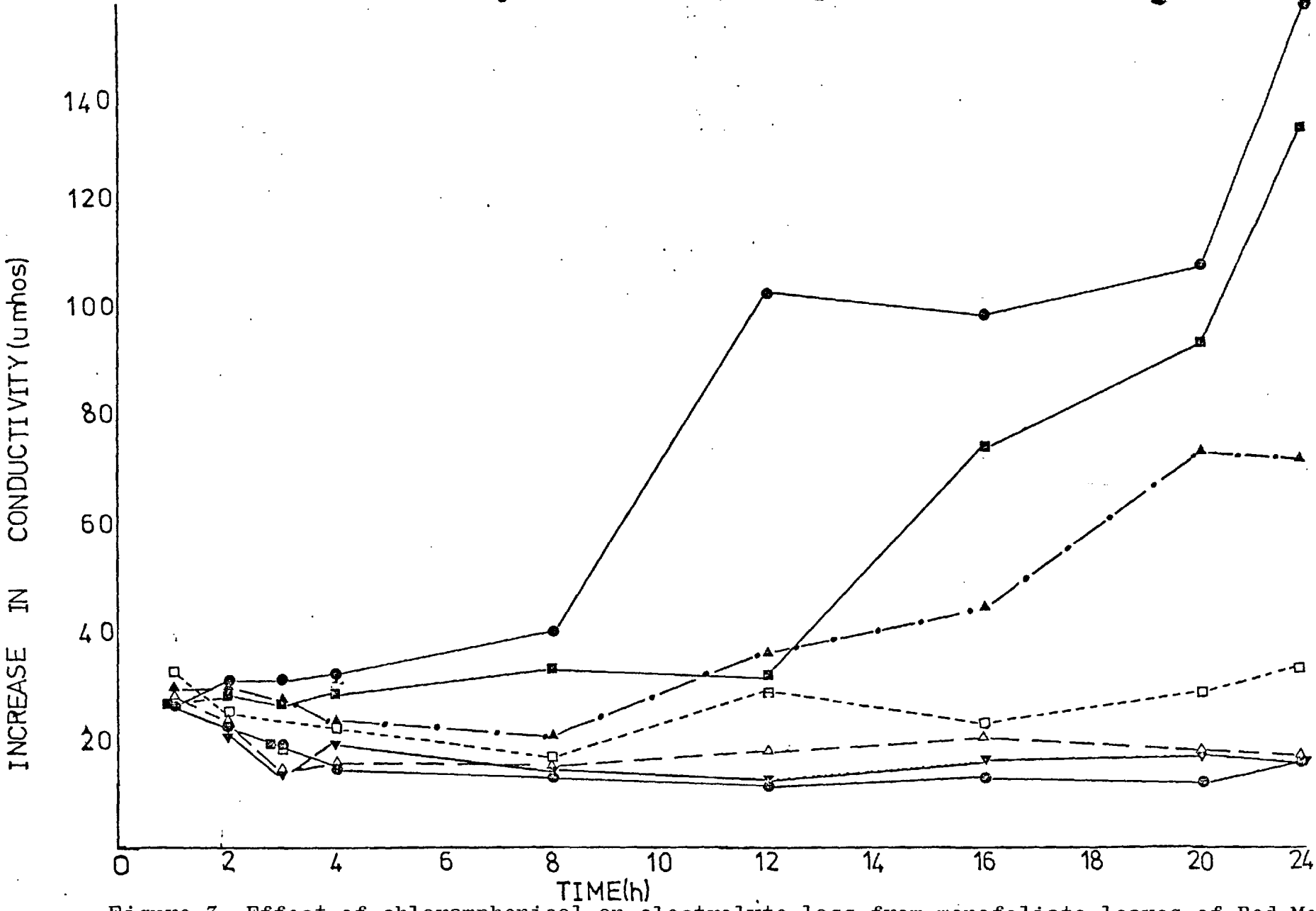


Figure 3. Effect of chloramphenicol on electrolyte loss from monofoliate leaves of Red Mexican following injection of P. mors-prunorum. 3-Collapse of inoculation area (HR). (●——●) Control I, Standard Inoculum (SI) 2.6 x 10^8 cells/ml P. mors-prunorum; (●——●) Control II, Chloramphenicol (CHL) 60 µg/ml; (▼——▼) Control III, Water; (■——■) CHL 5 µg/ml & SI; (▲-·-▲) CHL 15 µg/ml & SI; (□- - -□) CHL 30 µg/ml & SI; (△— —△) CHL 60 µg/ml & SI.

b) Effect of chloramphenicol on electrolyte loss from host cells (collapse period), following injection of P. mors-prunorum

An increase in permeability of host cells concomitant with the collapse of the inoculated area of the leaf is a characteristic feature of the collapse period of HR. (Cook and Stall, 1968; Goodman, 1968). In this experiment chloramphenicol was used over a wide range of concentrations in conjunction with P. mors-prunorum (2.6×10^8 cells/ml). The bacteria and antibiotic were injected simultaneously into the leaves of RM, and at various time intervals leaf disks (5.5 mm diam) were removed and put into de-ionized water and the conductivity taken. After 1 h conductivity of the water was retaken and results in Figure 3 are expressed as increases of conductivity in μmho. Collapse of leaf tissue in the inoculated area was evident at 16 h and is shown on Figure 3 as reaction grade 3.

Results from Figure 3 show that there was little release of ions from the host cells at concentrations of the inhibitor which prevented development of HR. However, the release of ions when the bacteria were injected with 15 μg/ml of chloramphenicol seems high in view of the complete inhibition of HR expressed at this concentration. Injection of water or chloramphenicol (60 μg/ml) without bacteria had little effect on electrolyte loss from leaf cells.

B) Effects of inhibitors on the susceptible reaction

The cultivar Canadian Wonder was used exclusively in

conjunction with the bacteria Pseudomonas phaseolicola races 1 and 2. An inoculum of 2.6×10^8 cells/ml was used throughout. Both bacteria caused susceptible reactions (SR) in this cultivar. Responses were noted fourteen days after injection of the bacteria, at which time in a normal susceptible reaction collapse of the inoculation zone in monofoliate leaves is complete and chlorosis of the trifoliates is pronounced. These stages are represented by the reaction grades 5 and 7.

a) Effect of chloramphenicol and rifampicin on the susceptible reaction

Experiments were set up to determine what effect a bacterially specific inhibitor of RNA (rifampicin) and inhibitor of protein (chloramphenicol) would have on the SR.

Monofoliate leaves of CW were injected with varying concentrations of either rifampicin or chloramphenicol together with the standard inoculum of bacteria. In Table 16, the effect of chloramphenicol and rifampicin on the collapse of the inoculation area over 336 h (14 d) after bacterial injection is shown, and in Table 17 the effect of the inhibitors on the development of chlorosis of trifoliate leaves by 14 d is shown. Reaction grades 3-5 represent the various stages of dehydration in the inoculation area following collapse of leaf tissue.

Results from Table 16 show that the inoculation areas on monofoliate leaves in a normal SR collapsed by three days (72 h). When the bacteria and chloramphenicol or rifampicin above 15 µg/ml were injected, the inoculation

Table 16. Effect of chloramphenicol and rifampicin on the development of the susceptible reaction in Canadian Wonder to Pseudomonas phaseolicola race 2

Time (h) after bacterial inoculation	Inhibitor	Molarity at 125 µg/ml	Reaction Grade [a] Concentration of inhibitor (µg/ml) 125	60	30	15	5	1	0
72	Chloramphenicol	4.0×10^{-4}	NIL[b]	NIL	NIL	2	3	3	3
144			3	3	3	3	5	5	5
216			4	4	4	4	5	5	5
288			5	5	5	5	5	5	5
336			5	5	5	5	5	5	5
72	Rifampicin	1.7×10^{-4}	NIL	NIL	NIL	NIL	3	3	3
144			NIL	NIL	2	3	5	5	5
216			NIL	3	3	4	5	5	5
288			NIL	4	4	5	5	5	5
336			NIL	5	5	5	5	5	5

a 4 plants/treatment

b NIL - no response
2 - light coloration in inoculation area
3 - collapse of inoculation area which is white/bronze in colour
4 - inoculation area light brown in colour
5 - " " mid brown " "

Table 17. Effect of chloramphenicol and rifampicin on the development of the susceptible reaction in Canadian Wonder to Pseudomonas phaseolicola

Bacterium	Inhibitor	Reaction Grade [a] Molarity at 125 µg/ml	Concentration of inhibitor (µg/ml) 125	60	30	15	5	1	0
Pseudomonas phaseolicola race 1	Chloramphenicol	4.0×10^{-4}	6[b]	7	7	7	7	7	7
	Rifampicin	1.7×10^{-4}	NIL	6	6	7	7	7	7
Pseudomonas phaseolicola race 2	Chloramphenicol	4.0×10^{-4}	6	7	7	7	7	7	7
	Rifampicin	1.7×10^{-4}	NIL	6	6	6	7	7	7

a 4 plants/treatment. Results taken 14 d (336 h) after bacterial injection

b NIL - no response

6 - mild interveinal chlorosis of trifoliate leaves

7 - severe " " " " "

areas did not collapse by 72 h. With chloramphenicol at the highest concentration, collapse of the inoculation area was complete by 144 h, but with rifampicin at the highest concentration collapse did not occur at all by 336 h. With rifampicin at 60 and 30 μg/ml collapse occurred by 216 h. Thus, the general effect of chloramphenicol and rifampicin was to delay the collapse of the inoculation area.

Results from Table 17 show that the overall effect of chloramphenicol and rifampicin by fourteen days (336 h), and particularly on development of chlorosis of trifoliate leaves, was less pronounced. The trifoliate leaves following monofoliate injection of bacteria and inhibitor all showed mild (reaction grade 6) to severe (reaction grade 7) chlorosis with the exception of when rifampicin at 125 μg/ml was injected. In this case no chlorosis of the trifoliate leaves developed. The development of chlorotic symptoms in trifoliate leaves was similar when monofoliates were injected with either P. phaseolicola race 1 or race 2.

The effect of chloramphenicol and rifampicin on the growth of P. phaseolicola race 2, over a fourteen day period, in the inoculation area in leaves of CW was determined. This was done by counting the numbers of viable bacteria when in the leaf together with the inhibitor. The method employed for counting bacterial numbers in vivo (dilution plating) has already been described (Materials and Methods 3). Results are expressed in Tables 18 and 19 as bacterial numbers/leaf disk.

Table 18. Effect of chloramphenicol on growth of P. phaseolicola race 2 in leaves of Canadian Wonder

Concentration of inhibitor (µg/ml)	Bacterial numbers/leaf disk [a]							
	Time (h)							
	0	12	24	48	96	192	288	386
0	5.5×10^5 [b]	4.5×10^6	3.0×10^6	7.2×10^6	7.8×10^6	4.2×10^7	7.0×10^6	7.0×10^6
60	4.8×10^5	3.5×10^6	3.0×10^5	1.1×10^6	6.5×10^6	4.7×10^7	7.0×10^6	6.0×10^6
125	3.5×10^5	2.4×10^5	1.8×10^5	9.0×10^5	7.5×10^6	7.5×10^7	1.2×10^7	8.0×10^6

a Leaf disk 4.5 mm diam

b Mean of 6 replicates

Table 19. Effect of rifampicin on growth of P. phaseolicola race 2 in leaves of Canadian Wonder

Concentration of inhibitor (µg/ml)	Bacterial numbers/leaf disk [a] Time (h) 0	12	24	48	96	192	288	386
0	5.5 x 10^5 [b]	4.5 x 10^5	3.0 x 10^6	7.2 x 10^6	8.0 x 10^6	4.2 x 10^7	7.0 x 10^6	7.0 x 10^6
60	2.1 x 10^5	2.5 x 10^4	1.2 x 10^4	5.6 x 10^4	8.5 x 10^3	3.0 x 10^4	7.5 x 10^5	5.2 x 10^5
125	1.4 x 10^5	1.8 x 10^4	3.8 x 10^3	6.5 x 10^3	1.0 x 10^4	1.2 x 10^4	4.2 x 10^3	8.5 x 10^3

a Leaf disk 4.5 mm diam

b Mean of six replicates

In SR the inoculation area enlarges in a radial manner usually visible as a watersoaked margin and chlorotic halo. An attempt was also made to determine the numbers of bacteria at the edge of the inoculation area during the period 96-386 h after injection of P. phaseolicola race 2 and chloramphenicol (watersoaking is not apparent before this time). Results are given in Table 20 as bacterial numbers/leaf disk.

The results in Tables 18 and 19 show that bacterial numbers without inhibitor increase by c. 10x/leaf disk over a fourteen day period (386 h). Numbers with chloramphenicol at first decrease slightly until 48 h and then increase again to a level of the control. In all cases (control, 60 and 125 µg/ml chloramphenicol) numbers were greatest at 192 h.

Bacterial numbers with rifampicin 60 µg/ml decrease until 96 h and then increase slightly, but overall the numbers decrease by c. 4x/leaf disk during the fourteen day period. With rifampicin 125 µg/ml numbers decrease until 96 h when a slight increase occurs, and then decrease again such that the overall decrease is c. 12x/leaf disk over fourteen days.

In Table 20 results show that numbers at the edge of the inoculation area with and without chloramphenicol do not differ greatly from numbers in the centre of the inoculation area (Table 18). Numbers are again greatest at 192 h.

The effect of chloramphenicol and rifampicin on the growth of P. phaseolicola race 2 in trifoliate leaves of

Table 20. Effect of chloramphenicol on growth of P. phaseolicola race 2 at the edge of the inoculation area in leaves of Canadian Wonder

Concentration (µg/ml)	Bacterial numbers/leaf disk [a] Time (h) 96	192	288	384
0	3.7 x 10^6 [b]	6.1 x 10^7	2.1 x 10^7	1.1 x 10^7
60	2.1 x 10^6	6.5 x 10^7	1.0 x 10^7	3.5 x 10^6
125	1.7 x 10^6	6.3 x 10^7	3.6 x 10^7	6.0 x 10^6

a Leaf disk 4.5 mm diam

b Mean of six replicates

Table 21. Effect of chloramphenicol and rifampicin on growth of P. phaseolicola race 2 in trifoliate leaves of Canadian Wonder following inoculation of monofoliate leaves

Inhibitor	Concentration of inhibitor (μg/ml)	Bacterial numbers/leaf disk [a] Time (h) 0	12	24	48	96	192	288	386
Chloramphenicol	0	8.5×10^{1} [b]	2.5×10^{2}	6.5×10^{2}	5.5×10^{2}	5.0×10^{3}	4.5×10^{4}	1.7×10^{4}	8.0×10^{3}
	60	1.0×10^{2}	5.0×10^{1}	1.7×10^{2}	5.0×10^{2}	2.5×10^{3}	4.0×10^{4}	1.0×10^{4}	7.0×10^{3}
	125	7.8×10^{1}	1.2×10^{2}	1.2×10^{2}	2.5×10^{2}	6.0×10^{2}	4.0×10^{4}	3.0×10^{3}	5.8×10^{3}
Rifampicin	60	6.2×10^{1}	1.4×10^{2}	1.1×10^{2}	1.4×10^{2}	1.3×10^{2}	6.0×10^{3}	2.2×10^{3}	1.3×10^{3}
	125	9.0×10^{1}	1.2×10^{1}	1.2×10^{2}	1.1×10^{2}	1.4×10^{2}	7.0×10^{3}	2.4×10^{2}	4.0×10^{2}

a Leaf disk 4.5 mm diam

b Mean of six replicates

CW, following inoculation of monofoliate leaves was determined. This was done by counting the numbers of viable bacteria present in the trifoliate leaves at different time intervals over fourteen days (386 h) by the method given (Materials and Methods 3). Results are given in Table 21 as bacterial numbers/leaf disk. From Table 21, it can be seen that numbers in trifoliates without inhibitor increase until 192 h and then decrease slightly, but overall an increase of c. 100x/leaf disk during the fourteen day period occurs. A similar trend is shown for numbers in trifoliate leaves when chloramphenicol at 60 and 125 μg/ml and P. phaseolicola were injected into monofoliates. In this case, however, the overall increase over 386 h is only c. 17x/leaf disk for both the chloramphenicol treatments.

With rifampicin at 60 and 125 μg/ml, numbers in trifoliate leaves again increase until 192 h and then decrease slightly. The overall increase during 386 h is c. 12x and c. 14x/leaf disk for rifampicin at 60 and 125 μg/ml respectively.

In summary it appears that the growth pattern of P. phaseolicola in monofoliate leaves, with and without chloramphenicol, and in trifoliate leaves following monofoliate inoculation is similar in that numbers increase until c. 192 h and then decrease slightly but in all cases numbers increase overall. With rifampicin in monofoliate leaves numbers continue to decrease from inoculation, but in trifoliate leaves the growth trend is similar to that of control and chloramphenicol treatments. In all cases numbers of bacteria in the leaves with rifampicin were

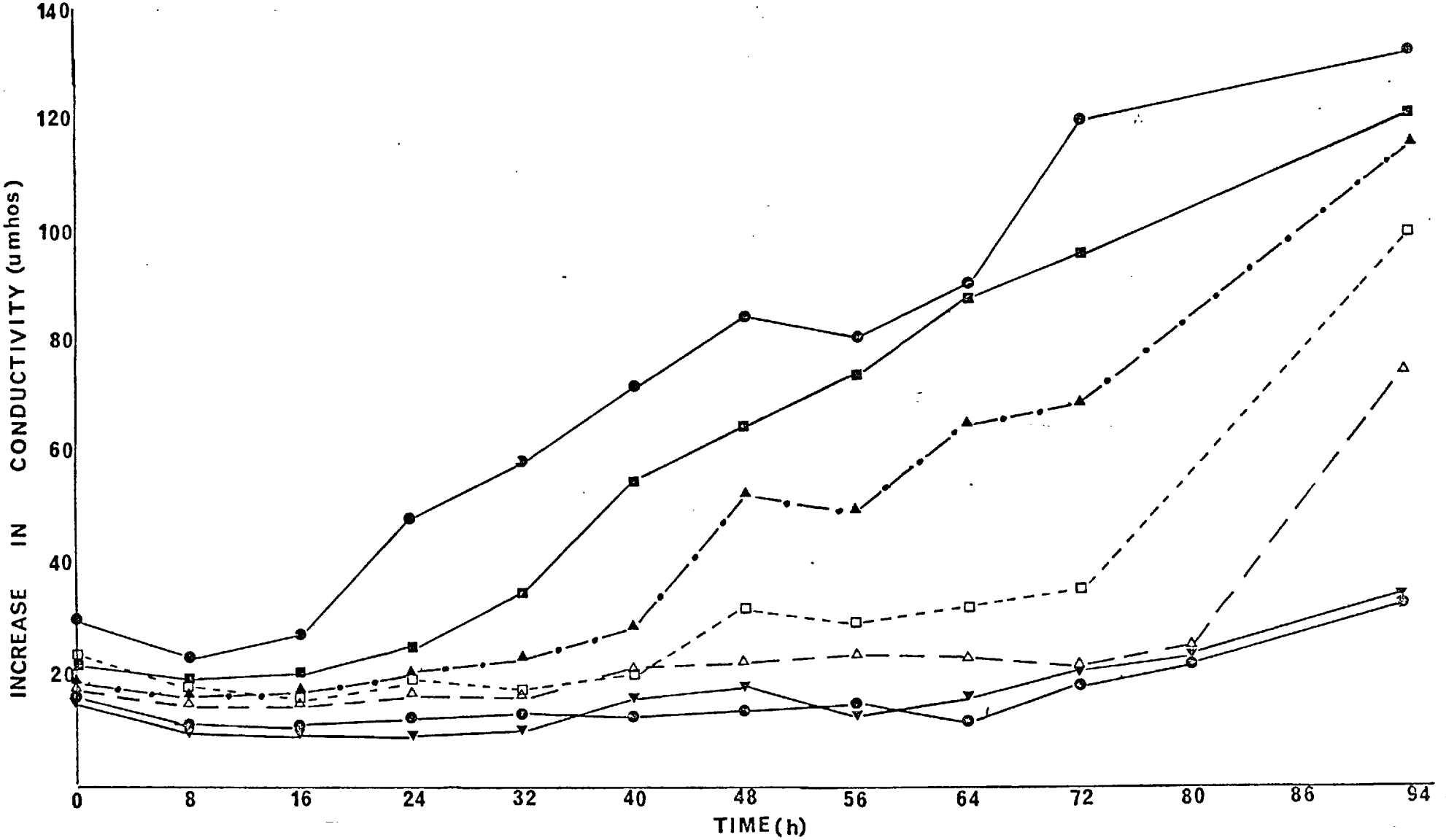


Figure 4. Effect of chloramphenicol on electrolyte loss from monofoliate leaves of Canadian Wonder following injection of P. phaseolicola race 2. 3-Collapse of inoculation area (SR). (●——●) Control I, Standard Inoculum (SI) 2.6 x 10^8 cells/ml P. phaseolicola; (●——●) Control II, Chloramphenicol (CHL) 60 µg/ml; (▼——▼) Control III, Water; (■——■) CHL 5 µg/ml & SI; (▲—•—▲) CHL 15 µg/ml & SI; (□----□) CHL 30 µg/ml & SI; (△— —△) CHL 60 µg/ml & SI.

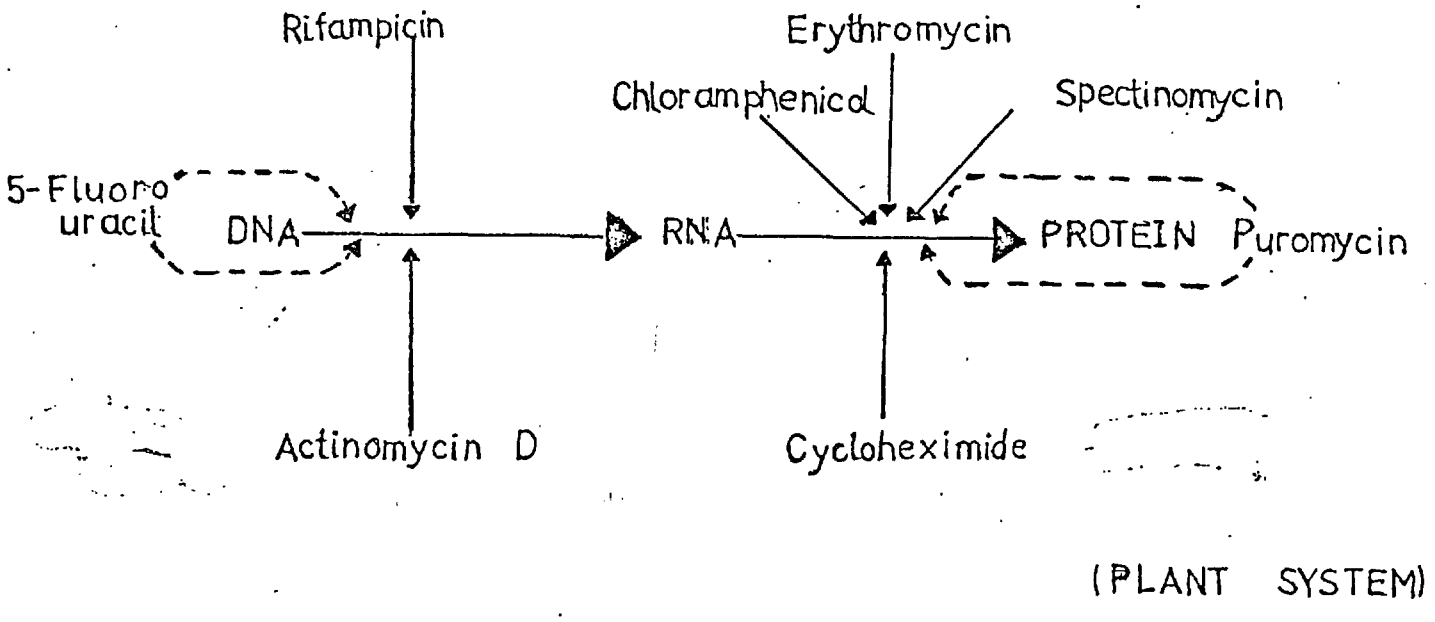
considerably less than with control or chloramphenicol.

The effect of plant specific inhibitors on SR was not determined primarily because of the long time lapse before development of symptoms (14 days) during which, specific inhibitors of protein and RNA synthesis would become extremely phytotoxic. Also, as chloramphenicol and rifampicin are two of the most bacterially specific inhibitors known and in view of their failure to prevent SR no attempt was made to use other inhibitors.

b) Effect of chloramphenicol on electrolyte loss from monofoliate leaves in the susceptible reaction

The effect of a wide range of concentrations of chloramphenicol on permeability changes in the monofoliate leaf of CW during the first 96 h of SR was determined. Leaves were inoculated with P. phaseolicola race 2 and chloramphenicol and at various time intervals leaf disks were removed from the inoculation area. Permeability changes (electrolyte loss) were measured as the increase in conductivity of deionized water that the leaf disks from the inoculation area were floated on (Materials and Methods 4). Results in Figure 4 are given as increases in μ mho/leaf disk and the collapse of the inoculation area is shown as reaction grade 3. Results show that the permeability of leaves inoculated with P. phaseolicola alone and P. phaseolicola with 5μg/ml chloramphenicol increases at an almost linear rate from 16-24 h after inoculation. By 72 h the inoculation areas in the leaves, with these two treatments, had collapsed. The permeability of host cells inoculated with P. phaseolicola and 15 and

RNA AND PROTEIN INHIBITORS



BACTERIAL CELL WALL INHIBITORS

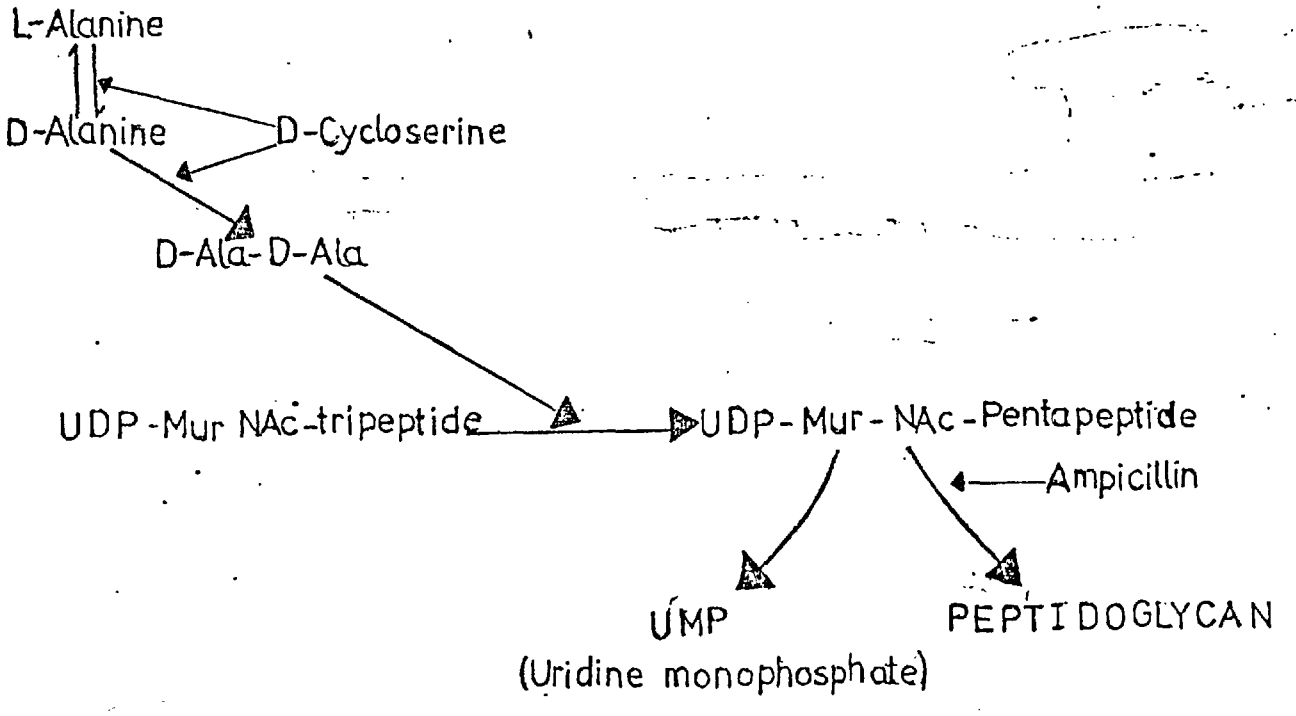


Figure 5. Diagram for site of action of inhibitors of RNA, protein and cell wall synthesis in bacterial and plant systems.

30 µg/ml of chloramphenicol starts to increase at 40 and 56 h respectively. In both of these cases the inoculation areas collapsed by 96 h. Inoculation with P. phaseolicola and chloramphenicol 60 µg/ml caused little increase in host permeability until 72 h and the inoculation area did not collapse by 96 h. There was no significant change in host cell permeability from inoculation of chloramphenicol 60 µg/ml or water without the bacteria. The results show that collapse of the inoculation area in SR is accompanied by large increases in electrolyte loss from host cells with the rate of electrolyte loss being approximately linear. The concentrations of chloramphenicol which delayed the collapse of the inoculation area (Table 16) also delayed the time at which host cell permeability started to increase.

Summary

A range of inhibitors have been used to determine their effects on the hypersensitive (HR) and susceptible reactions (SR). Bacterially specific inhibitors of protein and RNA synthesis prevented HR and delayed SR. Specific inhibitors of bacterial cell wall synthesis, the enzymes RNAase and trypsin and plant specific inhibitors of protein and RNA synthesis had no effect on HR. Use of chloramphenicol showed that collapse of the inoculation area in HR and SR correlated well with increases in host cell permeability. The effects of the inhibitors on the metabolism of the host and bacterium are summarised in Figure 5.

(3) Attempts to alter the hypersensitive and susceptible reactions

The hypersensitive (HR) and susceptible reactions (SR) of plants to bacteria can be suppressed or altered

by a variety of treatments (Ercolani, 1970; Lovrekovich and Farkas, 1965; McIntyre et al., 1973; Schroth and Vitanza, 1974). Obtaining methods to treat plants so that SR becomes HR and HR becomes a nil response is potentially of considerable practical importance. The ease by which host response to a pathogen can be altered naturally is best shown by cultivars of wheat containing the SR6 gene. At temperatures of 25°C and below these cultivars of wheat respond hypersensitively to Puccinia graminis var. tritici, but above 26°C wheat responds in a susceptible manner. Similarly with tobacco and P. tabaci, below 35°C HR is caused but above 36°C SR is caused.

In this investigation various bacterial and chemical treatments were used in attempts to alter host response.

A) Use of chemicals injected into leaves of Red Mexican and Canadian Wonder

By using high concentrations of metabolites common to either/both the bacterium and host it was hoped to alter host response by a mechanism of metabolic feedback, induction or repression (Jacob and Monod, 1961).

Metabolic feedback is responsible for adjusting the rate of synthesis of metabolic intermediates according to the demands of synthesis. Usually an early reaction in the synthesis of the metabolite is inhibited by an end product, thereby avoiding the accumulation of several intermediates which would be wasteful to the cell.

Metabolic repression is a mechanism which controls the concentration of cellular enzymes. Frequently an end product (metabolic repressor) inhibits the formation of an

enzyme by acting on an early stage in its synthesis. In this way amino acids for the synthesis of other enzymes become available to the cell.

Metabolic induction is a mechanism by which the rate of synthesis of an enzyme can be stimulated by the addition of the substrate of the enzyme. Both metabolic repression and induction are highly specific but inducers are substrates of the sequences whereas metabolic repressors are products of the sequences.

The following chemicals (metabolites) common to both bacterium and host were used: cyclic adenosine monophosphate (CAMP), glucose and sucrose. The other chemicals used which were mostly common to the bacterium or host were, various amino acids and deoxyribonucleic acid from various sources. Metabolites and chemicals at a concentration of 5 mM, except DNA (250 μg/ml), were injected into monofoliate leaves of RM and CW at various time intervals in relation to injection with Pseudomonas phaseolicola race 1 (2.6×10^8 cells/ml). In RM P. phaseolicola race 1 causes HR, but in CW it causes SR. The time intervals ranged from 24 h before to 4 h after the bacterium. In one treatment the bacteria and metabolite were mixed and then agitated together for 1 h on a reciprocal shaker before injection into the leaves. The results, taken 30 h after injection of bacteria into leaves for HR and after 72 h for SR, are given as reaction grades in Tables 22, 23 and 25. There were no phytotoxic effects after 3 d from injection of the chemicals without bacteria. Results from Table 22 show that little alteration of host response was achieved when the metabolites CAMP, glucose and sucrose

Table 22. Effect of metabolites on the hypersensitive and susceptible reactions to *Pseudomonas phaseolicola* race 1

Metabolite	Reaction Grade[a]													
	Metab. 24 h[c] then *P. phas.*		Metab. 4 h then *P. phas.*		Metab. 1 h then *P. phas.*		Metab. and *P. phas.*		*P. phas.* 1 h then Metab.		*P. phas.* 4 h then Metab.		*P. phas.* and metab. 1 h then injection	
	RM	CW[d]	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW
Cyclic AMP	[b]2	3*	3*	3*	3*	3	3	3	3	3	3	3	2	3
Glucose	2	3*	3*	3*	3	3	3	3	3	3	3	3	2	3
Sucrose	3	3	3	3	3	3	3	3	3	3	3	3	3	3

a. 4 replicates/treatment

b. 3-HR(RM) or SR(CW)
3*-incomplete collapse of inoculation area.

c. Metab. - Metabolite
P. phas. - *P. phaseolicola* race 1

d. RM - Red Mexican
CW - Canadian Wonder

Table 23. Effect of amino acids on the hypersensitive and susceptible reactions to P. phaseolicola race 1

Amino acid (L-isomers)	Reaction Grade[a]											
	A.A. 24 h[c] then P. phas		A.A. 4 h then P. phas		A.A. 1 h then P. phas		A.A. and P. phas		P. phas 1 h then A.A.		P. phas 4 h then A.A.	
	RM	CW[d]	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW
Alanine												
Arginine												
Asparagine												
Cysteine												
Glutamic acid	[b]3	3	3	3	3	3	3	3	3	3	3	3
Glycine												
Phenylalanine												
Tryptophan												
Proline												

a. 4 replicates/treatment

b. 3 - HR(RM) or SR(CW)

c. A.A. - Amino acid

d. RM - Red Mexican
CW - Canadian Wonder

were injected into leaves of RM and CW before, simultaneously with or after P. phaseolicola race 1. However, host response was delayed when C. AMP and glucose were injected 24 h before P. phaseolicola 1 and when the bacterium was incubated with the metabolites for 1 h before injection. Therefore, it appears that these metabolites play little or no part, either directly or through a feedback, repression or induction mechanism, in host response to the bacterium.

Results from Table 23 again show that no alteration in host response was achieved when a variety of amino acids were injected into leaves of RM and CW sequentially to P. phaseolicola race 1. All the amino acids, except glutamic acid, are naturally occurring L isomers and are actively involved in protein synthesis. Therefore, this result implies that either the amino acids are causing little alteration in protein synthesis, or that any new or altered proteins being produced have little effect on host response to the bacteria.

Table 24 shows the spectral data for DNA extracted from P. mors-prunorum and P. phaseolicola in comparison with that obtained for commercial nucleic acid and Marriur's original figures (Marmur, 1961). The method employed for extracting DNA from bacteria has already been given (Materials and Methods 6). Results from Table 24 show that the ratio of absorbances for the DNA extracted from P. mors-prunorum and P. phaseolicola are closely allied to that obtained for commercial DNA and Marmur's figures. The result therefore shows that the extracted DNA has a high degree of purity. In order to determine the total amount

Table 24. Spectral data of nucleic acids

Source of DNA	Ratio of Absorbances 280 mμ	260 mμ	230 mμ
Commercial DNA (*E. coli*)	.556	1.0	.458
Values per Marmur[a]	.515	1.0	.450
Observed *P. phaseolicola*	.520	1.0	.440
Observed *P. mors-prunorum*	.530	1.0	.455

a. Marmur 1961

Table 25. Effect of nucleic acid on hypersensitive and susceptible reactions to Pseudomonas spp.

	Reaction Grade[a]											
	DNA 24 h[c] then P. phas 1		DNA 24h then P. phas 2		DNA 24 h then P. m-p		DNA and P. phas 1		DNA and P. phas 2		DNA and P. m-p	
	RM[d]	CW	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW
Escherielia coli												
Calf thyrus												
P. mors-prunorum	[b]3	3	3	3	3	3	3	3	3	3	3	3
P. phaseolicola 1												
P. phaseolicola 2												

a. 4 replicates/treatment

b. 3 - HR(RM) or SR(CW) - P. phaseolicola race 1
3 - HR - P. mors-prunorum
3 - SR - P. phaseolicola race 2

c. P. phas 1 - P. phaseolicola race 1
P. phas 2 - " race 2
P. m-p - P. mors-prunorum

d. RM - Red Mexican
CW - Canadian Wonder

of DNA within a bacterial inoculum of 2.6×10^8 cells/ml, a calculation based on the amount of DNA within an Escherichia coli bacterium was carried out.

Calculation to determine the amount of DNA in inocula of 2.6×10^8 cells/ml of Pseudomonas spp.

Amount of DNA in E. coli bacterium 4.7×10^{-15} g (data from Strickberger (1968).

Inoculum concentration = 2.6×10^8 cells/ml

Total DNA in inocula = $2.6 \times 10^8 \times 4.7 \times 10^{-15}$ g/ml

= 1.22×10^{-6} g/ml

= 1.22 µg/ml.

The above calculation shows that an injection of DNA (250 µg/ml) contained c. 200 x more nucleic acid than was present in an inoculum of 2.6×10^8 cells/ml of Pseudomonas spp.

In Table 25 the effect of DNA (250 µg/ml) from various sources on HR and SR caused by the bacteria P. mors-prunorum and P. phaseolicola races 1 and 2 (2.6×10^8 cells/ml) is given. The results show that neither the commercially prepared (E. coli and calf thymus) nor the extracted DNA (P. mors-prunorum and P. phaseolicola races 1 and 2) had any effect on HR or SR when injected before, simultaneously with or after the bacteria. In view of the purity and concentration of the injected DNA, this result must imply that movement of DNA from the injected bacteria into host cells is not the direct inducer of HR or SR.

B) Use of systemic chemicals

A systemic chemical is one which is taken up and translocated by a plant such that a large proportion of the cells of the plant eventually come into contact with or absorb that chemical. Some systemic chemicals have been reported to have various effects on host cell metabolism ranging from changes in nucleic acid synthesis caused by growth regulators to changes in host cell membrane permeability caused by ionic solutions.

The growth regulators indole acetic acid, gibberellic acid and kinetin can affect host metabolism by directly affecting nucleic acid synthesis so forming new or increased amounts of protein. De novo synthesis of α-amylase in barley endosperm can be caused by external addition of gibberellin. Other enzymes such as proteinases and β-amylase also increase markedly on addition of gibberellin (Varner et al., 1965). In this case gibberellin causes a de-repression of the genes for the enzymes in the system, presumably by acting directly at the gene level. Jensen et al., (1964) reported that kinetin caused a doubling of RNA levels within 30 min in onion root tip cells although it reduced DNA.

An increase in electrolyte loss from leaves of host cells undergoing the final stages of HR and SR has been well reported. Suppression of electrolyte loss from inoculated leaves undergoing HR and SR can be obtained by addition of solutions of calcium, magnesium and strontium salts to the bacterial inoculum (Cook and Stall, 1971).

Thus, using a variety of systemic chemicals it was

Table 26. Type and use of commercial systemics

Chemical	Type	Use
Alar	Organic acid used as a translocated plant growth regulator	Reduces internode elongation, induces drought, frost and lodging resistance
CCC [a]	Chlorinated hydrocarbon used as a plant growth regulator	Increases tillering and reduces lodging, and stunts growth of ornamental plants
Ethrel	Organic phosphorus compound used as a growth regulator	Enhances fruit maturity and fruit abscission. Increases tillering and reduces lodging in some cereals
Gibberellic acid	Organic plant hormone used as a growth regulator	Promotes stem growth in pea, parthenocarpic fruit growth, flower initiation and seed germination
Off Shoot 'T'	Organic compound used as a growth regulator	Applied to tobacco to suppress sucker development

a. (2-Chloroethyl)-trimethyl-ammonium chloride

hoped that some alteration in host response (HR/SR) to bacterial injection would result.

The following chemicals were used: commercial systemics of known plant regulator effect, Ciba-Geigy experimental systemics, other growth regulators (plant hormones), various solutions of salts and solutions of DNA. The type and use of some of the commercial systemics is given in Table 26. The Ciba-Geigy experimental chemicals are of known systemic nature, but which are still under investigation for commercial viability. Their plant growth regulatory effect has not yet been fully determined.

Solutions of the above chemicals were taken up through the roots by whole plants of RM and CW for 24 h before injection of P. phaseolicola (2.6×10^8 cells/ml) into the monofoliate leaves. The results were taken 30 h after bacterial injection for HR and after 72 h for SR, and are given as reaction grades in Tables 27, 28, 29 and 30.

Results from Table 27 show that no alteration in host response was achieved when a variety of systemic growth regulators were taken up by the plant for 24 h before inoculation of P. phaseolicola into the monofoliate leaves. In view of the known growth regulatory effect of these chemicals (Table 26) and the visible effect of uptake and translocation through the plant (toxicity in the leaves), this result implies that the chemicals are not affecting the metabolism of either the host or pathogen in such a manner as to alter host expression to infection.

Results from Tables 28, 29 and 30 concern the effect

Table 27. Effect of commercial chemicals on the hypersensitive and susceptible reactions to Pseudomonas phaseolicola race 1

Chemical	Reaction Grade[a] Concentration chemical (µg/ml) 125 RM[c]	125 CW[c]	60 RM	60 CW	Comments on phytoxicity of chemical
Alar.					Toxic even at 60 µg/ml - leaves appeared flaccid
Alsol					Toxic at 60 µg/ml leaf roll apparent
C.C.C.[d]					No visible toxicity
Ethrel	3[b]	3	3	3	Leaf chlorosis and eventual abscession with petiole at stem
Gibberellic acid					No visible toxicity
Lasso					Some marginal leaf roll and flaccidity
Off Shoot T					Leaf roll and reddish discoloration of roots

a. 4 plants/treatment

b. 3 - HR(RM) or SR(CW)

c. RM - Red Mexican
CW - Canadian Wonder

d. (2-Chloroethyl)-trimethyl-ammonium chloride

Table 28. Effect of Ciba-Geigy experimental chemicals on the hypersensitive and susceptible reactions to P. phaseolicola race 1

Chemical	Concentration Chemical (µg/ml) 125		60		Comments on phytotoxicity of chemical
	Reaction Grade[a]				
	RM[c]	CW[c]	RM	CW	
CGA 45'072					Marginal leaf roll at 125 µg/ml. No toxicity at 60 µg/ml
CGA 17'048					Flaccidity in leaves at 125 µg/ml. No toxicity at 60 µg/ml
CGA 37'407	3[b]	3	3	3	Leaf roll in monofoliate and trifoliate leaves. Abscission of monofoliates
CGA 45'434					As above but with browning of trifoliates
CGA 22'640					No visible toxicity

a. 4 plants/treatment

b. 3 - HR(RM) or SR(CW)

c. RM - Red Mexican
CW - Canadian Wonder

Table 29. Effect of experimental chemicals on the susceptible reactions to P. phaseolicola 2

Chemical	Reaction Grade[a] Concentration Chemical (µg/ml) 125		60	
	RM	CW	RM	CW
45'072				
17'048				
37'407	3[b]	3	3	3
45'434				
22'640				

a. 4 plants/treatment
b. 3 - SR
c. RM - Red Mexican
CW - Canadian Wonder

Table 30. Effect of experimental chemicals on the hypersensitive reaction to P. mors-prunorum

Chemical	Reaction Grade[a] Concentration Chemical (µg/ml) 125		60	
	RM[c]	CW[c]	RM	CW
45'072				
17'048				
37'407	3[b]	3	3	3
45'434				
22'640				

a. 4 plants/treatment
b. 3 - HR
c. RM - Red Mexican
CW - Canadian Wonder

Table 31. Effect of plant hormones on the hypersensitive and susceptible reactions to *Pseudomonas phaseolicola* race 1

Plant hormone	Reaction Grade[a] Concentration (µg/ml)										Comments on phytotoxicity of hormone
	125		60		30		15		0		
	RM[c]	CW[c]	RM	CW	RM	CW	RM	CW	RM	CW	
Abscisic acid											No toxicity
Gibberellic acid	3[b]	3	3	3	3	3	3	3	3	3	No toxicity
Indole acetic acid											Some leaf flaccidity at 125 µg/ml
6-Methyl amino purine											No toxicity

a. 4 plants/treatment

b. 3 - HR(RM) or SR(CW)

c. RM - Red Mexican
CW - Canadian Wonder

on HR and SR of Ciba-Geigy experimental chemicals. In Table 28, HR and SR in RM and CW respectively was caused by P. phaseolicola race 1. In Tables 29 and 30 P. mors-prunorum and P. phaseolicola race 2 were each injected into both RM and CW, with P. mors-prunorum causing HR and P. phaseolicola 2 causing SR. Results in Table 28 were taken 30 h after bacterial injection for HR and after 72 h for SR. Results in Tables 29 and 30 were taken after 24 h for HR and after 72 h for SR. All results were recorded as reaction grades.

Results from Tables 28, 29 and 30 show that no effect on HR (caused by P. phaseolicola 1 in RM and P. mors-prunorum) or SR (caused by P. phaseolicola 1 in CW and P. phaseolicola 2) was achieved with the Ciba-Geigy chemicals. The chemicals were again shown to be systemic by toxicity in the leaves, and so it must be assumed that they are not altering the metabolism of either the host or pathogen in a manner which would alter host expression to infection.

Results from Table 31 again show that no alteration in host response was achieved when a variety of systemic growth regulators (plant hormones) were taken up by the plant for 24 h before inoculation of P. phaseolicola into the monofoliate leaves.

A synergism in plant response to indole acetic acid (IAA) and other plant hormones is known, e.g. IAA and gibberellin together promotes cell expansion faster than either regulator individually and IAA and kinetin (6-methyl amino purine) together promotes cell division

Table 32. Effect of simultaneous application of two growth regulators on the hypersensitive and and susceptible reactions to Pseudomonas phaseolicola race 1

Main hormone	Reaction Grade[a] Secondary hormone (125 µg/ml)					
	Abscisic acid		6-Methyl amino purine		Gibberellic acid	
	RM[c]	CW[c]	RM	CW	RM	CW
IAA 60 µg/ml	3[b]	3	3	3	3	3

a. 4 plants/treatment

b. 3 - HR(RM) or SR(CW)

c. RM - Red Mexican
CW - Canadian Wonder

faster than either individually. Therefore, an experiment was designed to investigate whether two hormones applied simultaneously would alter plant response to P. phaseolicola (Table 32). The concentration of the IAA was reduced to 60 µg/ml because some phytotoxicity was shown in the previous experiment.

Results from Table 32 show that simultaneous uptake of two hormones for 24 h caused no alternation in host response to P. phaseolicola.

The effect of ions in altering host response to bacterial pathogens has been reported (Cook and Stall, 1971). An experiment was set up in which a broad range of monovalent and divalent cations and anions were used. Preliminary experiments determined the molarity of the solutions to be used which in all cases was slightly below unacceptable toxicity. In view of the toxicity of some of the ions to bacteria, the number of bacteria in the leaf when the responses were noted was also determined. Bacterial numbers were determined by the method given (Material and Methods 3).

The results in Tables 33 and 34 show the effect on HR and SR in RM and CW of uptake, through the roots, of ionic solutions for 24 h before bacterial injection into the monofoliate leaves. In Table 33 HR and SR in RM and CW respectively was caused by P. phaseolicola race 1. In Table 34, P. mors-prunorum and P. phaseolicola race 2 were each injected into both RM and CW with P. mors-prunorum causing HR and P. phaseolicola 2 causing SR. In Table 33 host response was noted 80 and 72 h after inocula-

Table 33. Effect of uptake of ions on hypersensitive and susceptible reactions to Pseudomonas phaseolicola race 1

Ion (molarity and source)	RM Reaction[a] Grade	RM Bacterial No Leaf disk[b]	CW Reaction[a] Grade	CW Bacterial No Leaf disk[b]	Comments on phytotoxicity of chemical
Ca^{2+} (0.1M) $Ca(NO_3)_2$	NIL[c]	4.2×10^5	NIL	7.0×10^6	Prevention less apparent at 0.05M. Little toxicity.
Mg^{2+} (0.1M) $Mg(NO_3)_2$	NIL	4.7×10^5	NIL	6.6×10^6	As above.
K^+ (0.1M) (KNO_3)	3	5.2×10^5	3	7.2×10^6	Little toxicity.
Sr^{2+} (0.05M) $SrCl_2$	NIL	6.0×10^5	NIL	4.6×10^6	Prevention less apparent at 0.01M. Some necrotic flecking of foliage.
Ba^{2+} (0.05M) $BaCl_2$	NIL	4.0×10^5	NIL	6.0×10^6	As above.
Be^{2+} (0.05M) $BeSO_4$	NIL	4.5×10^5	NIL	4.5×10^6	As in Sr.

Table 33. (cont'd.)

Ion (molarity and source)	Reaction[a] Grade	RM Bacterial No Leaf disk[b]	Reaction[a] Grade	CW Bacterial No Leaf disk[b]	Comments on phytotoxicity of chemical
Fe^{3+} (0.005M) ($FeCl_3$)	3	5.0×10^5	3	6.6×10^6	Some necrotic flecking of foliage.
Zn^{2+} (0.005M) $ZnCl_2$	NIL	1.5×10^5	NIL	3.0×10^6	Some browning of veins in leaves.
Mn^{2+} (0.005M) ($MnCl_2$)	3	5.0×10^5	3	4.6×10^6	No toxicity.
Mo (0.005M) MoO_3	NIL	4.0×10^5	NIL	7.2×10^6	Prevention less apparent at 0.001M. Some inter-veinal chlorosis.
BO_3^- (0.001M) H_3BO_3	3	5.0×10^5	3	8.5×10^6	Necrotic flecking.
Cu^{2+} (0.001M) $CuCl_2$	3	5.5×10^5	3	5.5×10^6	No toxicity.
Nil (H_2O)	3	5.2×10^5	3	7.5×10^6	

a. 4 plants/treatment.
b. Leaf disk (4.5 mm diam)
c. NIL - No response.
3 - HR (RM) or SR (CW).

Table 34. Effect of uptake of ions on the hypersensitive and susceptible reactions to P. mors-prunorum and P. phaseolicola race 2

Ion (molarity and source)	P. mors-prunorum Reaction Grade [a] RM	CW	P. phaseolicola race 2 Reaction Grade [a] RM	CW
Ca^{2+} (0.1M) $Ca(NO_3)_2$	NIL[b]	NIL	NIL	NIL
Mg^{2+} (0.1M) $Mg(NO_3)_2$	NIL	NIL	NIL	NIL
K^{+} (0.1M) KNO_3	3	3	3	3
Sr^{2+} (0.05M) $SrCl_2$	NIL	NIL	NIL	NIL
Ba^{2+} (0.05M) $BaCl_2$	NIL	NIL	NIL	NIL
Be^{2+} (0.05M) $BeSO_4$	NIL	NIL	NIL	NIL
Fe^{3+} (0.005M) $FeCl_3$	3	3	3	3
Zn^{2+} (0.005M) $ZnCl_2$	NIL	NIL	NIL	NIL
Mn^{2+} (0.005M) $(MnCl_2)$	3	3	3	3
Mo^{3+} (0.005M) MoO_3	NIL	NIL	NIL	NIL
BO_3^{-} (0.001M) H_3BO_3	3	3	3	3
Cu^{2+} (0.001M) $CuCl_2$	3	3	3	3
H_2O	3	3	3	3

a 4 plants/treatment

b NIL - No response
3 - HR (P. mors-prunorum)
3 - SR (P. phaseolicola 2)

tion for HR and SR respectively, and in Table 34 after 24 and 72 h.

Results from Tables 33 and 34 show that ionic solutions of varying strengths of calcium, magnesium, strontium, barium, beryllium, zinc and molybdenum inhibited HR and SR when the solutions were taken up through the roots for 24 h before inoculation of P. phaseolicola races 1 and 2 and P. mors-prunorum into monofoliate leaves. Effective uptake of the ions was again shown by toxicity in the leaves. Numbers of bacteria in the leaves (Table 33) when inhibition of HR and SR occurred did not fall significantly below the numbers in leaves when water alone was taken up. This shows that the inhibition of plant response was not due to a reduction of bacterial numbers to a level below which visible collapse of the inoculation area occurs. From Tables 33 and 34, it further appears that inhibition of HR and SR is not specific to the divalent cations belonging to group IIa of the periodic table since zinc and molybdenum, which are not members of group IIa, were also effective in inhibiting HR and SR. However, neither of the monovalent ions used prevented HR or SR.

In another experiment on uptake of chemicals, cut ends of hypocotyls of RM and CW bearing the primary and first set of trifoliate leaves were placed in solutions of nucleic acid (250 µg/ml) for 24 h before bacterial inoculation (2.6×10^8 cells/m) into the monofoliate leaves. Nucleic acids were obtained from the same source as in the previous section (3A). Results were recorded as reaction grades in Table 35 and were taken 30 and 72 h after inocula-

Table 35. Effect of uptake of DNA on the development of the hypersensitive and susceptible reactions to Pseudomonas species

Source of DNA	Reaction Grade[a]					
	DNA 24 h then P. phas. 1[c]		DNA 24 h then P. phas. 2		DNA 24 h then P. m-p	
	RM	CW	RM	CW	RM	CW
Calf Thymus						
P. mors-prunorum	3[b]	3	3	3	3	3
P. phaseolicola 2						

a. 6 plants/treatment

b. 3 - HR (RM) or SR (CW) - P. phaseolicola race 1
3 - HR - P. mors-prunorum
3 - SR - P. phaseolicola race 2

c. P. phas. 1 - P. phaseolicola race 1
P. phas. 2 - P. phaseolicola race 2
P. m-p - P. mors-prunorum

tion for HR and SR respectively.

Results in Table 35 show that the DNA of calf thymus, P. mors-prunorum and P. phaseolicola did not cause any alteration in host response to the bacteria P. phaseolicola races 1 and 2 and P. mors-prunorum. Even when the DNA of P. mors-prunorum or P. phaseolicola was used against the same bacterium no alteration in host response occurred. This result implies that the DNA of P. mors-prunorum and P. phaseolicola is not the active principle which is transported across the host/pathogen interface to cause host response. This result supports the conclusion reached, in the previous section, when DNA from P. phaseolicola and P. mors-prunorum was injected into monofoliate leaves of RM and CW before, simultaneously with or after the live bacterium.

C) Use of rapid alteration of air temperature as an inducement of shock

Predisposition is the tendency of nongenetic conditions, acting before infection, to affect the susceptibility of plants to disease (Yarwood, 1959).

Past work has shown that when plants are exposed to high or low temperature before inoculation, their susceptibility to pathogens may be increased or decreased. The most common effect is an increase in susceptibility with heating - Botrytis on apple (Vasudeva, 1930), Erysiphe polygoni on bean (Yarwood, 1956), Erwinia on potato (Gregg, 1952). Heating of hosts can also decrease infection. Keyworth and Dimond (1952) found that dipping tomato roots in hot water reduced subsequent infection

Table 36. Effect of rapid alteration of air temperature on the hypersensitive and susceptible reactions to Pseudomonas phaseolicola race 1

Temperature °C	Reaction Grade[a] Time in the altered environment (min)					
	5		10		15	
	RM[c]	CW	RM	CW	RM	CW
60						
50	[b]3	3	3	3	3	3
40						
22	Normal Environment					
10						
0	3	3	3	3	3	3
-2						
-5						

a. 6 plants/treatment

b. 3 - HR (RM) or SR (CW)

c. RM - Red Mexican
CW - Canadian Wonder

with Fusarium lycopersici. Exposure of plants to low temperatures also affects their susceptibility to disease. Frost injury is reported to increase the susceptibility of broad beans (Moore, 1944), lettuce (Kerling, 1952) to Botrytis; of birch and spruce to Nectria (Gäumann, 1950).

Thus by altering the environment (temperature) around the plant it was hoped that some alteration in host response would occur.

Whole plants of RM and CW grown in a controlled environment ($22^{o} \pm 1^{o}C$) were removed and subjected to a rapid change in air temperature for brief periods, returned to the controlled environment, and injected with Pseudomonas phaseolicola race 1 (3.0×10^{8} cells/ml). The temperature range used varied between $27^{o}C$ below to $38^{o}C$ above the controlled environment with the periods the plants spent in the altered temperature ranging from 5 - 15 mins. Results were noted 30 and 72 h after bacterial injection for HR and SR respectively and were expressed as reaction grades in Table 36.

Results in Table 36 show that rapid alteration of air temperature by as much as c. $40^{o}C$ for brief periods had no effect on altering host response to P. phaseolicola race 1. The severest treatment (15 min at $-5^{o}C$) resulted in considerable frost damage to the foliage but the inoculation areas still collapsed within the allotted time for HR and SR. This result implies that the treatments used were not sufficient to alter the part of the hosts' metabolism involved in the response to pathogens.

D) Use of cross protection

Cross protection is a term used to explain the effects obtained when host response to a pathogen is altered by inoculation (before, simultaneously with or after) of other pathogens or non-pathogens into the same host plant.

In view of the reported success in this field by other workers (Leben 1963; Averre and Kelman 1964; Lovrekovich and Farkas 1965; and Hsu and Dickey 1972), an experiment was set up using a wide range of species of bacteria from different genera including virulent, avirulent and saprophytic types. The challenging bacteria were 24 h cultures of live and heat killed cells (Materials and Methods 7B) which were injected into the same areas of monofoliate leaves of RM and CW before, simultaneously with or after P. phaseolicola race 1 (3.0×10^8 cells/ml). The live cells of the challenging bacteria were injected at 5.0×10^6 cells/ml and the heat killed cells at 3.0×10^8 cells/ml. Results were recorded after 30 and 72 h for HR and SR respectively and are given as reaction grades in Table 37.

In a similar but smaller experiment the bacteria Pseudomonas mors-prunorum and Pseudomonas phaseolicola race 2 were injected into both RM and CW to cause HR and SR respectively against a range of challenging bacteria. In this experiment, the challenging bacteria were injected as live cells (5.0×10^6 cells/ml) simultaneously with or 24 h before P. mors-prunorum or P. phaseolicola (3.0×10^8 cells/ml). Results were recorded after 24 h and 72 h for HR and SR respectively and are given as reaction grades in Table 38.

Table 37. Effect of live and heat killed bacterial cells on the hypersensitive and susceptible reactions to Pseudomonas phaseolicola race 1

Challenging Bacterium	Reaction Grade[a]											
	Bacterium 24 h[c] then P. phas 1		Bacterium 4 h then P. phas 1		Bacterium & P. phas 1		P. phas 1 4 h then Bacterium		Bacterium (Dead) 24 h then P. phas 1		Bacterium (Dead) & P. phas 1	
	RM[d]	CW	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW
Pseudomonas fluorescens	[b]NIL	3	3*	3	3	3	3	3	3*	3*	3	3
Pseudomonas lachrymans	3*	3	3	3	3	3	3	3	3*	3	3	3
Pseudomonas mors-prunorum	3*	3	3	3	3	3	3	3	3*	3	3	3
P. phaseolicola 1									3	3	3	3
P. phaseolicola 2	3		3		3		3		3	3	3	3
Pseudomonas solanacearum	NIL	3	3*	3	3	3	3	3	3*	3	3	3
Pseudomonas tabaci	3*	3	3	3	3	3	3	3	3	3	3	3

Table 37 cont'd.

Challenging Bacterium	Reaction Grade[a]											
	Bacterium 24 h[c] then *P. phas* 1		Bacterium 4 h then *P. phas* 1		Bacterium & *P. phas* 1		*P. phas* 1 4 h then Bacterium		Bacterium (Dead) 24 h then *P. phas*. 1		Bacterium (Dead) & *P. phas* 1	
	RM[d]	CW	RM	CW	RM	CW	RM	CW	RM	CW	RM	CW
Erwinia atroseptica	3	3	3	3	3	3	3	3	3	3	3	3
Erwinia caratovora	3	3	3	3	3	3	3	3	3	3	3	3
Xanthomonas cassava	3	3	3	3	3	3	3	3	3	3	3	3
Xanthomonas manihotis	3	3	3	3	3	3	3	3	3	3	3	3
Xanthomonas phaseoli	3	3	3	3	3	3	3	3	3	3	3	3

a. 4 plants/treatment

b. 3 - HR (RM) or SR (CW)
3*- incomplete collapse of inoculated area

c. Bacterium - challenging bacterium
P. phas 1 - *P. phaseolicola* race 1

d. RM - Red Mexican
CW - Canadian Wonder

Table 38. Effect of live and heat killed bacterial cells on the hypersensitive reaction to Pseudomonas mors-prunorum and susceptible reaction to Pseudomonas phaseolicola race 2

Challenging Bacterium	Reaction Grade[a]							
	Bacterium 24 h[c] then P. m-p		Bacterium 24 h then P. phas 2		Bacterium & P. m-p		Bacterium & P. phas 2	
	RM[d]	CW	RM	CW	RM	CW	RM	CW
Pseudomonas fluorescens	NIL[b]	NIL	3	3	3	3	3	3
Pseudomonas lachrymans	3*	3	3	3	3	3	3	3
Pseudomonas mors-prunorum	3*	3*	3	3			3	3
Pseudomonas phaseolicola 2	3	3	3		3	3		
Pseudomonas solanacearum	NIL	NIL	3	3	3	3	3	3
Pseudomonas tabaci	3*	3*	3	3	3	3	3	3

Table 38 cont'd.

Challenging Bacterium	Reaction Grade[a]							
	Bacterium 24 h[c] then *P. m-p*		Bacterium 24 h then *P. phas* 2		Bacterium & *P. m-p*		Bacterium & *P. phas* 2	
	RM[d]	CW	RM	CW	RM	CW	RM	CW
Erwinia atroseptica	3	3	3	3	3	3	3	3
Xanthomonas cassava	3	3	3	3	3	3	3	3

a. 4 plants/treatment

b. NIL - No response
3 - HR (*P. mors-prunorum*)
3 - SR (*P. phaseolicola* 2)
3* - incomplete collapse of inoculated area.

c. Bacterium - challenging bacterium
P. m-p - *P. mors-prunorum*
P. phas 2 - *P. phaseolicola* race 2
P. phas 1 - *P. phaseolicola* race 1

d. RM - Red Mexican
CW - Canadian Wonder

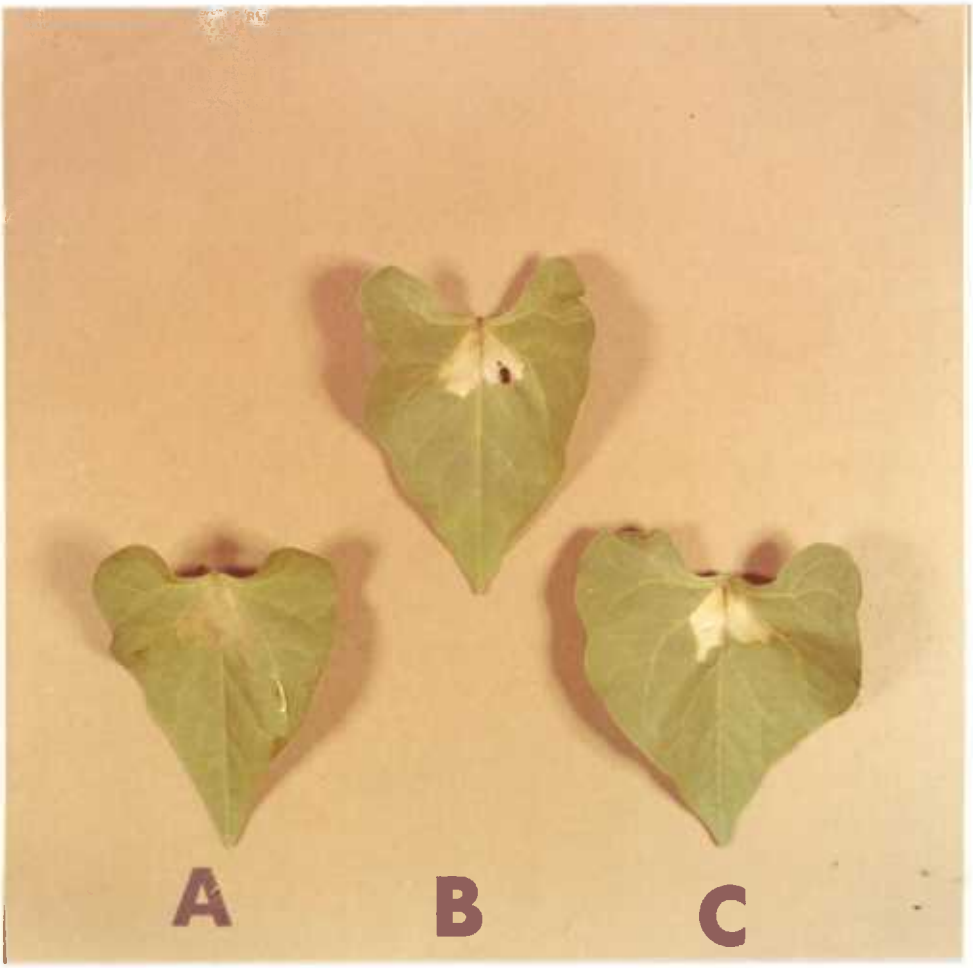


Plate 3a. Cross protection against HR

A. A RM leaf showing cross protection against HR from inoculation of P. fluorescens (5.0×10^6 cells/ml) 24 h before inoculation of P. mors-prunorum (3.0×10^8 cells/ml)

B. A RM leaf showing HR caused by injection of P. mors-prunorum (3.0×10^8 cells/ml) 24 h after injection of water

C. A RM leaf showing HR caused by injection of P. mors-prunorum (3.0×10^8 cells/ml)

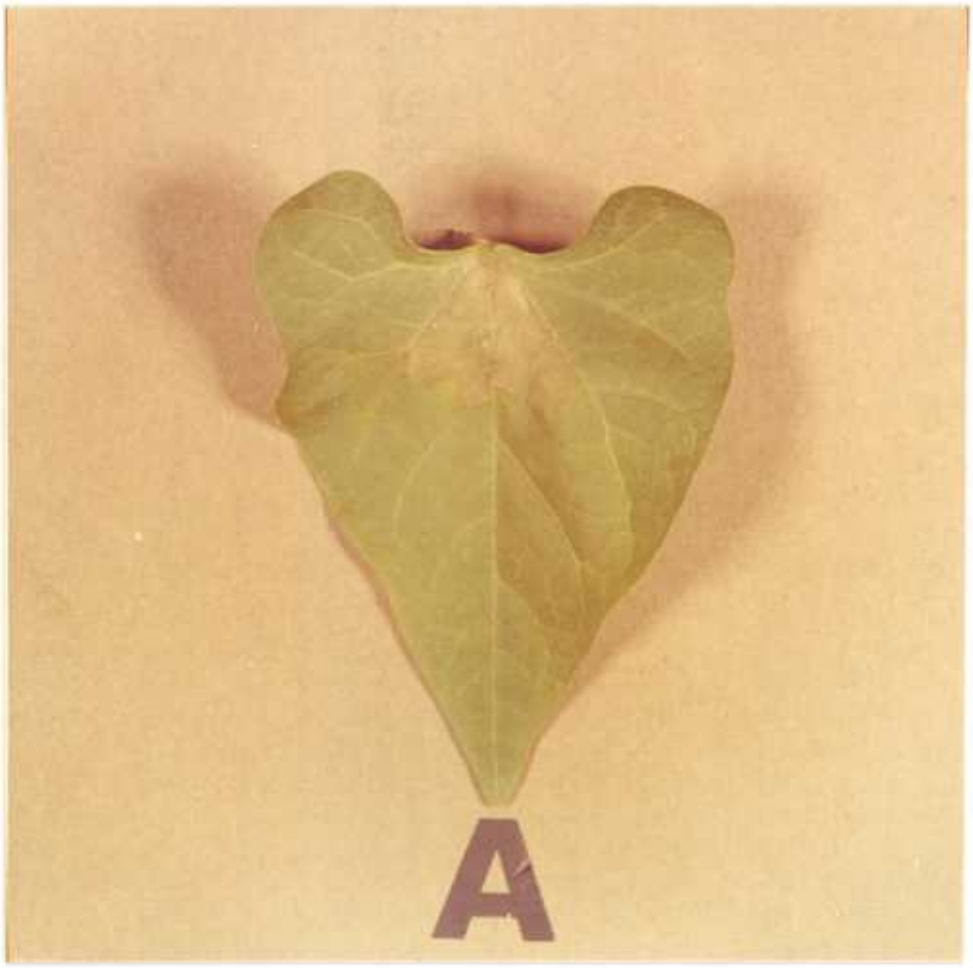


Plate 3b. As in Plate 3a.A but x 2 magnification

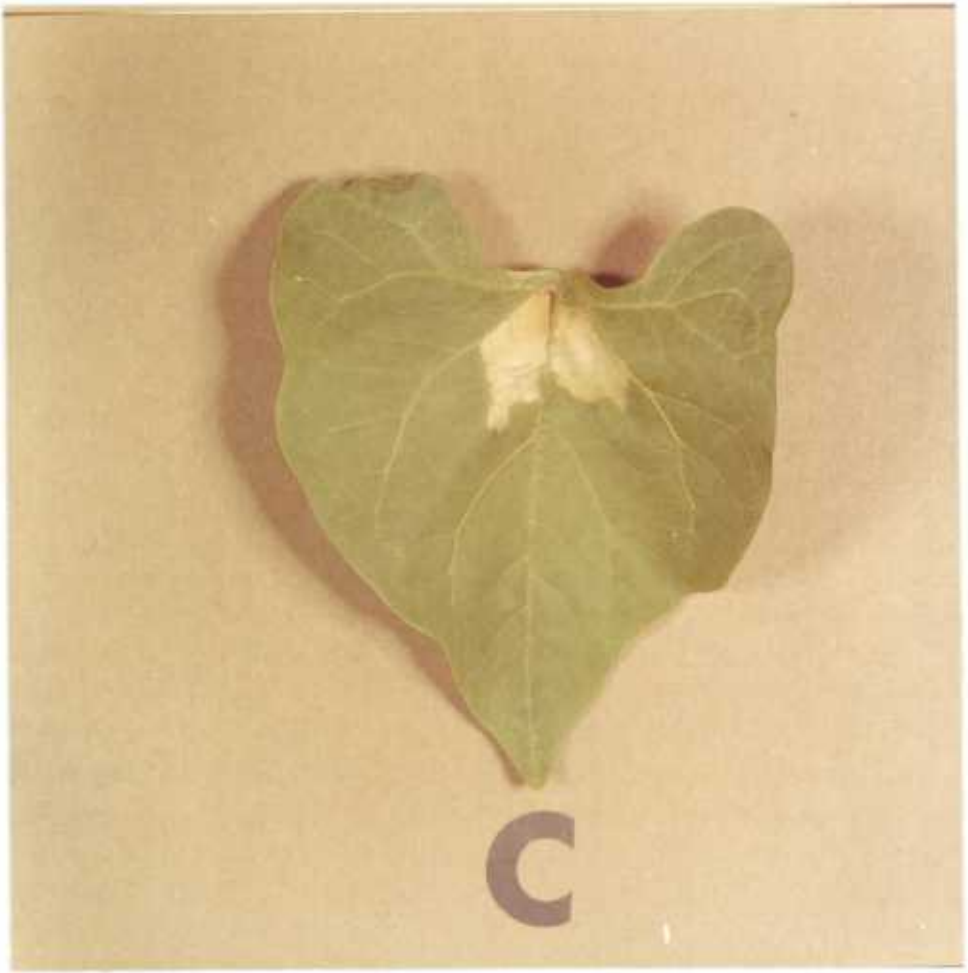


Plate 3c. As in Plate 3a.C but x 2 magnification

Results from Tables 37 and 38 show that only P. fluorescens and P. solanacearum inhibited HR when injected 24 h before either P. phaseolicola race 1 or P. mors-prunorum. It can be further seen that P. fluorescens and P. solanacearum failed to inhibit HR when inoculated simultaneously with P. mors-prunorum or P. phaseolicola race 1 (Tables 37, 38) or when inoculated as heat killed cells (Table 37). In some cases the challenging bacteria P. mors-prunorum, P. lachrymans, P. tabaci when inoculated as live cells 24 h before either P. phaseolicola or P. mors-prunorum partially inhibited HR. None of the challenging bacteria when inoculated as live or heat killed cells altered the SR evoked by P. phaseolicola races 1 and 2. The cross protective effect against HR is shown in Plates 3a, 3b and 3c.

In summary, a wide variety of chemicals and treatments has been used to try to significantly alter the host response to bacteria. The treatments included using injected chemicals, systemic chemicals, shock inducement and cross protection. Positive results were obtained only when some systemic chemicals (ionic solutions) were used and in a few cases of cross protection.

(4) Analysis of bacterial cell walls

The cultivars RM and CW differ by a single gene for resistance (Coyne et al., 1966). In RM P. phaseolicola race 1 causes HR but in CW it causes SR. Since the cultivars respond differently to the same bacterium then a recognition reaction must take place at the host-pathogen interface to determine whether HR or SR is to be caused.

The possible role of proteins (antigens), which are common to the plant and bacterium, as recognition factors, has been suggested (Devay et al., 1972; Doubly et al., 1960; Schnathorst and Devay, 1963). In this section bacterial cell wall proteins of compatible, incompatible and saprophytic bacteria were examined to see whether common proteins exist in compatible bacteria which are not found in incompatible bacteria and whether pathogens contain common proteins which non-pathogens do not. Use of the protein composition of microbial cell walls as a means of classification has been reported for mycoplasmas (Rottem and Razin, 1967) and for Streptococcus group A (Larsen et al., 1969).

A) Polyacrylamide gel electrophoresis of bacterial cell wall proteins

Cell walls of P. mors-prunorum (incompatible in RM and CW), P. phaseolicola race 1 (incompatible in RM but compatible in CW), P. phaseolicola race 2 (compatible in RM and CW) and P. fluorescens (saprophyte) were obtained (Materials and Methods 8), and the amount of total protein in each was determined (Materials and Methods 9). Cell walls were solubilised using phenol : acetic acid : water (2 : 1 : 0.5 w/w/v) and to each sample sucrose was added to a final concentration of 5% (w/v).

Polyacrylamide gels, pH 2.3, containing 7.5% acrylamide, 35% (v/v) acetic acid and 6 M urea were prepared. The electrophoresis buffer was 10% acetic acid. To each gel 80 µl of solubilised preparations containing 90 µg protein was added, and electrophoresis was carried out for 3.5 h from anode to cathode at a constant current of 5 ma/tube.

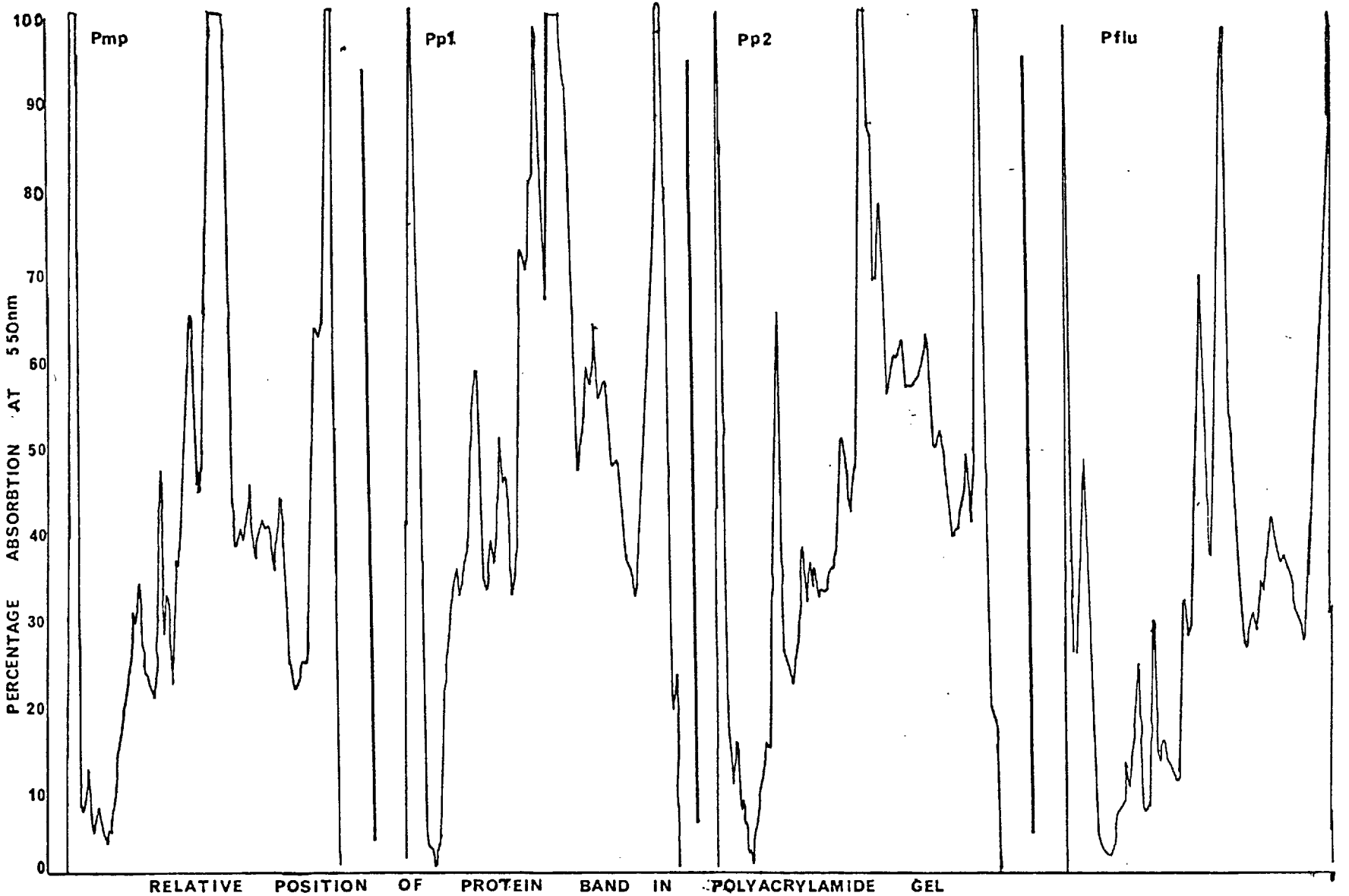


Figure 6. Electrophoretic and densitometry profiles of the proteins of bacterial cell walls obtained by polyacrylamide gel electrophoresis. Each gel was loaded with c. 90 µg/ml of solubilised protein sample. PMP - P. mors-prunorum; Pp1 - P. phaseolicola race 1; Pp2 - P. phaseolicola race 2; P.flu - P. fluorescens.

Table 39. Polyacrylamide-gel electrophoresis analysis of bacterial cell walls

Protein band number	Position of protein bands in units from top of gel: Bacterium			
	P. phaseolicola 1	P. phaseolicola 2	P. mors-prunorum	P. fluorescens
1	13	5	3	12
2	16[a]	11	14	14
3	19	15[a]	16[a]	16[a]
4	22[b]	22[b]	19	20
5	32[a]	28	23[b]	29
6	36	33[a]	25	33[a]
7	39[a]	39[a]	33[a]	40
8	44[b]	42	40[a]	46[a]
9	45	44[b]	44[b]	48[a]
10	46[a]	46[a]	46[a]	54
11	48[a]	48[a]	48[a]	56
12	52	50	55	68[a]
13	58[c]	58[c]	56	71

Table 39. (cont'd.)

Protein band number	Position of protein bands in units from top of gel — Bacterium			
	P. phaseolicola 1	*P. phaseolicola* 2	*P. mors-prunorum*	*P. fluorescens*
14	60[c]	61[c]	66[b]	73[a]
15	66[b]	63	69a	
16	69[a]	65	72[a]	
17	71	66[b]		
18	72[a]	67		
19		69[a]		
20		73[a]		

a. Proteins common to all four bacteria
b. " " " the plant pathogens
c. " " " *P. phaseolicola*

Gels were stained in 2% w/v Coumaisse brilliant blue in 7% (v/v) acetic acid. Stained gels were scanned in a Gilford spectrophotometer at 550 nm, such that 1 cm of gel was equivalent to 1 cm on the chart paper. Each protein band in the gel gave a peak on the paper. The position of the bands in the gels were compared by measuring the distance from the top of the gel (on chart paper) to the various bands down the gel. Those bands which appeared at the same distance from the top were assumed to be similar proteins. Using this method any similarity or dissimilarity in cell wall proteins of compatible, incompatible and saprophytic bacteria could be compared. The results are given in Table 39 and are recorded as units. In Figure 6 the electrophoretic and densitometry pattern of the bacterial species examined is shown. Plate 4 compares the protein bands of P. mors-prunorum and P. fluorescens obtained by photographing the gels after electrophoresis.

Results in Table 39 show that some proteins were present in the cell walls of all four bacteria at 15-16, 32-33, 39-40, 46, 48, 68-69, 72-73 units, some were common to the pathogens (P. phaseolicola races 1 and 2, P. mors-prunorum) at 44, 66 units and others were common to P. phaseolicola (races 1 and 2) at 50, 58 and 60-61 units. Accuracy is c. $\pm$ 1 unit. The bands (proteins) which all four bacteria possessed were probably proteins characteristic of Pseudomonas spp. generally. Differences within the genus are shown by the bands common to the pathogens which the non-pathogen (P. fluorescens) did not have and at another level by bands which P. phaseolicola races 1 and 2 had but which were absent in the other two bacteria.

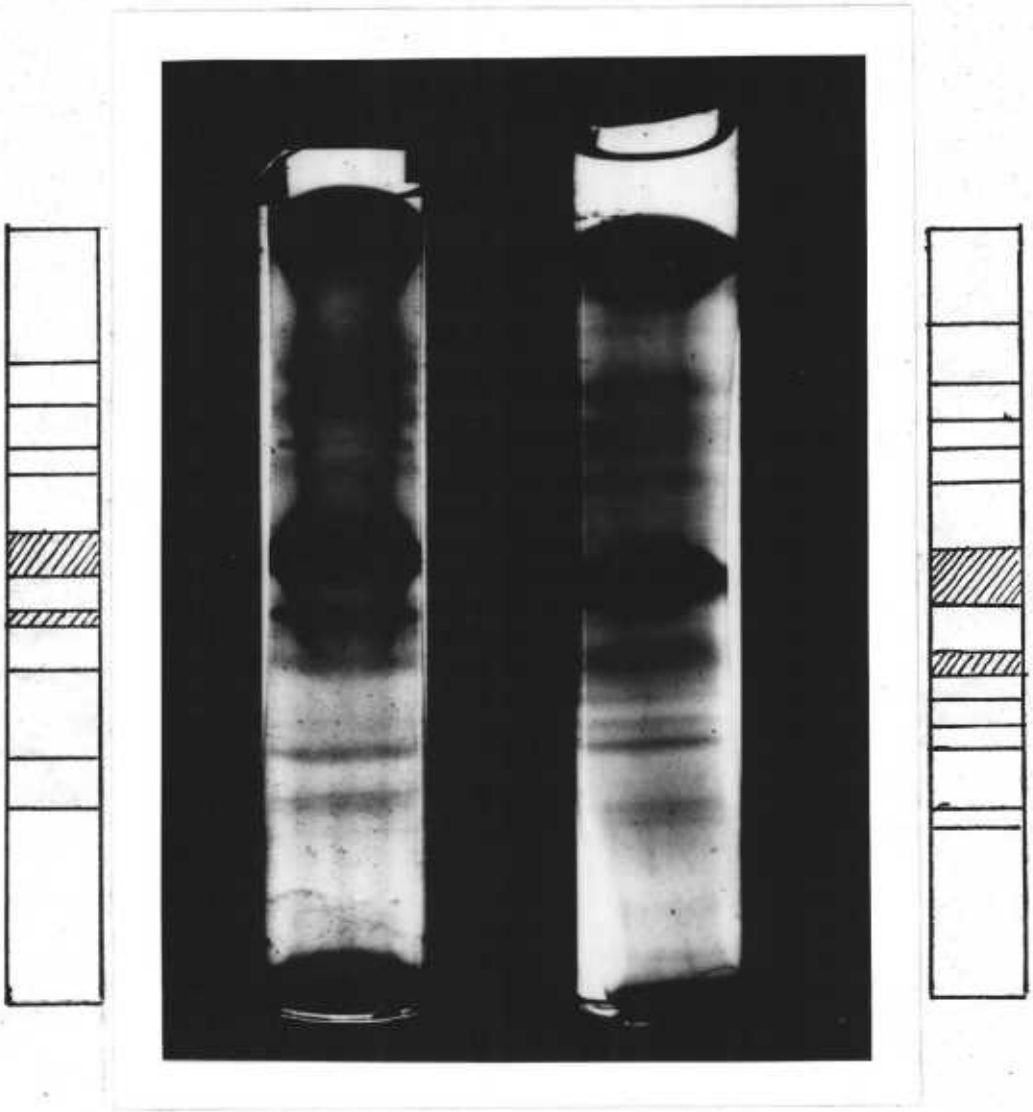


Plate 4. Polyacrylamide gel electrophoresis of proteins from bacterial cell walls. Both gels were loaded with c. 90 µg protein from solubilised preparation. Electrophoresis was conducted for 3.5 h at 5 ma/tube running from anode to cathode. Gel (left) shows separation pattern of proteins in the cell wall of P. fluorescens. Gel (right) shows separation pattern of proteins in the cell wall of P. mors-prunorum.

Further, differences at the race level are apparent in that P. phaseolicola race 1 has a band common with P. mors-prunorum at 19 units which P. phaseolicola race 2 does not have, and that P. phaseolicola race 1 also has bands at 36 and 52 units which are not common to the other three bacteria.

It is generally considered that SR is more specific than HR and that HR is more specific than no response. These views are supported by the fact that P. phaseolicola race 2 (compatible in both RM and CW) has the most protein bands with P. phaseolicola race 1 (incompatible in RM) the next, followed by P. mors-prunorum (incompatible in RM and CW). The saprophyte, P. fluorescens, which causes no visible host response has the fewest protein bands. Of the twenty different proteins in P. phaseolicola race 2 only twelve are shared with P. phaseolicola race 1 and only ten are shared with P. mors-prunorum. Thus P. phaseolicola race 2 has eight proteins, or c. a third of the cell wall complement, which are different from those of P. phaseolicola race 1.

B) Amino acid analysis

In order to further analyse the differences in cell wall composition, amino acid analysis of the cell walls of four bacteria was carried out. However, in this case the saprophyte, P. fluorescens, was replaced with another incompatible bacterium, P. lachrymans.

Hydrolysates of dried bacterial cell walls of P. lachrymans, P. mors-prunorum and P. phaseolicola races 1 and 2 were prepared using 6 N HCl (Materials and Methods 10). The hydrolysed samples were then analysed on an

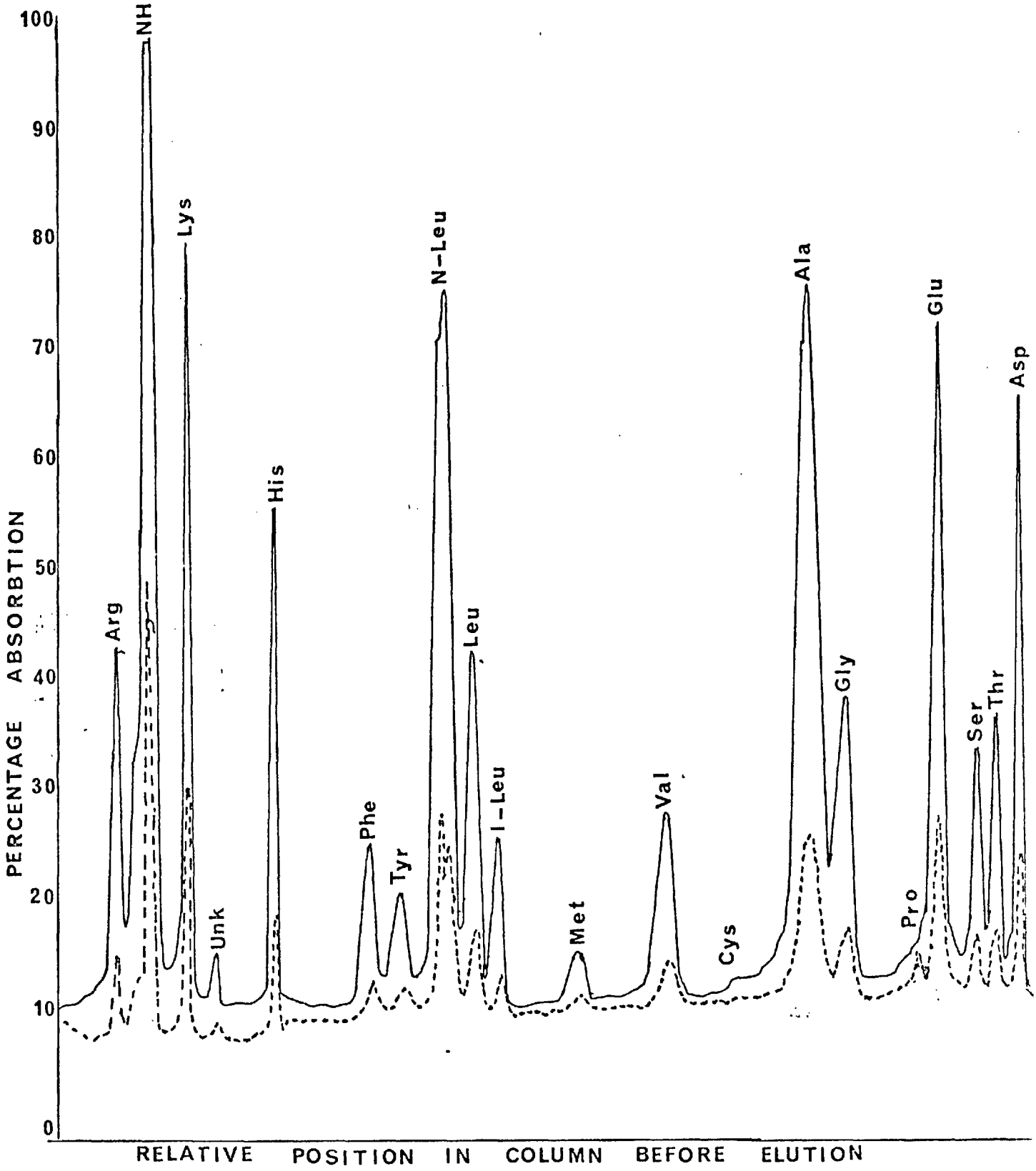


Figure 7. Amino acid analysis of bacterial cell wall of P. phaseolicola race 1. (——) Absorption of amino acids at 550 nm; (- - - -) Absorption of amino acids at 420 nm.

Table 40. Amino acid composition of bacterial cell walls

Amino acid	Amino acid content[a] (Gram %) Bacterium			
	P. phaseolicola 1	P. phaseolicola 2	P. mors-prunorum	P. lachrymans
Asparagine	7.18	9.99	12.02	9.12
Threonine	4.15	4.69	5.54	3.83
Serine	3.89	4.01	4.81	4.59
Glutamic acid	9.00	7.97	10.41	8.99
Proline	3.36	2.34	2.90	2.97
Unknown 1		-	-	1.45
Glycine	6.59	4.95	5.77	7.56
Alanine	19.68	6.60	8.63	14.86
Cysteine	-	0.96	-	-
Valine	5.23	7.18	7.91	5.93
Methionine	-	4.55	3.72	
Unknown 2	-	1.39	-	
Iso-leucine	3.06	6.88	4.21	3.76
Leucine	6.98	8.47	8.34	7.66

Table 40. (cont'd.)

Amino acid	Amino acid content[a] (Gram %) Bacterium			
	P. phaseolicola 1	P. phaseolicola 2	P. mors-prunorum	P. lachrymans
Tyrosine	4.26	5.14	4.18	6.06
Phenylalanine	3.65	4.47	4.57	5.63
Unknown 3	-	1.63	-	-
Unknown 4	-	1.83	-	1.43
Histidine	6.60	2.92	1.97	2.58
Lysine	8.85	6.52	6.59	6.59
Ammonia	1.75	1.39	1.62	2.09
Unknown 5	-	-	-	2.68
Arginine	5.69	6.01	7.01	3.59

a. Recorded from chart at 420 nm

amino acid analyser which used the ninhydrin reaction. Nor-leucine was added to the sample and used as a marker (Materials and Methods 10). Results were measured spectroscopically on two charts at 550 and 420 nm. Two charts were required since at 550 nm the absorbance of proline is poor. Results are given in Table 40 and are recorded as gram % amino acid. Figure 7 shows the chart readings for P. phaseolicola 1 at 420 and 550 nm.

Results in Table 40 show that the cell walls of P. mors-prunorum, P. phaseolicola races 1 and 2 and P. lachrymans contained most of the amino acids for which the analyser was programmed. However, cysteine occurred only in the cell wall of P. phaseolicola race 2 and methionine was absent from the cell walls of P. phaseolicola race 1 and P. lachrymans. In addition to cysteine and methionine other amino acids, for which the analyser was not programmed, were found to be present in the cell walls of some but not other species. These amino acids are referred to as 'unknowns' 1-5 in Table 40. Unknowns 1 and 5 occurred only in the cell wall of P. lachrymans and unknowns 2 and 3 occurred only in the cell wall of P. phaseolicola race 2. Unknown 4 was found in the cell walls of P. phaseolicola race 2 and P. lachrymans.

Apart from qualitative differences in amino acid composition of the cell walls of the bacteria, quantitative differences were also found. The cell wall of P. phaseolicola race 1 contained three fold more alanine than that of P. phaseolicola race 2 and two fold more alanine than that of P. mors-prunorum. Similarly the cell wall of P. phaseolicola race 1 contained c. three fold more histidine

than the cell walls of the other three species. Also, the cell wall of P. phaseolicola race 2 contained c. two fold more iso-leucine than cell walls of the other three bacteria and the cell wall of P. lachrymans contained c. half the arginine of the cell walls of the other three bacteria.

The qualitative and quantitative differences in amino acid composition of the bacterial cell walls probably reflects the differences in the protein composition of the same cell walls as revealed by electrophoresis.

(5) Microscopy and analysis of bean plant tissue undergoing the susceptible reaction caused by P. phaseolicola

Halo blight of beans (Phaseolus vulgaris) is characterised by the appearance on leaves of watersoaked translucent spots, usually with a chlorotic halo, by systemic interveinal chlorosis of developing leaves and by the general dwarfing of plants.

There is some evidence that chlorosis in upper leaves is caused by phaseotoxin, an exotoxin produced by the causal organism P. phaseolicola in lower leaves. Treatment of leaves with culture filtrates mimics disease symptoms (Rudolph and Stahmann, 1966). Phaseotoxin specifically inhibits activity of ornithine carbamoyltransferase (OCT) and this leads to an accumulation of ornithine which is thought to be causally related to chlorosis (Patil and Gnanamanickam, 1976).

Past work has not established a clear relation between

the spread of the bacterium and development of trifoliate chlorosis (Waitz and Schwartz, 1956). However, Hildebrand and Schroth (1971) recovered the bacterium from 39 out of 40 trifoliate leaves following inoculation of monofoliate leaves.

There are only a few studies of ultrastructural modifications of plant tissues induced by toxic substances produced by plant pathogens. Victorin, from Helminthosporium victoriae, causes breakages in plasma membranes, disruption of chloroplast lamellae and abnormally large vesicles to develop in Golgi apparatus (Hanchey et al., 1968; Luke et al., 1966). Toxin from Corynebacterium sepedonicum damaged membranes of chloroplasts, mitochondria, the plasma membrane and the structural integrity of the cell wall of tomato plants (Strobel and Hess, 1968). Leseman and Rudolph (1969) reported that the toxin from P. phaseolicola caused a helix like aggregation of ribosomes but that other organelles were hardly affected. Other work showed that infection of tobacco leaves with P. tabaci causes a swelling and then decrease in the stroma of the chloroplasts with no starch accumulation. Also groundplasm of the mitochondria decreased and the plasma membrane separated from cell walls (Goodman and Plurad, 1971). Infection of bean leaves with P. phaseolicola showed disruption of chloroplast granae and the accumulation of large starch grains, although membranes remained intact. Other cellular organelles appeared normal (Bajaj et al., 1969). Siegge and Epton (1976) reported that in monofoliate bean leaves infection by P. phaseolicola caused breakdown of the chloroplast and an increase in the cytoplasmic ribosomes. In view of the above a transmission

and scanning electron-microscopical study was undertaken to try to determine the role of P. phaseolicola and phaseotoxin in halo blight.

A) Transmission electron-microscopy and analysis of trifoliate leaves following inoculation of monofoliate leaves with P. phaseolicola

Monofoliate leaves of bean cv. Canadian Wonder were inoculated by injection with suspensions of Pseudomonas phaseolicola (c. 3.0×10^8 cells/ml). Following inoculation, bacterial numbers in the site of inoculation and in trifoliate leaves at 1, 2, 4, 8, 12, 16 days were determined (Materials and Methods 3). In the monofoliate leaves, disks were taken from the inoculation zone; in trifoliate leaves they were removed from the junction of the petiole and lamina, with one disk per lateral and two per terminal leaflet. Results are given in Table 41 as viable bacterial numbers/leaf disk.

Chlorophyll from trifoliate leaves was extracted by the method of MacKinney (1941) at 3, 7, 10 and 13 d after inoculation of monofoliate leaves (Materials and Methods 12). Results are given in Figure 8 as mg chlorophyll/g fresh wt leaf. Total phenolics were also extracted from trifoliate leaves at 3, 10 and 14 d after inoculation of monofoliate leaves. Total phenolics were estimated by the Folin method (Materials and Methods 11) and results are expressed in Table 42 as catechol equivalents in µg/g fresh wt. of leaf.

Trifoliate leaves were also examined by light and electron microscopy at 3, 10 and 14 days after inoculation

Table 41. Multiplication of Pseudomonas phaseolicola race 2 in bean leaves following inoculation of monofoliate leaves

Time (d) after inoculation	Bacterial Numbers/Leaf Disk[a]	
	Monofoliate Leaf	Trifoliate Leaf
0	5.2×10^5[b]	8.2×10^1[b]
0.5	4.5×10^5	2.2×10^2
1	3.0×10^6	6.5×10^2
2	1.5×10^7	5.5×10^2
4	1.5×10^8	5.0×10^3
8	3.0×10^7	2.5×10^4
12	1.0×10^7	1.3×10^4
16	6.5×10^6	4.5×10^3

[a] Leaf disk 4.5 mm diameter

[b] Mean of 6 replicates

of monofoliate leaves. For light microscopy 5 μ sections of fresh leaf material was taken and stained for phenolic compounds using the nitrose stain, ferric chloride stain, ammonia vapour; for protein using coumaisse brilliant blue, bromophenol blue; and for lipids using Sudan IV reagent (Materials and Methods 13). For electron-microscopy, leaf material was fixed in glutaraldehyde and osmium tetroxide, embedded in eopn 1A : 1B resin, sectioned on an ultramicrotome to c. 600 Å and the sections stained in uranyl acetate and lead citrate (Materials and Methods 13).

The results in Table 41 show that bacterial numbers in monofoliate and trifoliate leaves increase at a similar rate for about eight days and then decrease when chlorosis normally begins to appear. In general, numbers in the monofoliate leaves were at least a thousand fold/disk greater than in the trifoliate leaves over the fourteen day period.

Three days after injection the inoculation area of monofoliate leaves had collapsed and was pale bronze in colour. At this stage no effects were observed in trifoliate leaves. After ten days monofoliate leaves had a watersoaked margin, usually with a chlorotic halo, around the inoculated area and a mild interveinal chlorosis appeared in trifoliate leaves. Ultrastructurally a marked change in the trifoliate leaves had occurred as shown in Plate 6. Chloroplasts now contained large starch grains (Plate 6c) and the vacuoles contained one or more of three types of darkly staining inclusions. Type 1 (Plates 6c, 7) appeared as a darkly staining, agglutinated amorphous matrix within the vacuole. This was different from the

ABBREVIATIONS USED IN PLATES

CHL, chloroplast; CW, cell wall; ER, endoplasmic reticulum; G, golgi body; GI, granular inclusion; M, mitochondrion; NS, normal vacuolar inclusion; T1-3, three types of vacuolar inclusion; SG, starch grain; VAC, vacuole.

Plate 5a. Trifoliate choroplasts 14 d after inoculation of monofoliate leaves with water (x 12,300).

Plate 5b. As plate 1a but showing the normal vacuolar inclusion (NS) (x 35,000).

Plate 6a. Trifoliate cells 10 d after inoculation of monofoliate leaves with P. phaseolicola showing granular inclusions in the middle lamella (GI) and vacuolar inclusion (T1) (x 20,190).

Plate 6b. As plate 2a showing high power of granular inclusion (GI) and vacuolar inclusion (T3) (x 41,700).

Plate 6c. As plate 2a showing chloroplast containing numerous starch grains (SG) but with chloroplast membranes and granae intact (x 27,100).

Plate 7. Trifoliate leaf 14 d after inoculation of monofoliate leaves with P. phaseolicola. Note large and numerous starch grains in chloroplasts, vacuolar inclusions T1 and NS in some vacuole; also T2 inclusion in

different vacuole (x 12,300).

Plate 8. As plate 3, showing high power of chloroplasts with no degeneration of membrane or granae, golgi body (G) and mitochondrion (M) and vacuolar inclusion on tonoplast (T2-T3) (x 85,800).

Plate 9. As plate 3, showing high power of two cells. Note osmiophilic properties of T3, the high incidence of ER and ribosomes, the intact tonoplast and plasma membrane (x 106,800).

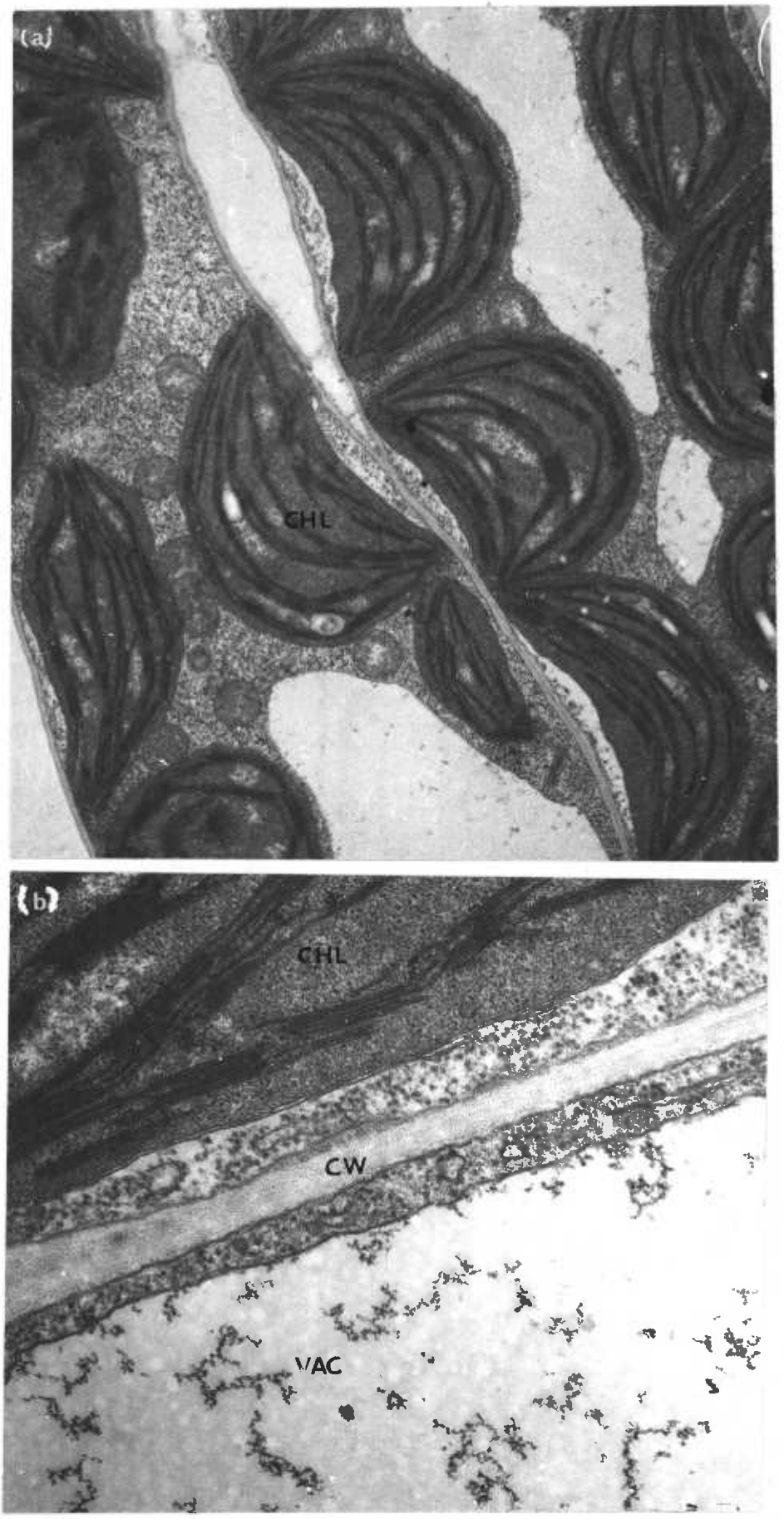


Plate 5.

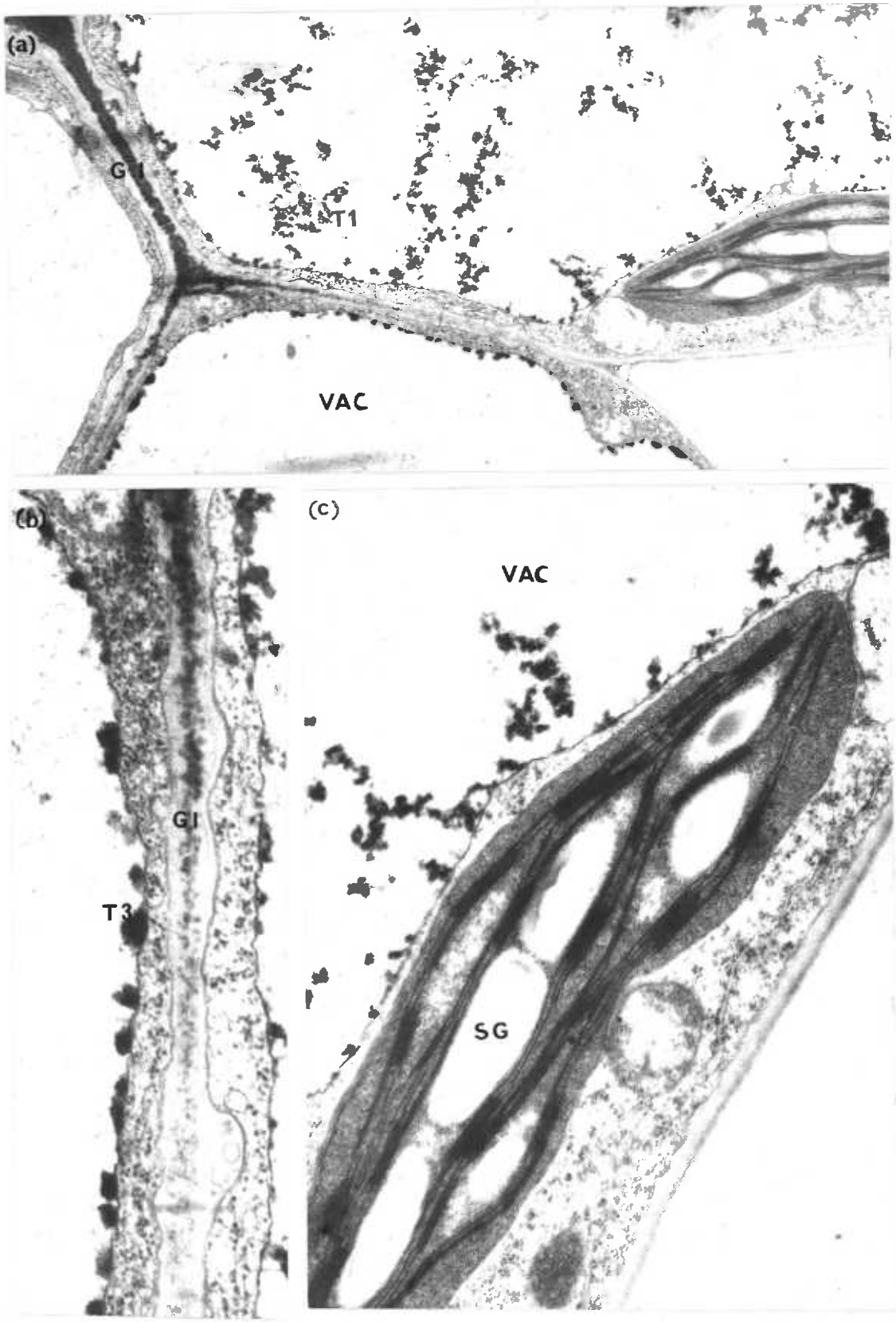


Plate 6.

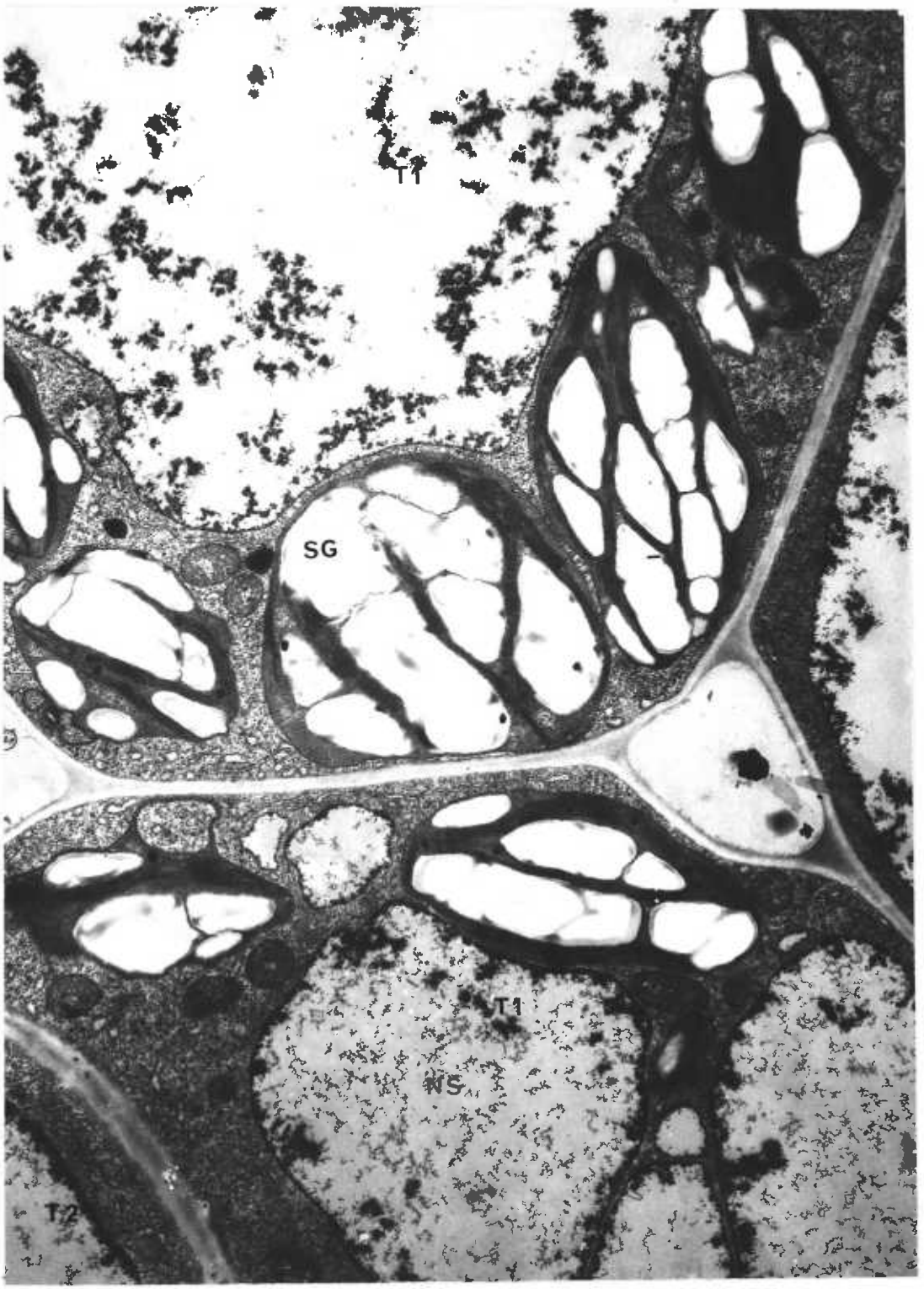


Plate 7.

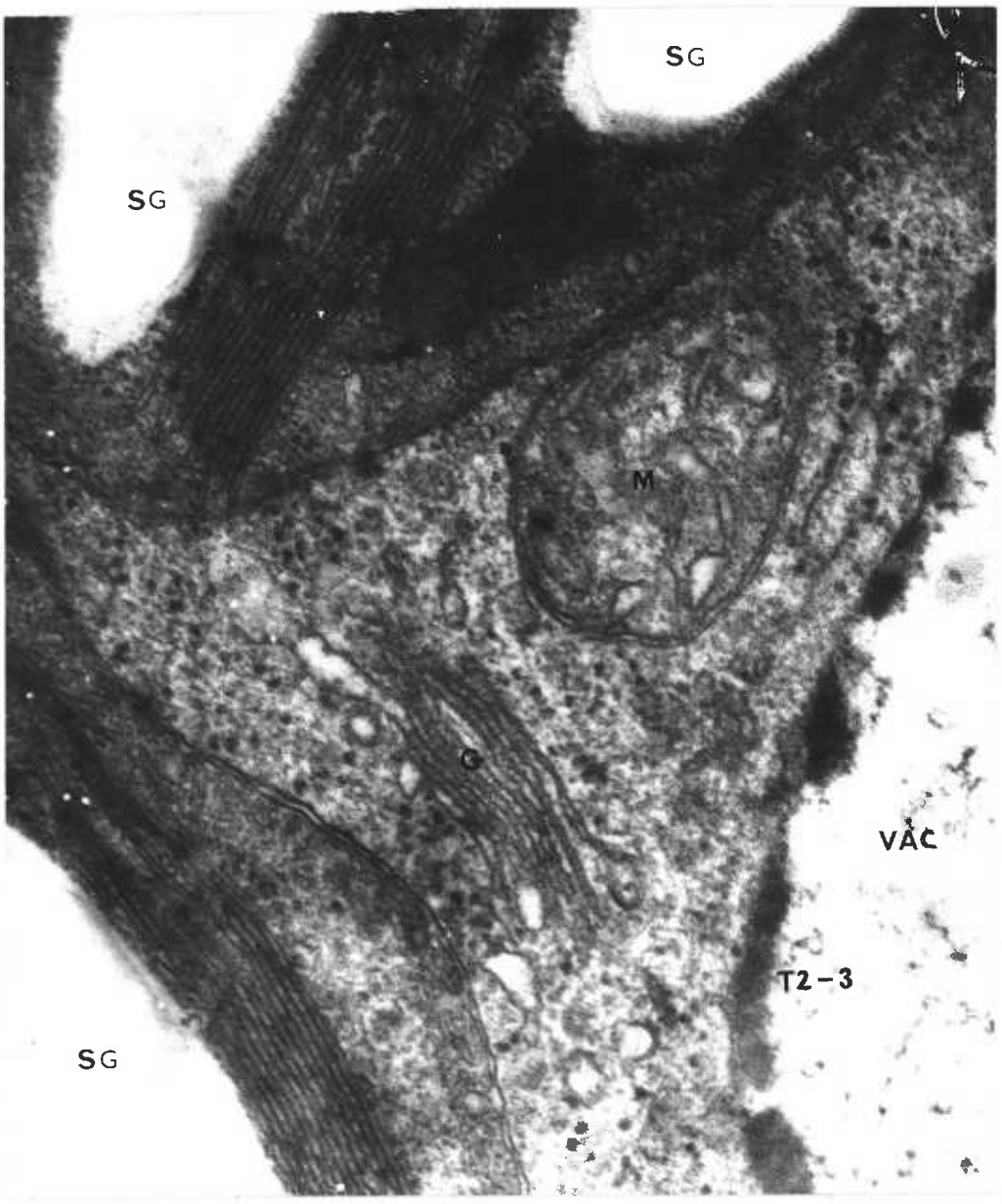


Plate 8.

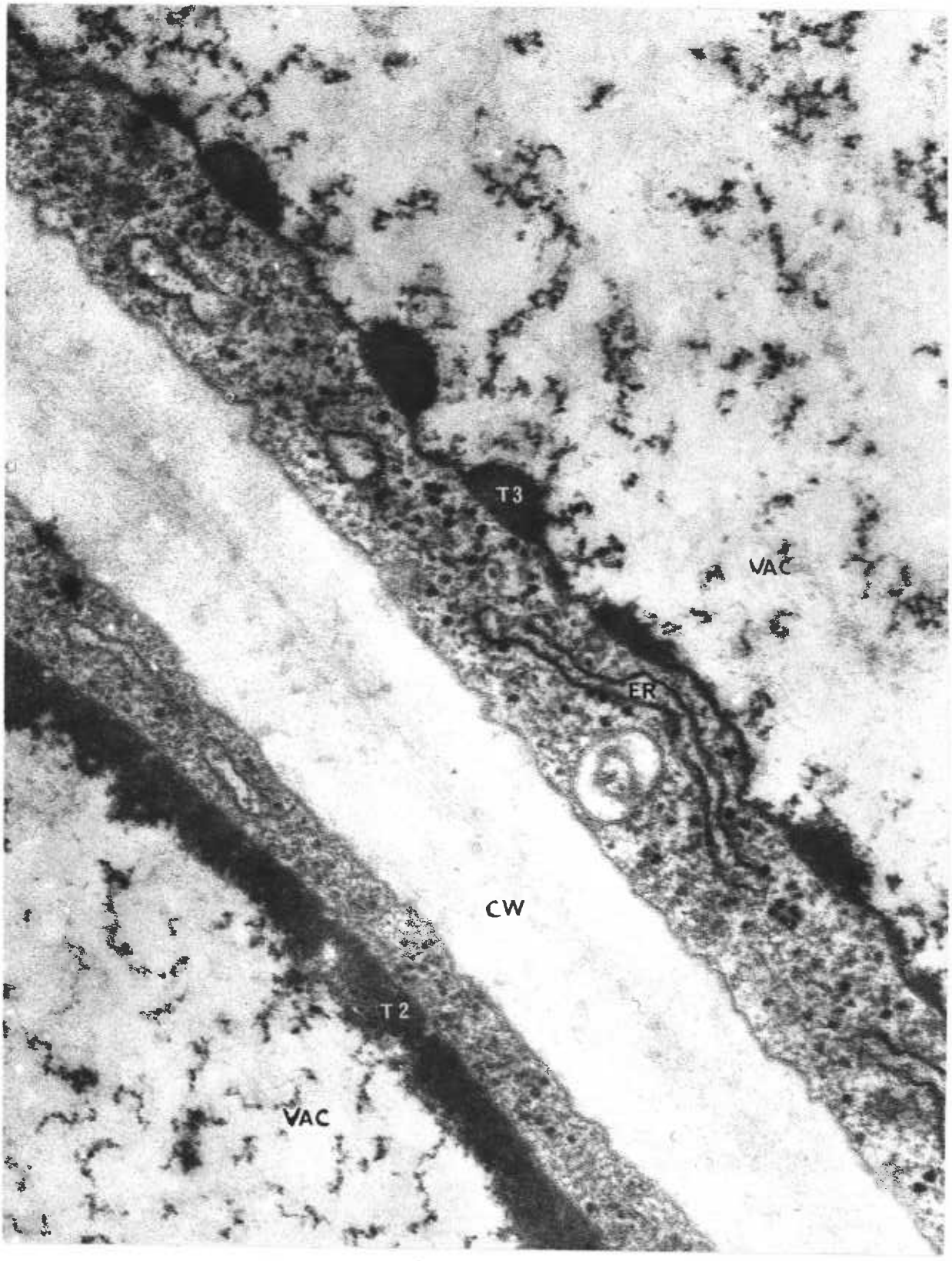


Plate 9.

contents normally present in the vacuole (NS) which were less darkly stained and more fibrous (Plates 5b, 7). Type 2 occurred as a densely fibrous matrix lining the tonoplast membrane (Plates 7, 8, 9), and Type 3 was a globular coalescent deposit lying on the tonoplast (Plates 6b and 9). In Plate 9, type 3 seems to directly adjoin the tonoplast membrane suggesting a lipoid content.

Additional to the vacuolar inclusions, a granular inclusion (GI) occasionally occurred within the middle lamella region of cell walls (Plates 6a, 6b). Other organelles in cells containing one or more of these inclusions appeared normal.

After fourteen days monofoliate leaves had died or become very chlorotic and desiccated, and the trifoliate leaves exhibited extreme interveinal chlorosis. Ultrastructurally the chloroplasts of trifoliate leaves were so full of starch as to displace the granae, although structurally the chloroplast appeared normal (Plate 8). Vacuolar inclusions (Types 1-3) were now more common and membranes less distinct as the groundplasm stained more intensely. Other organelles appeared normal except for the higher incidence of endoplasmic reticulum and increased concentration of ribosomes. In both cases amounts appeared to be at least double that of the controls.

The effects of inoculation on chlorophyll levels in trifoliate leaves are shown in Figure 8. After fourteen days levels were approximately half those of leaves in plants injected with water or suspensions of avirulent bacteria. In all cases there was a steady decline in chlorophyll levels but the rate was c. twice as fast after

Figure 8.

Chlorophyll content of trifoliate leaves following monofoliate inoculation of Pseudomonas spp. (●——●), water control; (△----△), P. mors-prunorum; (○—○), P. phaseolicola race 1; (■——■), P. phaseolicola race 2. Bacterial suspensions 3.0 x 10^8 cell/ml. All readings mean of three replicates.

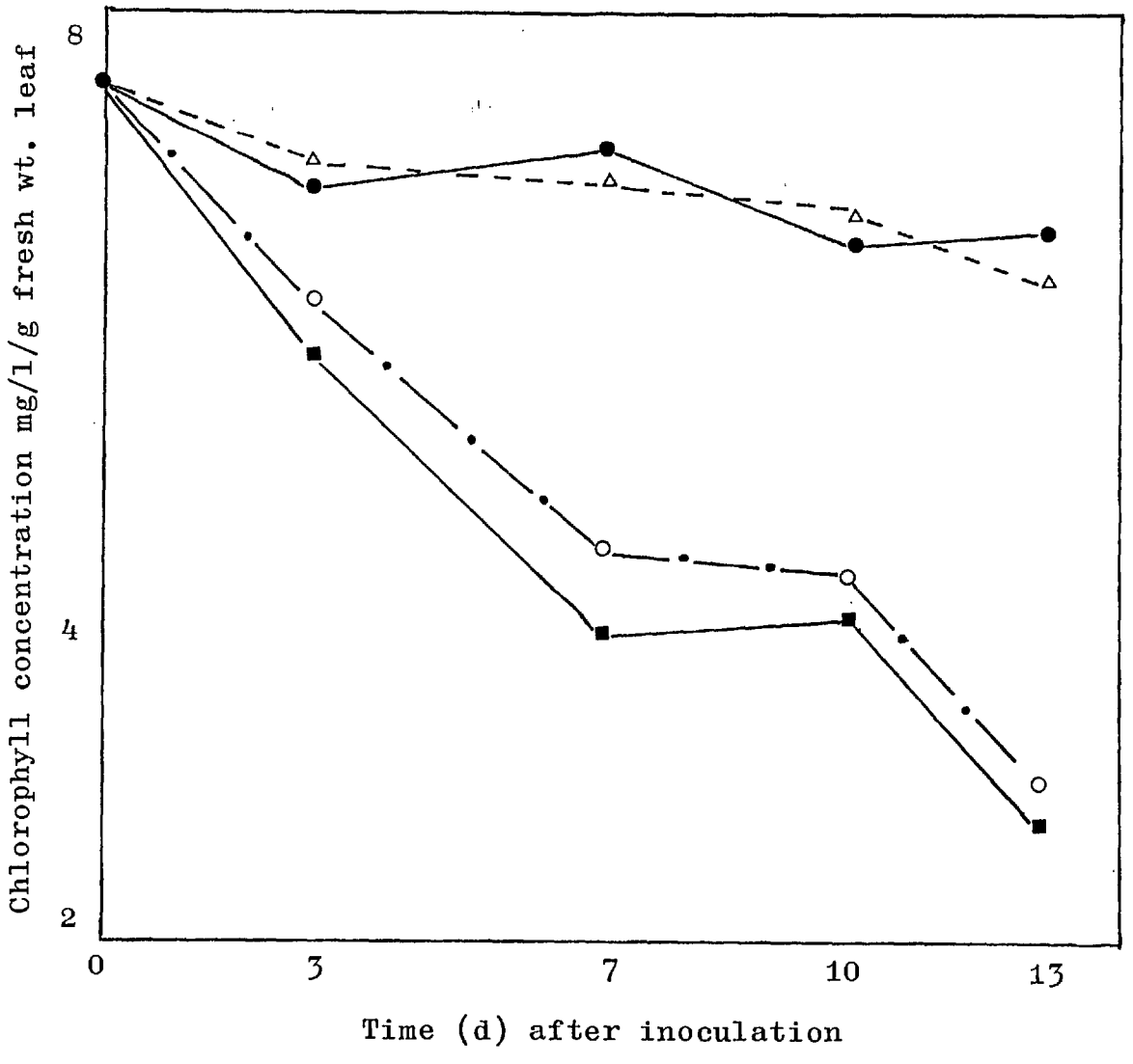


Table 42. Total phenolic compounds in trifoliate leaves following inoculation of monofoliate leaves by Pseudomonas phaseolicola race 2

Time (d) after inoculation	Total phenolic content µg/g fresh wt. leaf	
	Water	P. phaseolicola race 2
0	[a]401(388,414)	[a]416(380,432)
3	412(405,419)	408(430,386)
10	397(364,430)	452(475,429)
14	428(399,457)	539(566,512)

[a] Mean for two replicates in brackets

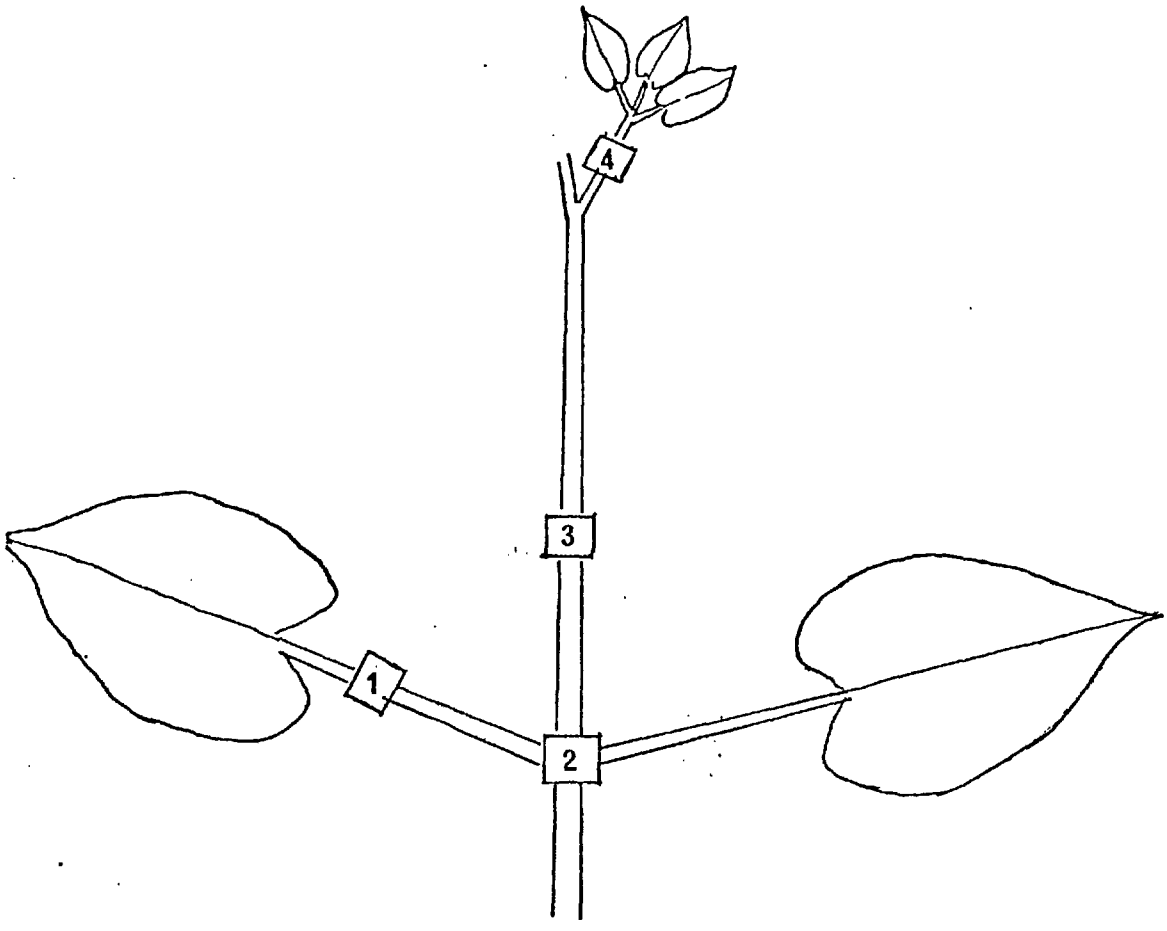
inoculation with virulent bacteria.

Histochemical tests on tissue from chlorotic trifoliate leaves to determine the nature of the vacuolar inclusion (Type 1-3) were carried out. Tests for phenolic compounds gave positive reactions only in the phloem parenchyma. Reactions for lipids (Sudan IV) and proteinaceous material (Coumaisse and Bromophenol blue) were no different from those in the controls.

In Table 42 the amount of total phenolic in trifoliate leaves over fourteen days was compared after inoculation of monofoliate leaves with P. phaseolicola race 2 or water. From Table 42 it can be seen that there were no increases in total phenolic compounds until ten days after inoculation when c. 30 µg is noted, but by fourteen an increase of c. 100 µg over the original concentration has occurred.

B) Scanning electronmicroscopy of bean plant tissue following inoculation of monofoliate leaves with P. phaseolicola

In the previous section, transmission electronmicroscopy and analysis of bean plant tissue undergoing the susceptible reaction showed that numbers of bacteria in the trifoliate leaves increased c. 1000 fold, eight days after inoculation of the monofoliate leaves. However, past work has shown that P. phaseolicola could not be isolated regularly from chlorotic trifoliate leaves following inoculation of monofoliate leaves (Omer and Wood, 1969; Waitz and Schwarz, 1956). In this section, scanning electron-microscopy of plant tissue at 4 regions between



□ POSITION OF SEGMENTS TAKEN FOR SCANNING ELECTRON MICROSCOPY

Figure 9. Diagram of position of segments taken from bean plants following inoculation of monofoliate leaves with P. phaseolicola race 2.

the monofoliate and trifoliate leaves was undertaken to seek further evidence of bacterial presence, to determine paths by which the bacteria move between monofoliate and trifoliate leaves and to relate the role of the bacteria and its toxins such as phaseotoxin in halo blight.

Monofoliate leaves of bean cv. Canadian Wonder were inoculated by injection with suspensions of Pseudomonas phaseolicola race 2 (c. 3.0×10^8 cells/ml). After sixteen days petiole and stem segments (c. 1 cm long) from four regions were taken and examined. Segment 1 was taken from the petiole just below a monofoliate leaf; segment 2, from the node formed by the junction of petioles of the two monofoliate leaves; segment 3, from stem tissue intermediate between the monofoliate node and the first set of trifoliate leaves and segment 4, from the petiole just below a lateral trifoliate leaf, (Figure 9). Stem and petiole segments were dehydrated in an ethanol series before being transferred to acetone and finally immersed in liquid carbon dioxide and critical point dried. Segments were then coated with a gold film c. 480Å thick and viewed in a Cambridge Stereoscan microscope (Material and Methods 13).

Results showed that in segment 1 (Plates 10, 11, 12, 13) P. phaseolicola was mainly confined to the xylem vessels. From Plates 10 and 11 it can be seen that the phloem elements, lying adjacent to the xylem in Plates 12 and 13, contain only one or two bacteria. However, the vessels of the xylem (Plates 12, 13) contain numerous bacteria which in Plate 13 are seen to be covered by an extracellular coating. The internal walls of the vessels

of the xylem (Plate 13) and the sieve tubes of the phloem (Plate 11) also appear to be coated with material similar in structure and electron density to that enveloping the bacteria.

In segment 2, the bacteria are now more numerous in the phloem sieve tube elements (Plates 14, 15). The extracellular material (ED), presumably produced by the bacteria, is clearly seen coating the internal wall of the phloem sieve tube near the bacteria whereas areas of the phloem wall distant from the bacteria appear to have no ED deposit. In the vessels of the xylem (Plate 16), numbers of bacteria appear (by visual assessment) to have increased c. 3-4 fold over numbers in the xylem vessels of segment 1. Thus in both segments 1 and 2 the greatest number of bacteria appears to be moving in xylem. A few bacteria were now also found in the parenchyma cells of the cortex (Plate 17) but the parenchyma cells appear devoid of ED.

In segment 3, numbers of P. phaseolicola in the parenchyma cells of the cortex also appeared to have increased by c. 3 fold and the internal walls of the cells now have thin interlaced strands of ED (Plate 18). Finally in segment 4, the coating of ED in the phloem sieve tubes (Plates 19, 20) is more clearly shown. Overall (including the evidence in Plates 19, 20) it appears that the amount of ED in a cell is related to the number of bacteria present. Thus in cells where large numbers of bacteria are found the coating appears as a dense web covering both the bacteria and the wall of the cell. However, when few bacteria are found within the cells ED appears as interlocking strands thinly covering the internal

ABBREVIATIONS USED IN PLATES

B, bacteria; ED, extracellular material; PC, parenchyma cell; Ph, phloem sieve tube cell; Xy, xylem vessel.

Plate 10. Distribution of phloem and xylem in segment 1 (x 1,050).

Plate 11. Phloem cell adjacent to xylem vessel in Plate 10, showing absence of bacteria but slight deposit of ED (x 2,300).

Plate 12. Low power of xylem vessel in segment 1, showing widespread occurrence of bacteria (x 2,300).

Plate 13. High power of Plate 12 showing bacteria and ED deposit (x 5,800).

Plate 14. Phloem sieve tube cell in segment 2 showing dense deposit of ED around bacteria. Note absence of ED in upper region of cell with few bacteria (x 2,450).

Plate 15. High power of Plate 14 showing the concentration of ED in relation to bacterial size (x 6,200).

Plate 16. Xylem vessel in segment 2 showing an apparent 4-5 fold increase in bacterial numbers over vessel in segment 1 (x 1,100).

Plate 17. Parenchyma cells of cortex in segment 2 showing a few bacteria but no ED (x 1,050).

Plate 18. Parenchyma cell in segment 3 showing c. 3 fold increase in numbers of bacteria over segment 2. Note also the presence of strands of ED now in cell (x 2,450).

Plate 19. Phloem sieve tube cell in segment 4 showing bacteria and deposit of ED as thin strands along the wall (x 5,750).

Plate 20. Another phloem cell in segment 4 showing a higher concentration of ED and bacteria. Note (that in the case cf. above) the change of form of ED from thin interlocking strands to a dense weft covering both the bacteria and the internal wall of the plant cell (x 5,750).

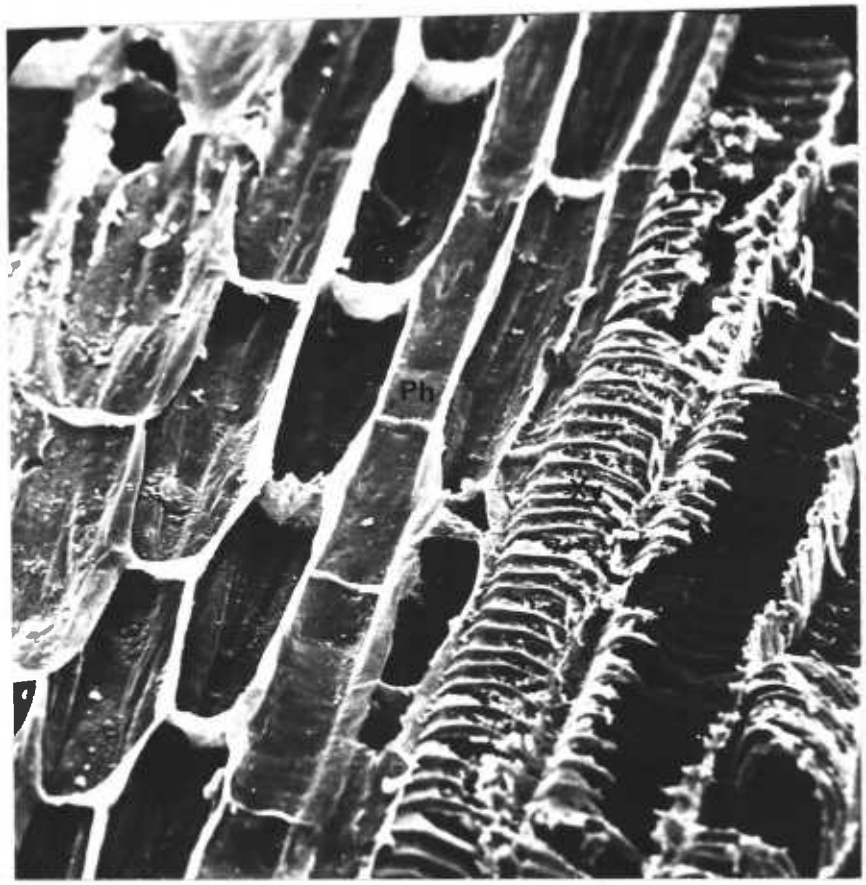


Plate 10.

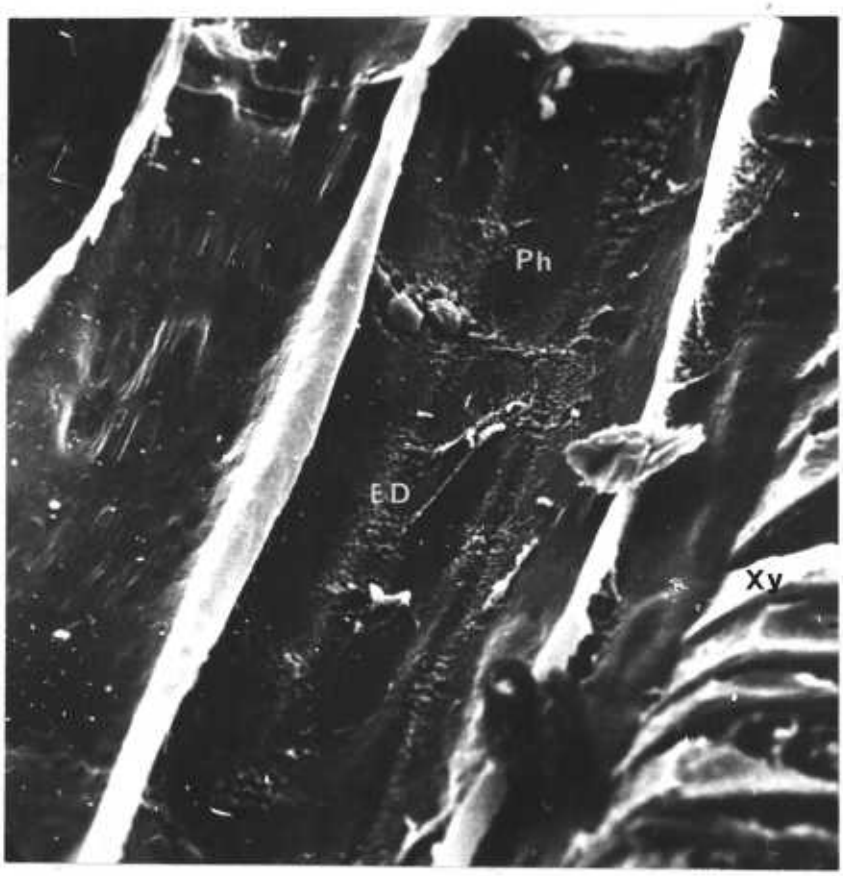


Plate 11.

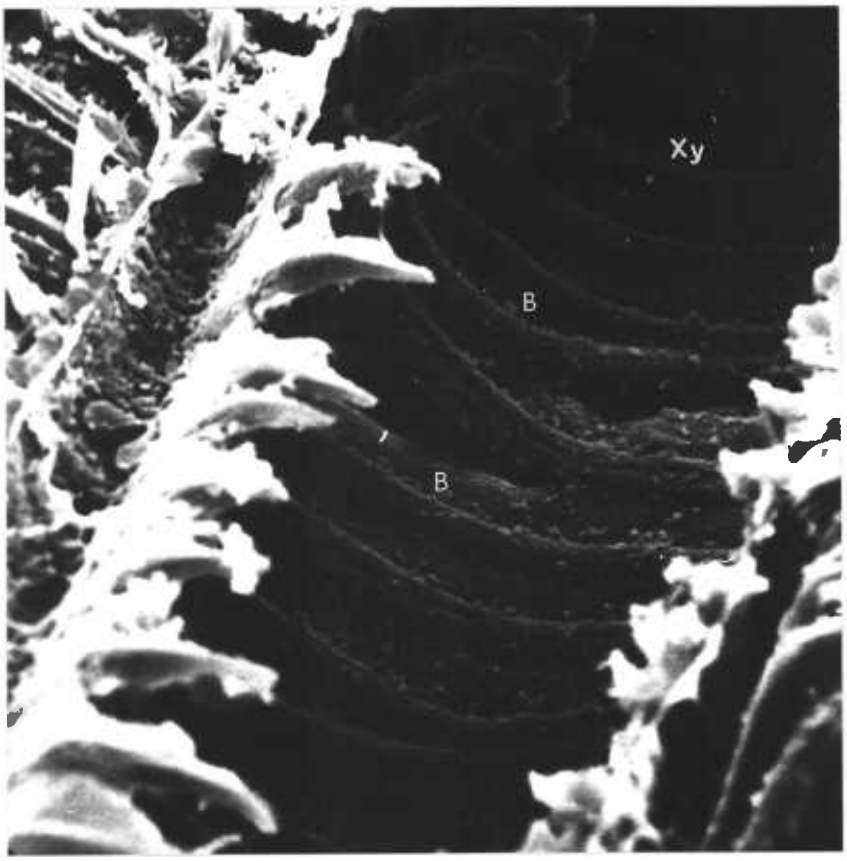


Plate 12.

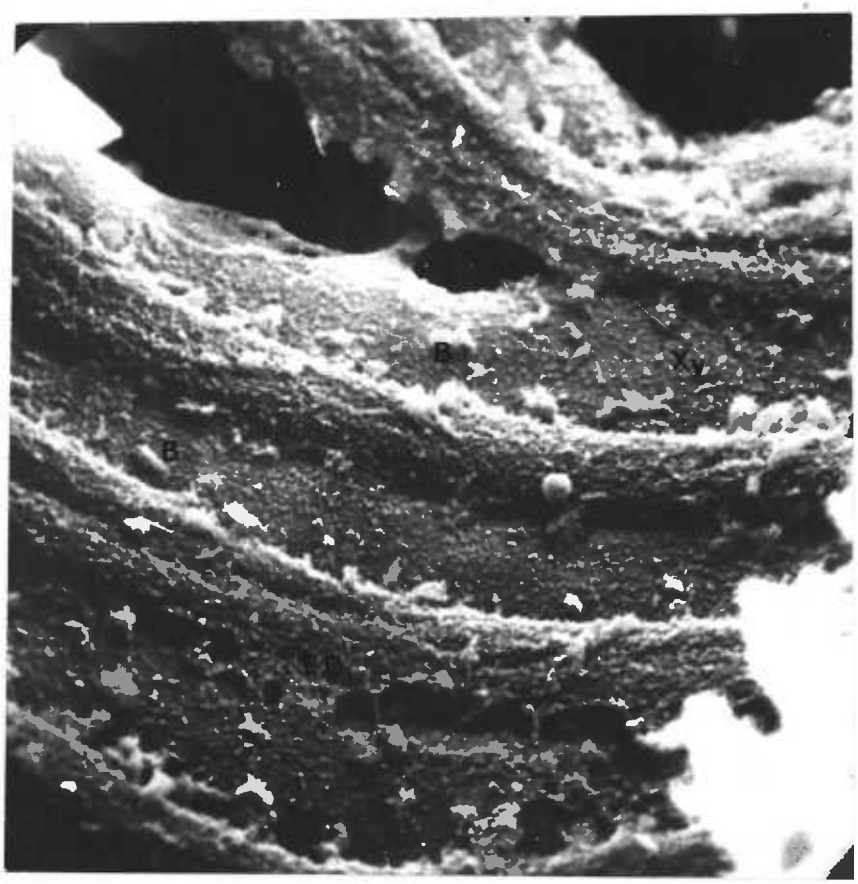


Plate 13.

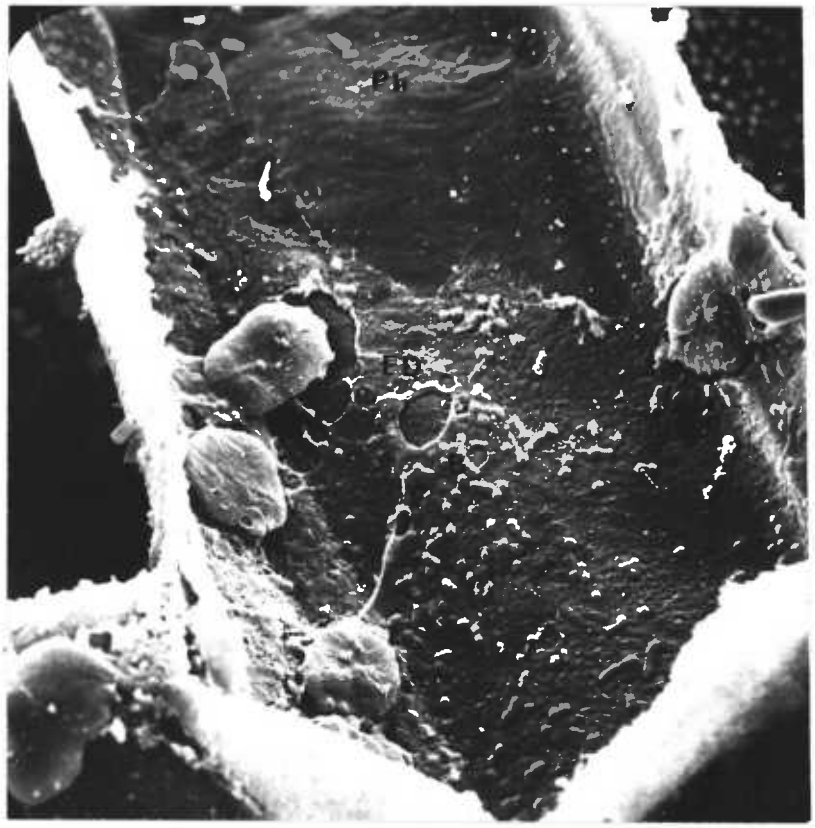


Plate 14.

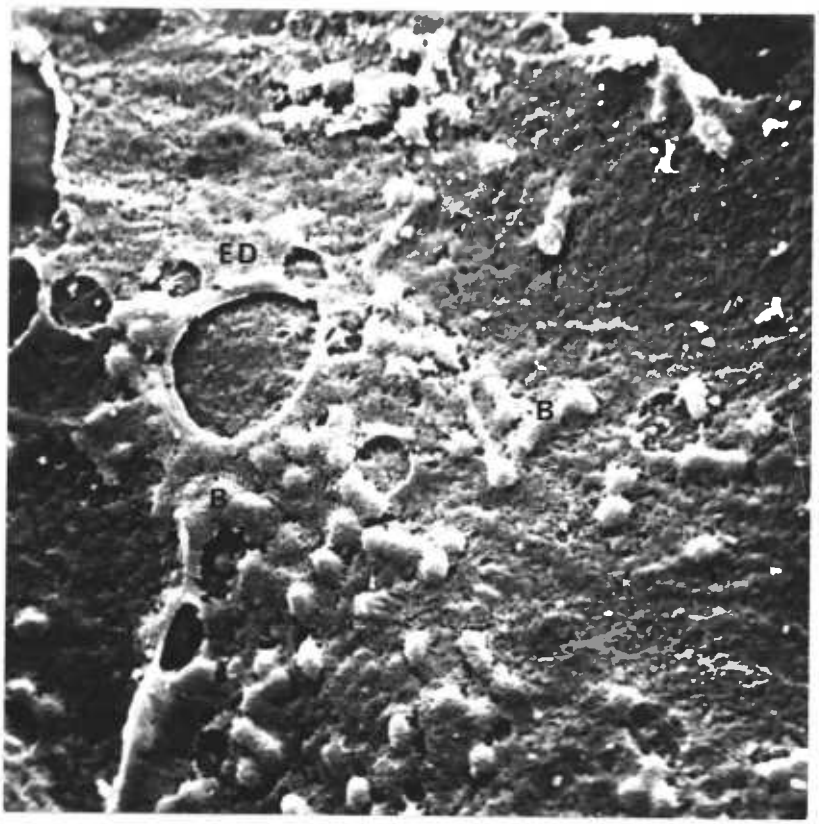


Plate 15.

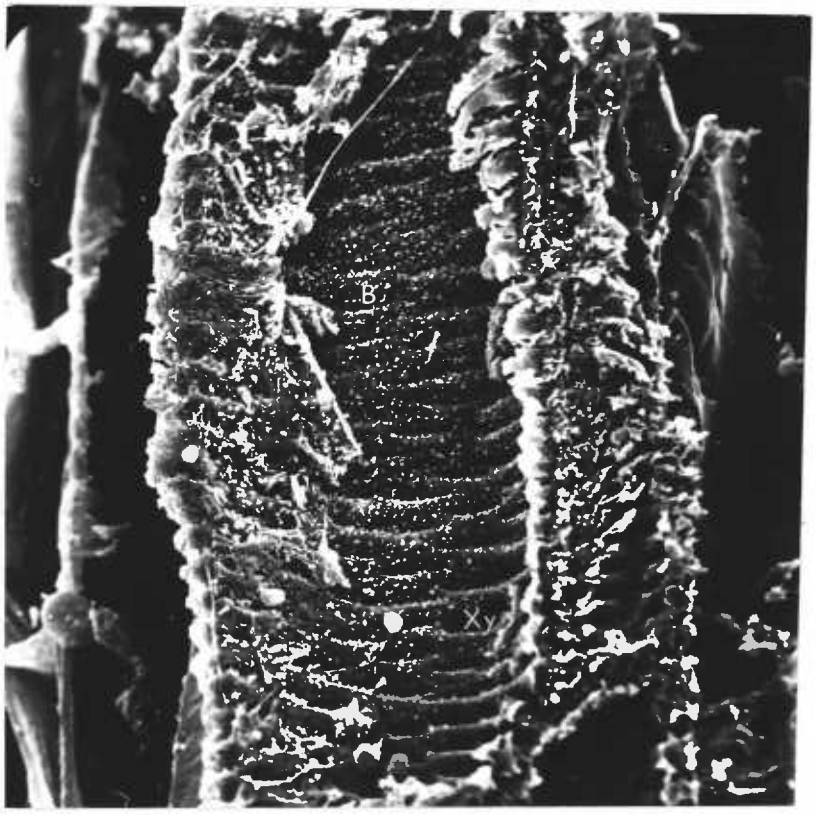


Plate 16.

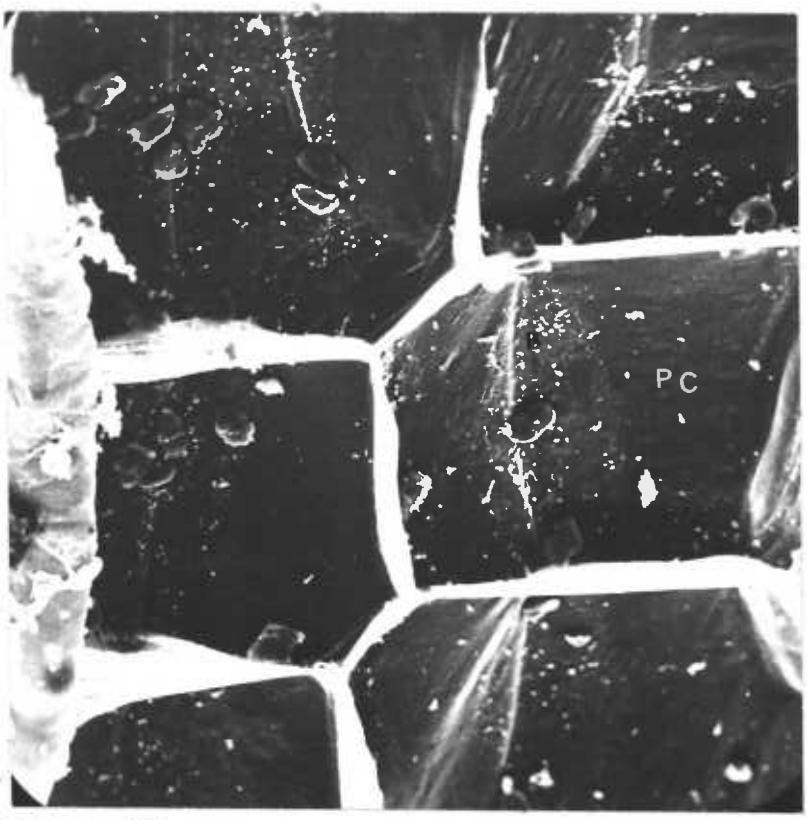
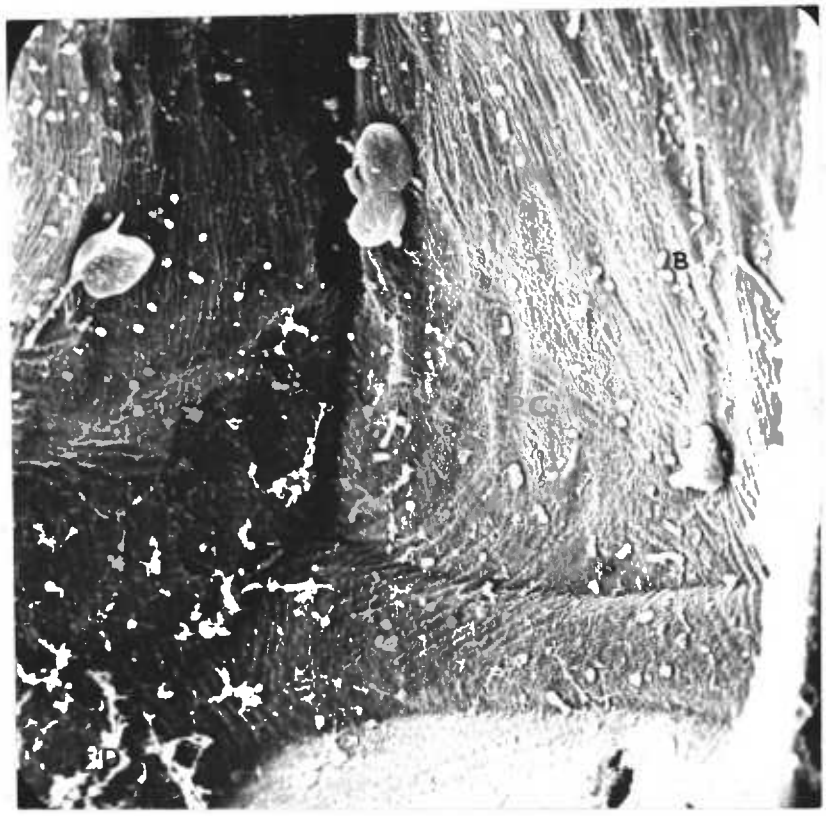


Plate 17.

[redacted]



P[redacted].

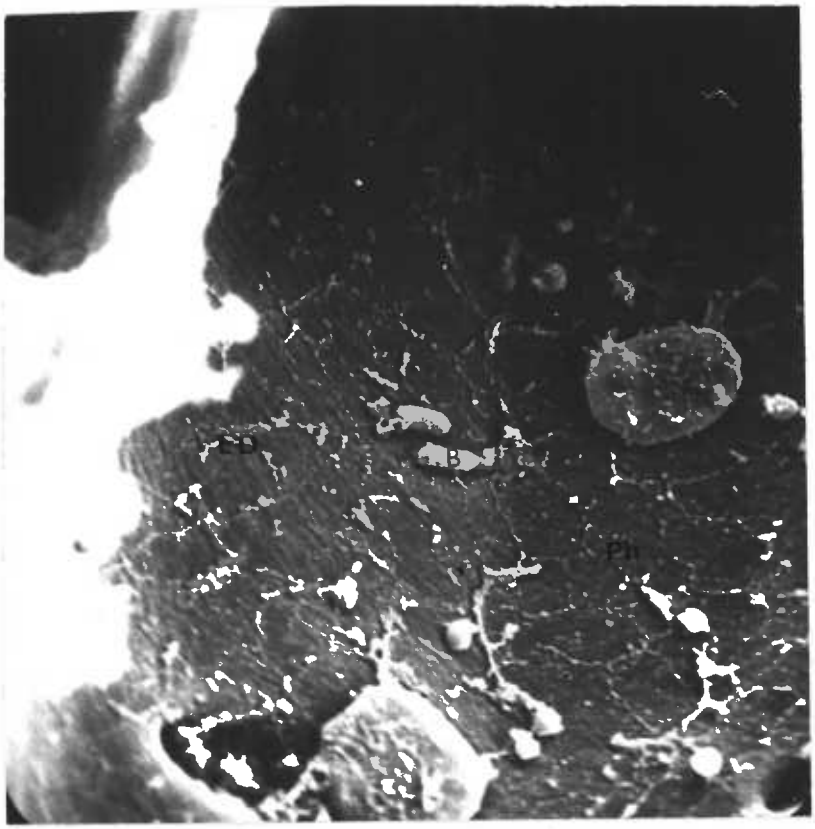


Plate 19.

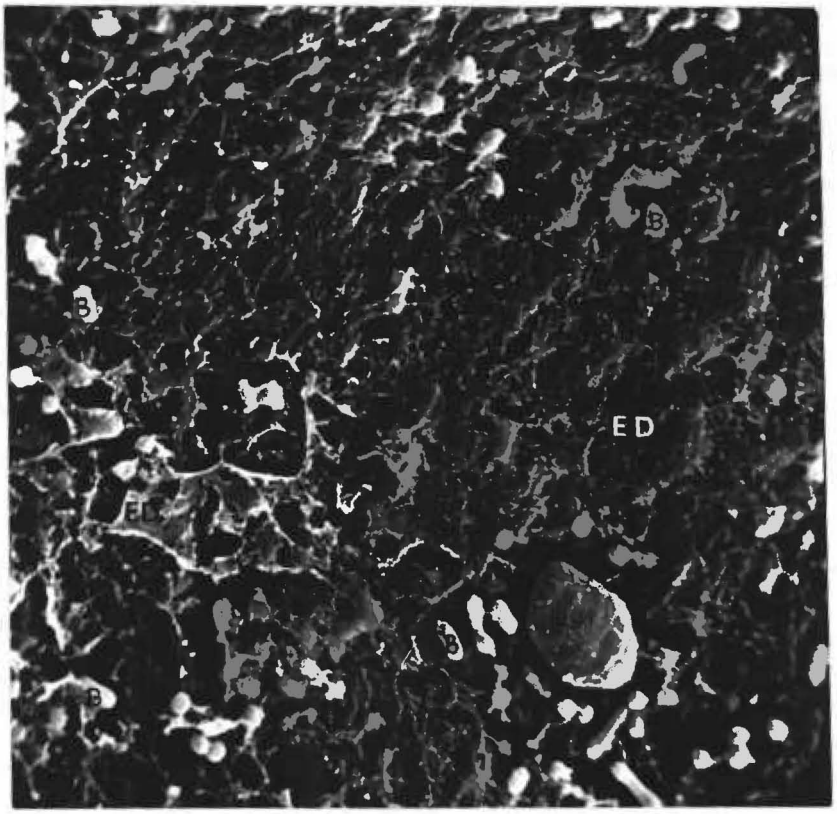


Plate 20.

wall of the cell. A reasonable inference from these observations is that ED is a product of the bacterium.

(6) Microautoradiography of bacterial cells

Autoradiography is a technique whereby metabolites within an organism or organelle are radioactively labelled such that translocation of those metabolites or derivatives to different cells or the same or different organisms can be monitored by photography. Monitoring the passage of radioactively labelled metabolites by photography is possible because photographic emulsion (film) is as sensitive to radioactive emission as to visible light. The advantage of electron-microscopy over light microscopy in autoradiography is that the resolution can be greatly increased. Using this technique, translocation of specifically labelled metabolites from the bacterium to the host plant during HR or SR could be detected.

However, the high cost of materials, the time needed for development of the autoradiograms and the difficulties and uncertainties of the experimental procedure limited the study to incorporating and photographing the label within the bacterial cells. At a later date, the technique applied may be put to further use in providing more information on the interactions between pathogen and host and on the passage of metabolites which occur at the host-parasite interface during the earliest stages of interaction.

A) Whole cell and thin section autoradiography

In these experiments the RNA of P. mors-prunorum was

labelled with radioactive uridine. To label the bacteria, P. mors-prunorum was grown for c. $2\frac{1}{2}$ h in a medium containing 41 μ Ci of tritiated uridine. The bacteria were then washed 7 times by centrifugation in distilled water and finally fixed in glutaraldehyde for 2-4 h and post fixed in osmium tetroxide for 1 h. In whole cell autoradiography, fixed cells were then washed again by centrifugation and pipetted directly onto formvar coated copper grids which were dried in an oven before being coated with K5 photographic emulsion. The grids were then incubated at $5^{o}C$ for 10 days. In thin section autoradiography, fixed cells were added to 4% (w/v) agar which was cooled, dehydrated in an alcohol series and embedded in Epon 1A : 1B epoxy resin. Sections of the bacteria in the agar were then put on to formvar coated copper grids and coated with K5 photographic emulsion before being incubated at $5^{o}C$ for 2 months.

After incubation the whole cell and thin section autoradiograms were processed in two types of developer. The chemical developer (D19) reduced the silver halide crystal to give a long filament of silver which grew out of the surface and coiled randomly. The physical developer dissolved the activated silver halide crystal to form a smaller grain.

In Plates 21a and 21b, autoradiograms of whole cells of P. mors-prunorum developed in D19B developer are shown. Around the bacteria several silver grains can be seen. These grains were formed from the silver halide crystals in the photographic emulsion which covered the bacteria during the incubation period (Figure 10). Once a beta particle from the tritium source in the bacterium hits a

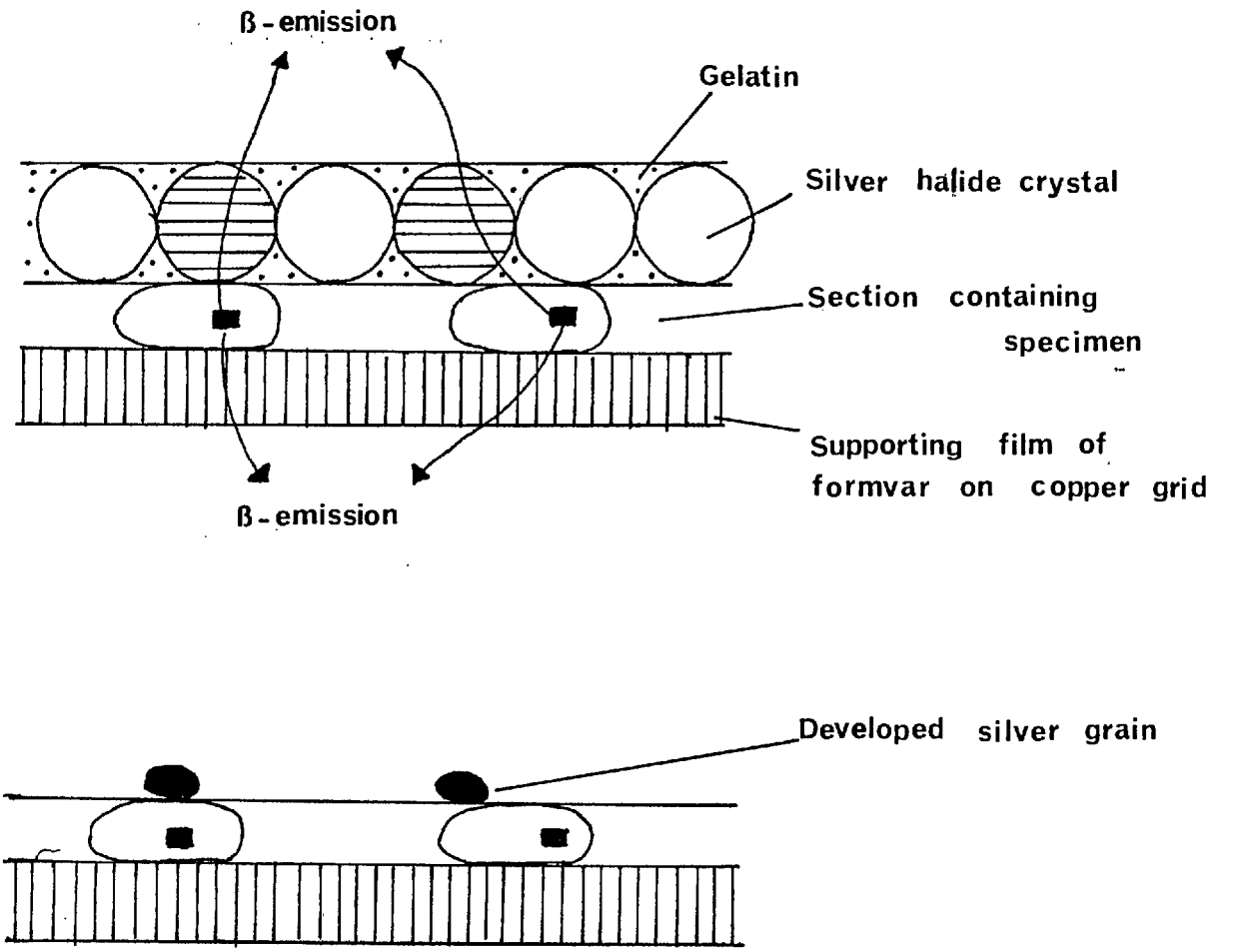


Figure 10. Diagram of a microautoradiograph preparation

(a) Relative positions of the preparation during incubation. The silver halide crystal is activated by the passage of a beta particle through it.

(b) Relative positions of the preparations after developing. The activated silver halide crystals now form elongated silver grains directly above the specimen containing the tritium source.

silver halide crystal then the crystal becomes activated and on subsequent development is reduced to silver. The effect of the chemical developer in forming a large randomly coiled silver grain is clearly shown in Plate 21c. The smaller dots interspersed over the radiogram are unactivated silver halide crystals which were not dissolved during development. Comparison of a silver grain and the silver halide crystal gives some measurement of how much the silver grain enlarges during the development process.

In Plates 22a, b, c, the effect of developing whole cell autoradiograms in a physical developer is shown. In this case the silver grains are considerably smaller because the developer inhibits the enlargement of the silver from the activated silver halide grain. The resolution of the whole cell autoradiograms (distance from specimen to silver grain) can be approximately determined knowing that the rods of P. mors-prunorum are c. 1.0-1.5 μ in length and 0.5 μ wide. From Plates 21a, b and 22 a, b, c, it can be seen that all the silver grains, in the autoradiograms developed in either the chemical or physical developer, are to be found within a radius of one bacterial length from the source of the label. Thus the resolution of whole cell autoradiography is c. 0.75 μ.

In Plates 23a and 23b, micrographs of thin section autoradiograms developed in the chemical developer are shown. In these plates the silver grains are more easily seen since the specimen containing the tritium source is c. 10 x thinner than in whole cell autoradiograms. However the size of the silver grains is the same as those found in the whole cell autoradiograms since only one beta

ABBREVIATIONS USED IN PLATES

B, bacterium; CW, cell wall of bacterium; NP, nucleic acid and protein; PS, periplasmic space; SG, silver grain; V, vacuole.

Plate 21a. Autoradiogram of whole cells of *P. mors-prunorum* labelled with tritiated uridine and developed in a chemical developer, D19B. Note presence of the large randomly coiled silver grains and the interspersion of the non-activated silver haliole crystals which have not been entirely dissolved during the development process (x 41,700).

Plate 21b. As Plate 21a but at higher magnification (x 52,500).

Plate 21c. High magnification of coiled silver grain. Note the difference in size between silver grain and silver haliole crystals around it (x 106,800).

Plate 22a. Autoradiogram of whole cells of *P. mors-prunorum*, developed in a physical developer. Note the size of the silver grains in comparison with those in Plates 21a, b (x 41,700).

Plate 22b. As Plate 22a (x 41,700).

Plate 22c. As Plate 22a (x 41,700).

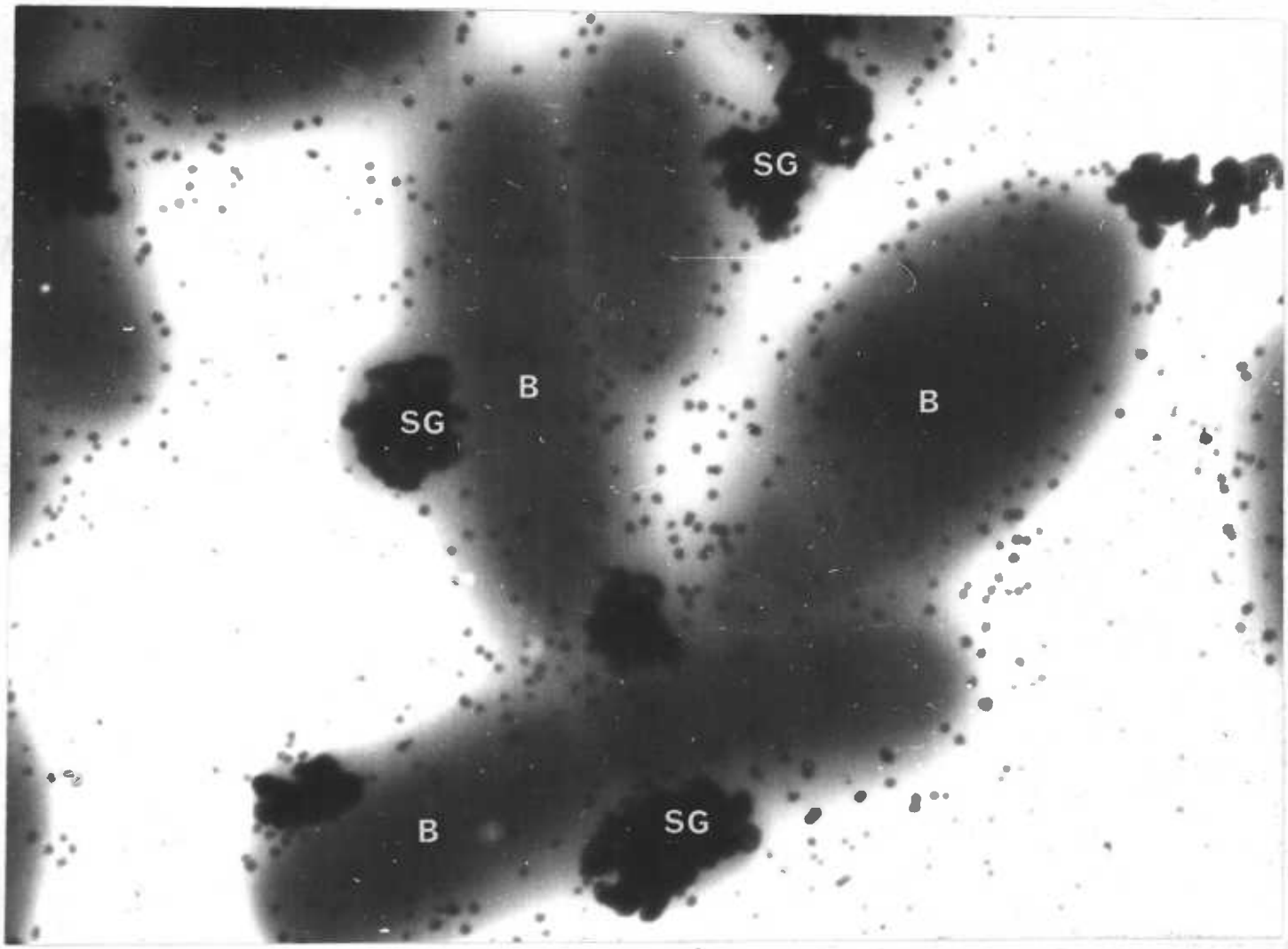
Plate 23a. Autoradiogram of thin section of cells of *P. mors-prunorum* developed in a chemical

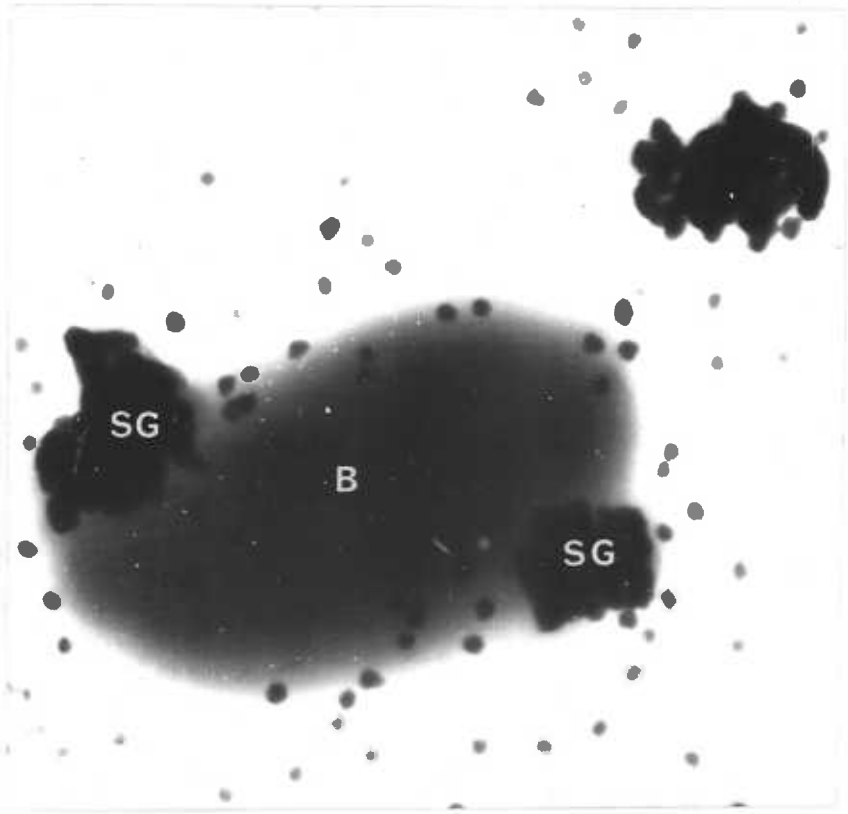
developer. Note detailed ultrastructure of the cells not seen in whole cell autoradiogram (x 52,500).

Plate 23b. As Plate 23a but higher magnification (x 85,800).

Plate 24a. Autoradiogram of thin section of cell of P. mors-prunorum developed in a physical developer. Note the presence of several adjacent silver grains which have agglutinated to appear as one (x 52,500).

Plate 24b. As Plate 24a but higher magnification (x 85,800).





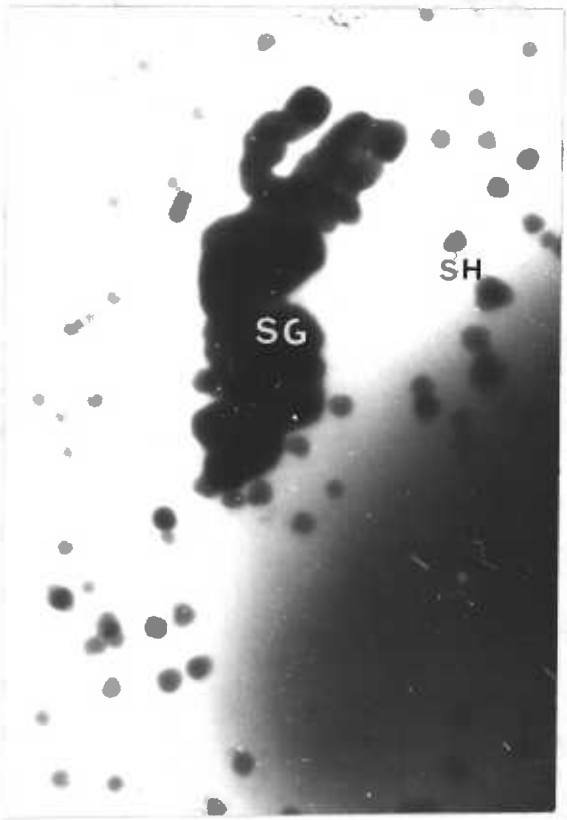


Plate 21.

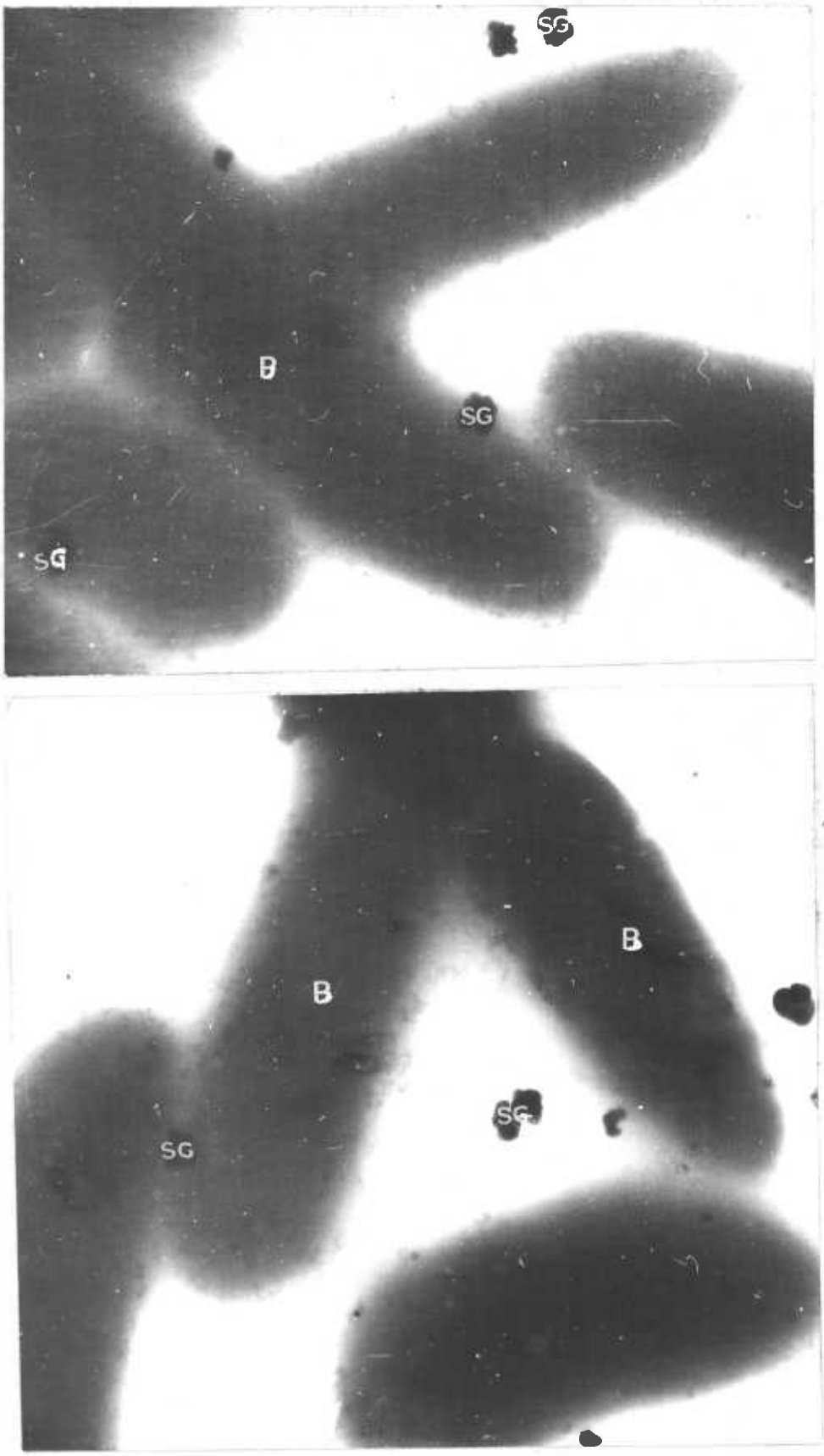


Plate 22.

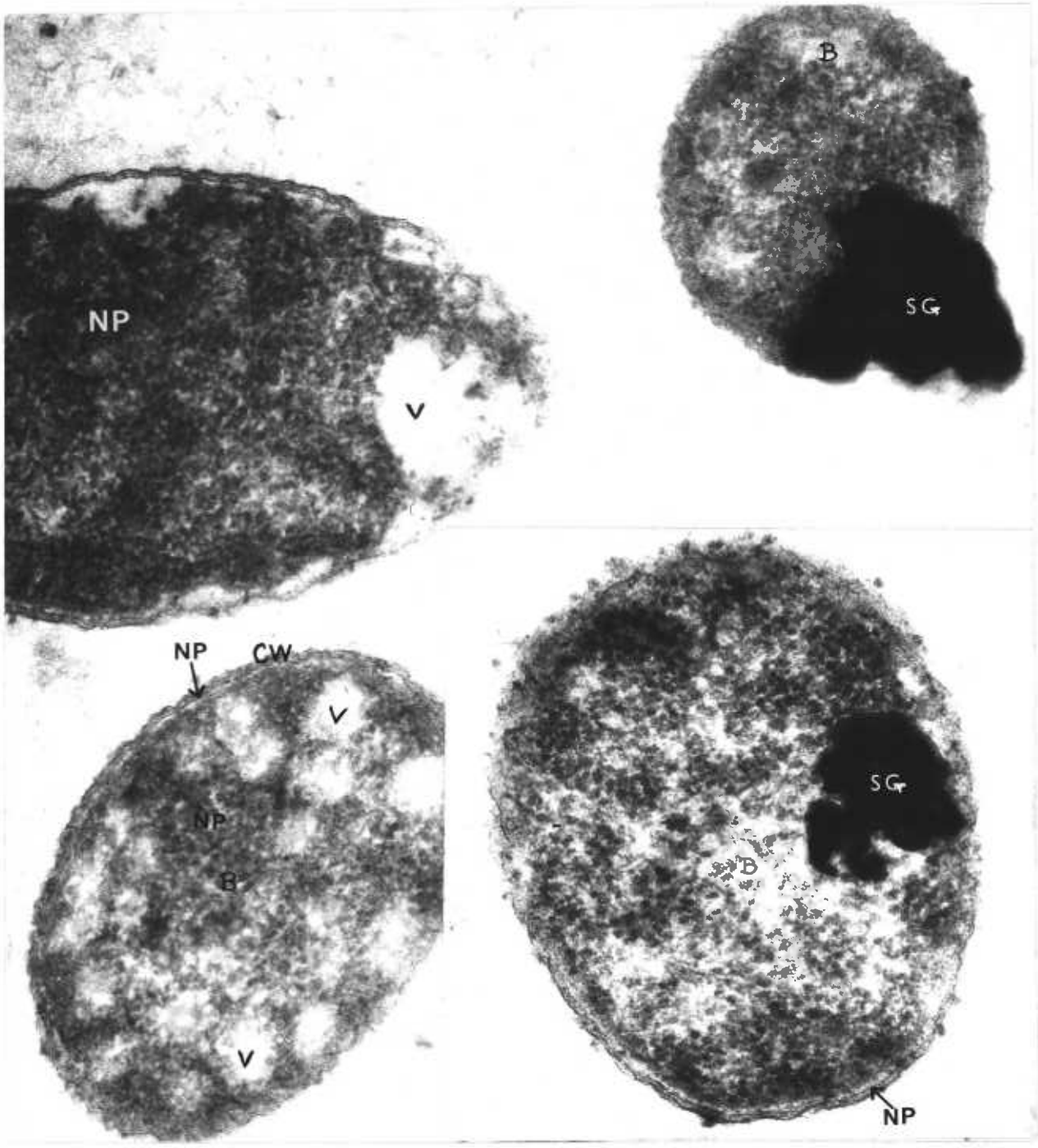
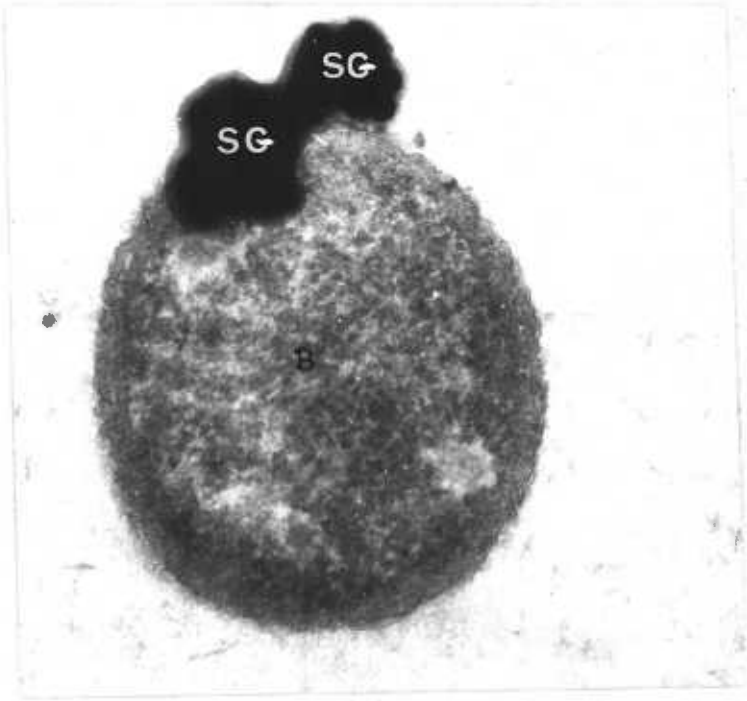


Plate 23.



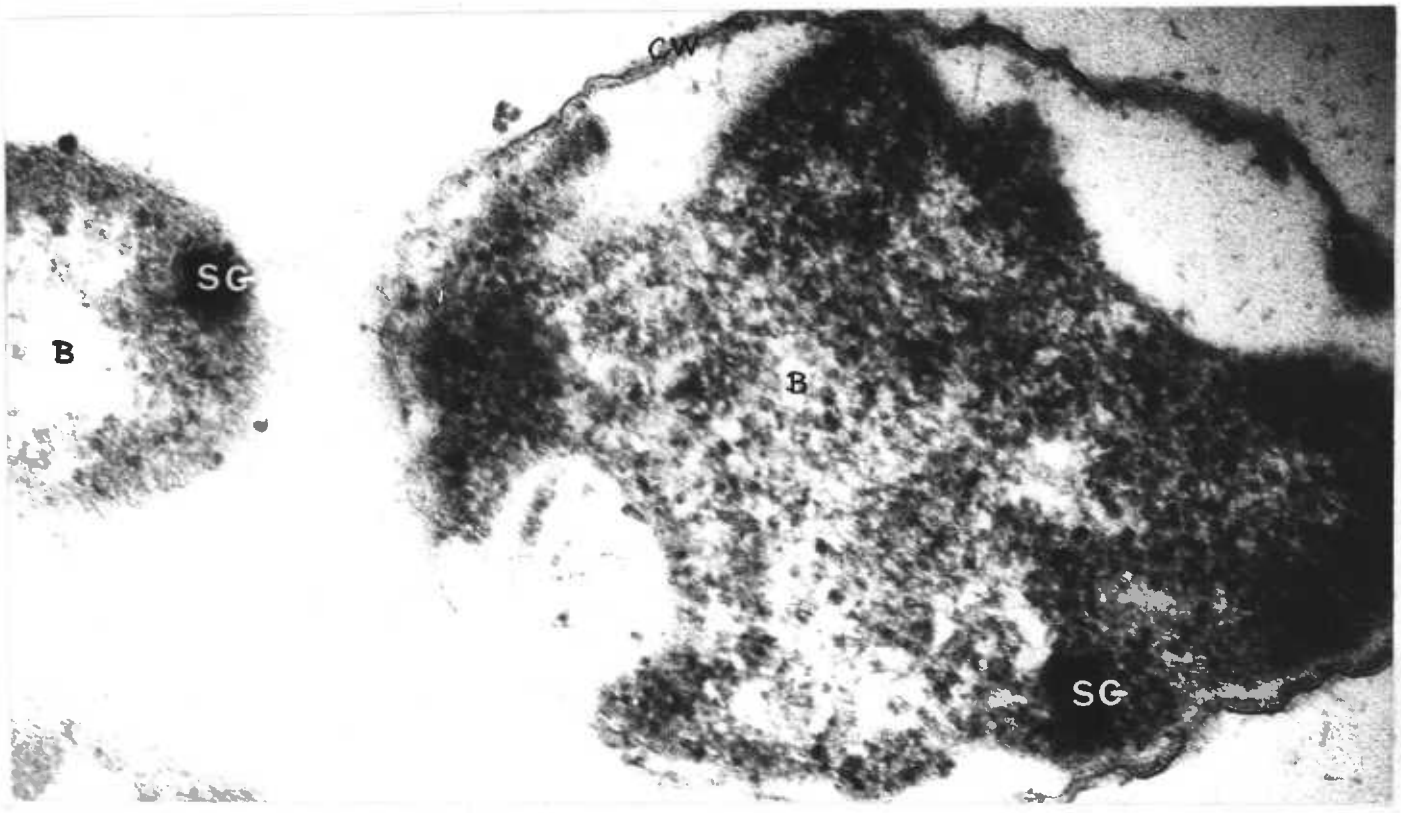


Plate 24.

particle is required in either case to activate the silver halide crystal.

In Plates 24a and 24b, micrographs of thin section autoradiograms developed in the physical developer are shown. From these micrographs it can be seen that the size of the silver grains is considerably smaller than those shown in Plates 23a and 23b. Therefore these results support those obtained when the whole cell autoradiograms were developed in the chemical and physical developers. In Plate 24a it can be seen that at least two silver halide crystals in close proximity to one another have become activated by a tritium labelled RNA within the bacterial cell.

From the micrographs of the thin section autoradiograms (Plates 23a, b; 24a, b) several features not observed in the whole cell autoradiograms (Plates 21a, b; 22a, b, c) can be noted. Firstly it can be seen all the silver grains are now either directly above or slightly to the side of the bacterial specimen. Thus the resolution has increased to within c. 0.2 μ. Secondly the resolution for determining the fine structure of the specimen has also increased such that the cell wall of the bacterium, the periplasmic space directly underneath the cell wall, the vacuoles within the cell, and the darkly staining nucleic acid and cytoplasmic protein can now be observed. Thirdly because the specimen is now much thinner the distance the beta particle travels, from its point source within the specimen to the silver halide crystal, is considerably reduced. This fact not only improves the overall resolution but also reduces the error of interpre-

tation involved when two or more sources of tritium labelled metabolite lie in close proximity to one another within the specimen.

B) Effect of labelled bacteria on host response

In this experiment the RNA of P. mors-prunorum and P. phaseolicola race 2 was labelled with tritiated uridine as for autoradiography. The bacteria were then adjusted turbidmetrically to a concentration of 3.0×10^8 cells/ml and injected into monofoliate leaves of RM and CW. After 20 and 72 h, HR and SR respectively were caused. These host responses were microscopically identical to responses evoked by a similar concentration of bacteria which had not been labelled. Thus the incorporation of tritiated uridine into the RNA of P. mors-prunorum and P. phaseolicola does not affect the pathogenicity of the bacteria.

DISCUSSION

Responses of bean leaves to bacteria

Klement (1971) distinguished three types of interaction between plants and bacteria; compatibility (susceptible reaction) and incompatibility (resistant reaction) in respect of plant pathogens, and no visible response following inoculation with saprophytic bacteria.

In bean leaves, compatible reactions were caused by the pathogens P. phaseolicola and X. phaseoli. Typical disease symptoms in both Red Mexican (RM) and Canadian Wonder (CW) were observed as reported by Burkholder (1926) and Patel and Walker (1963) for P. phaseolicola and Smith (1897) for X. phaseoli. The incompatible reaction was caused by P. lachrymans, P. mors-prunorum and P. tabaci in both cultivars and by P. phaseolicola race 1 in RM only. These incompatible reactions, as defined by Klement (1971), were caused by pathogenic bacteria in the non-host plant and by avirulent bacteria in a resistant plant (P. phaseolicola in RM).

No visible response was noted when the saprophyte P. fluorescens or when the soft rot bacteria E. atrosep-tica, E. caratovora or when P. solanacearum were inoculated into bean leaves. A slight yellowing of the inoculation area was noted when X. manihotis and X. cassava were injected into the leaves.

Kelman and Sequeira (1972) distinguished five types of interaction between plants and bacteria largely because it was difficult to envisage that incompatibility (excluding saprophytes), which in some cases produced macroscopic

changes in the host but in others did not, was governed by a single mechanism. Under the Kelman and Sequeira scheme the five types of interactions were divided into two plant responses, the incompatible (resistant/hypersensitive reaction) and the susceptible reaction. The interaction types were non-pathogens or saprophytes of plants, pathogens of organisms other than higher plants, plant pathogens in non host plants, plant pathogens in susceptible or resistant cultivars of a host plant and avirulent mutants of a pathogen in susceptible or resistant cultivars. However the host-pathogen interactions caused by E. atroseptica, E. caratovora, P. solanacearum, X. manihotis and X. cassava still do not fit adequately into any of the five types, defined by Kelman and Sequeira, and fit even less adequately to the hypothesis of Klement and Goodman (1967) that all incompatible host pathogen interactions (excluding saprophytes) cause HR.

Other cases where incompatible bacterium did not cause HR in the host have been reported. Lelliot et al. (1966) showed that certain soft rot Pseudomonas spp did not elicit HR in tobacco and Lasko and Starr (1970) also showed that a number of soft rot Erwinia spp did not induce HR in tobacco and bean leaves. From this it appears that the soft rot bacteria probably constitute a special category in that they all do not elicit HR in the incompatible host. Kelman (1969) came to a similar conclusion when dividing pathogenic bacteria into three groups based on their primary effect on host cells.

Two other cases where the incompatible bacterium did not cause HR have been reported. Lozano and Sequeira (1970)

have shown that an isolate of P. solanacearum, pathogenic to potato but non-pathogenic to tobacco, did not induce HR in tobacco leaves. Also, Stholasuta et al. (1971) showed that when pea leaves were inoculated with P. phaseolicola at c. 10^8 cells/ml no HR resulted.

Thus in the incompatible interaction the problem remains of how some bacteria cause visible host response (HR) whereas in others no response is shown.

Ercolani (1970) postulated a'unified hypothesis of induced resistance against bacterial infection'. Under this concept, 'multiplication sites' present in the host provide both 'sensitivity' and 'susceptibility' determinants'. Activation of the former will result in HR and activation of the latter SR. Additionally all bacterial pathogens contain factors for pathogenicity and virulence where the former controls the ability of the pathogen to grow in plant tissue and trigger HR and the latter to elicit the specific SR.

Evidence now exists to show that the pathogenicity factor, contained by all plant pathogens, may be responsible for eliciting HR by the production of a proteinaceous HR inducer (Sleesman et al., 1970; Gardner and Kado, 1972). The inability of the pathogens E. atroseptica, E. caratovora, P. solanacearum, X. manihotis and X. cassava to evoke HR may be due to their inability to produce the HR inducer, but at the same time the pathogenicity factor will enable them to multiply in plant tissue.

The concentration of incompatible bacteria in the inoculum does not influence HR directly (Klement and

Lovrekovich, 1961; Klement et al., 1964) but it does affect the formation of confluent visible necroses (Klement and Goodman, 1967). In bean, all the incompatible bacteria which caused HR, with the exception of P. phaseolicola race 1, produced confluent necroses in leaves when injected as an inoculum of 7.5×10^7 cells/ml. Below this concentration collapse of leaf tissue within the inoculation area was incomplete. However, with P. phaseolicola race 1 an inoculum of 1.5×10^8 cells/ml was needed to cause a confluent HR and at 5.0×10^7 cells/ml no visible reaction was shown. In tobacco leaves injected with P. tabaci 5.0×10^6 cells/ml caused a confluent HR (Klement and Goodman, 1967), but when tobacco leaves were injected with P. phaseolicola an inoculum of 1.0×10^8 cells/ml was required (Sule and Klement, 1971).

Ercolani (1973) hypothesised that HR was due to the 'cooperative action' of several of the inoculated bacterial cells, all of which contributed equally to overcome the defenses of the plant. It was suggested that the 'cooperative action' reflected a cumultive effect of some bacterial component in the plant. This component would presumably be the HR inducer. The view that HR develops only after large inocula are injected is supported by Logan (1960) and Hildebrand and Riddle (1971). Stall and Cook (1973) reported that a ratio of 100 cells of P. cichorii/tobacco leaf cell were required to give confluent necrosis. However, Turner and Novacky (1974) reported that one bacterium per host cell could cause HR at the microscopic level.

In summary, it appears that the most specific host/

bacterial reaction is SR while the incompatible reaction may produce HR or no visible response. Of the pathogens which do not cause HR in the incompatible host, the 'soft rot' bacteria are probably a special category. It is probable that several bacteria/host cell are required to cause confluent HR.

Effect of inhibitors on the hypersensitive and susceptible responses

Effect of inhibitors on the hypersensitive response

The results show that when specific inhibitors of bacterial RNA and protein synthesis are injected into leaves of RM simultaneously with P. mors-prunorum and P. phaseolicola race 1 the HR can be prevented, but when similar host plant specific inhibitors are used HR is not prevented. The induction period for HR was shown to be c. 3 h and the specific inhibitors of bacterial RNA and protein synthesis will not inhibit HR when injected after this time, relative to the bacterium.

Actinomycin D and cycloheximide (inhibitors of RNA and protein synthesis respectively) failed to inhibit HR at concentrations up to 125 μg/ml. Further, neither of these two inhibitors prevented the HR when injected into the leaf 24 or 4 h before the bacterium or 1 h after the bacterium.

Previous work has shown that actinomycin D (50 μg/ml) completely blocked the synthesis of peroxidase in sugar cane (Gayler and Glasziou, 1968), and in the mustard

(Sinapsis alba L.) seedling totally blocked anthocyanin production at 10 μg/ml (Lange and Mohr, 1965). In both cases, inhibition of the metabolites occurred through the blocking of new messenger RNA synthesis. In bacteria, actinomycin D has been reported to have powerful bacteriostatic effects on Gram-positive species (Kirk, 1960), but with Escherichia coli growth continues for several generations and synthesis of DNA, RNA and protein have been reported normal at concentrations up to 100 μg/ml. It is suggested that E. coli and other Gram-negative organisms are resistant to actinomycin because the inhibitor fails to enter the cell (Hurwitz et al., 1962; Reich and Goldberger, 1964).

As actinomycin fails to penetrate Gram-negative bacterial cells and as Pseudomonas spp are Gram-negative then the effects of this inhibitor will be directed entirely on the host. Cycloheximide, however, is known to be non-inhibitory to all bacteria (Ennis and Lubin, 1964; Glasziou, 1969) and therefore could only inhibit the synthesis of plant protein.

The failure of cycloheximide and actinomycin D to inhibit HR suggests that de novo synthesis of host plant protein does not occur during the induction period of HR in this system. Tani et al. (1973) has shown that the resistant response of oats to Puccinia coronata did not involve quantitative changes in the M-RNA levels of the leaves, thus supporting the results obtained with actinomycin D and cycloheximide. However, evidence from other work, which although conflicting, does appear to show

that the plant takes a more active role in disease resistance after the induction period. Pinkas and Novacky (1971) delayed HR by 18 h when cycloheximide (70 μg/ml) was injected with P. pisi into tobacco leaves. O'Brien (1973) also obtained a delayed HR when cycloheximide (20 μg/ml) was sprayed into leaves of RM simultaneously with P. phaseolicola race 1. However, Barbara and Wood (1975) reported that actinomycin D (10 μg/ml) applied to cucumber plants, normally resistant to CMV, produced susceptible symptoms. It was suggested that synthesis of antiviral compounds produced by the cucumber plant in response to infection was, in this case, suppressed. This contrasts with the work of Keen (1975) who reported that actinomycin D stimulated the production of hydroxyphaseolin in soya bean following inoculation with Phytophthora megasperma so enhancing disease resistance. Similarly, Byther and Steiner (1975) showed that actinomycin D and cycloheximide induced resistance to Helminthosporium sacchari in sugar cane. An enhancement of HR with cycloheximide was reported by Dougany (1973) when the inhibitor was injected into bean leaves with P. tabaci. It was further suggested by Dougany (1973) that HR is not dependent upon plant enzymes induced in response to infection by an incompatible bacterium.

Puromycin and 5 fluorouracil (5-FU) are known to be effective against eucaryotic and procaryotic systems. Two photomorphogenic reactions, the production of anthocyanin and phenylalanine ammonialyase have been shown to be completely inhibited by 100 μg/ml of puromycin. Cycloheximide (5 μg/ml) has a similar effect on the enzyme (Lange and

Mohr, 1965). Both anthocyanin and phenylalanine ammonialyase were shown to be produced by de novo synthesis in the plant (Rissland and Mohr, 1967). Puromycin has also been extensively used in analysis of protein synthesis in bacterial systems (Hille et al., 1967; Cundliffe and McQuillen, 1967).

5-FU has been used by Glasziou (1969) to inhibit RNA and protein synthesis in soybean and radish cotyledon. He found that although a 50% RNA inhibition could be achieved, cell elongation and nitrate reductase synthesis could not be inhibited thus showing that de novo synthesised metabolites were not implicated in these processes. 5-FU also has considerable antibacterial activity and is known to be bacteriostatic for a large number of Gram-negative bacteria (Cohen et al., 1958; Heidelberger et al., 1957). Aronson (1961) has shown that 5-FU inhibits RNA synthesis of Escherichia coli but that synthesis of protein appeared normal. It was suggested that 5-FU had a differential inhibition of RNA synthesis with m-RNA being the most resistant.

From the results it is clearly shown that puromycin and 5-FU are preventing HR by inhibiting the production of protein synthesis on 70S ribosomes of bacteria.

Thus far, the two host plant specific inhibitors (actinomycin D and cycloheximide) failed to inhibit HR and the two inhibitors of dual sites of action (puromycin and 5-FU) which did inhibit HR were shown to be doing so by acting on the bacterial system. The other inhibitors of protein and RNA synthesis used were bacterially specific

and all prevented the HR.

Chloramphenicol and erythromycin inhibit protein synthesis in organisms having 70S ribosomes. Both inhibitors have a similar site of action, the 50S ribosomal subunit, and do not inhibit protein synthesis on 80S ribosomes (Brock and Brock, 1959; Vasquez, 1966). The difference between the two is that erythromycin is more effective on Gram-positive organisms. In neither case is nucleic acid synthesis affected and in both cases bacteriostasis is reported (Brock and Brock, 1959).

Rifampicin is a highly specific inhibitor of bacterial transcription. Once the DNA dependent RNA polymerisation is initiated then rifampicin becomes ineffective as it is only transcription of *de novo* RNA which is inhibited (Sippel and Hartmann, 1968; Wehrli and Staehlin, 1971; Weisblum and Davies, 1968). Thus the blocking of HR by this inhibitor implicates that the HR inducer is a *de novo* synthesised bacterial metabolite.

Spectinomycin is another bacterially specific protein inhibitor which has no effect on RNA synthesis and exposure of *E. coli* to 1000 μg/ml has been reported to produce little killing (Davies *et al.*, 1965).

Before inhibition of HR by specific inhibitors of bacterial metabolites can be attributed to *de novo* synthesis, the effects of these inhibitors on multiplication of bacteria *in vivo* must be considered. If the bacteria, when in the leaf together with the inhibitor, are being reduced in numbers to a level below that required to give confluent necrosis of host cells, then the results become less significant. However, results showed that concentra-

tions of the inhibitors which prevented HR did not reduce bacterial numbers below the concentration required to give confluent necrosis. Thus, this result strongly implicates de novo synthesis of a bacterial metabolite is the inducer of HR.

Further evidence showing that inhibition of HR was specifically related to inhibition of bacterially directed synthesis of protein and RNA, was provided by using ampicillin and D-cycloserine. Both these inhibitors are specific for blocking the synthesis of peptidoglycan , a component of the bacterial cell wall, and act on growing cells only. Neither inhibitor at 1000 μg/ml inhibited HR, though the effects of these inhibitors in reducing bacterial numbers in vitro was shown to be more pronounced than that of rifampicin or chloramphenicol at 15 μg/ml, the concentration which inhibited HR.

Three important points emerge from the results obtained with inhibitors of bacterial cell wall synthesis. Firstly that the metabolites D-alanyl-D-alanine and UDP-Mur-NAC-pentapeptide implicated in the peptidoglycan component of the bacterial cell wall are not implicated in HR induction. Secondly, it is unlikely that peptidoglycan itself (the component of the bacterial cell wall which prevents lysis) is implicated in HR induction, as once the bacteria are exposed to the inhibitors, the metabolite becomes quantitavely reduced. Sequeira et al. (1972) came to a similar conclusion about the peptidoglycan component when dealing with the 'HR protection factor' of P. solanacearum. Thirdly, from all the results of inhibitors on bacterial growth in vitro and in vivo, it can be

seen that bacterial multiplication is not a critical factor in HR induction, since in nearly all cases numbers are either static or are decreasing over the first 5 h period. It can further be seen from the effects of chloramphenicol and rifampicin (15 μg/ml) over 24 h that the rate at which bacterial numbers decrease is increased outside the induction period thus providing further evidence that HR can be completed while bacterial numbers are being decreased. This conclusion was also reached by Klement (1971).

Phytopathogenic bacteria occur in the intercellular spaces between host plant cells and only penetrate the cell walls after mechanical damage. Recent evidence suggests that in an avirulent (incompatible) reaction the bacteria do not move great distances from the point of inoculation but agglutinate on the cell wall of the host in localised clumps (Goodman, 1974; Sequeira, 1976; Patil - personal communication). Assuming that de novo synthesis of a bacterial metabolite is the HR inducer, then the metabolite has to pass from the bacterial cell across the intercellular space and then into the plant cell. Evidence presented in the results suggests that the induced bacterial metabolite is a protein, though the possibility of de novo synthesised bacterial RNA being implicated can not be ruled out.

The results show that neither RNAase or protease (1000 μg/ml) when injected into the intercellular space during the induction period prevented the HR. This anomaly may be explained from the work of Tongur et al. (1968) who showed that in E. coli newly transcribed RNA

complexes with DNA, using nucleo-protein as a stabiliser, and becomes resistant to RNAase attack. Thus de novo synthesised RNA is resistant to nuclease attack until released from the DNA template. Once released the RNA becomes ensheathed in ribonucleoprotein as shown by protein specific staining (Lewin, 1970), thus again making it resistant to nuclease attack.

The inability of a protease to prevent HR may be explained in a two fold manner. Firstly, if the bacteria are agglutinated on the surface of the host cell wall, then the proteinaceous HR inducer may pass directly through the host cell wall, avoiding the intercellular space, and either bind to the plasma membrane and alter its permeability, as proposed by Klement and Goodman (1967), or enter the host cell directly and cause collapse and necrosis through a secondary mechanism. In this case the protein will avoid attack from the enzyme in the intercellular space. Secondly, a problem of substrate specificity may exist in that the protease (bovine) may not be able to attack and digest the bacterial protein.

The implication that bacterial protein is involved in HR induction is supported by similar results obtained from previous workers (Sequeira and Ainslie, 1969; Gardner and Kado, 1972; Sleesman et al., 1970). The evidence further suggests that the HR protectant (a factor which suppresses HR) is also a protein or glycoprotein (Sequeira et al., 1972; Wacek, 1974). Sequeira et al. (1972) postulated that pathogenic bacteria when inoculated into plants release small amounts of both these proteins but during HR induction, the protectant becomes masked by the inducer.

In summary it is hypothesised that induction of HR is a result of de novo synthesis of a bacterial protein which passes from the bacterium, agglutinated on the host cell wall in the intercellular space, into the plant cell and from there effects collapse of the host cell. It is further suggested, from the results with actinomycin D and cycloheximide, that the plant plays no significant role during the induction period but rather acts as a stimulus of recognition for induction of a specific bacterial cistron.

The use of changes in host cell permeability to monitor the process of infection by pathogens is well reported (Sadasivan, 1959; Wheeler and Black, 1962; Ghabmal, 1967). The three stages of HR development in bean, as defined by Klement (1971) for tobacco, are shown from the results of permeability changes in leaves of RM following inoculation of P. mors-prunorum.

The induction period (0-3 h after bacterial injection), defined as being dependent on the bacterium but independent of the host, is shown by little or no electrolyte loss. The latent period (3-8 h), which is dependent on the host but independent of the bacterium, shows a small increase in host cell permeability. The collapse period (8-24 h) is recognisable by an almost linear increase in host cell permeability giving rise to confluent collapse of the host cells in the inoculation area by 16 h. This pattern of increase in host cell permeability is similar to that reported by Goodman (1968) for tobacco and Stall et al. (1973) for pepper.

The effect of chloramphenicol (5 μg/ml) when injected with P. mors prunorum is to delay the commencement of electrolyte loss by c. 4 h, which in turn delays the collapse of the inoculation area over control by a similar period. However, the rate at which electrolytes are lost from cells, once permeability begins to increase, is similar to the rates for control leaves. As chloramphenicol is effective against the bacterium only, this delay must represent a slower rate of production of the HR inducer. The large differences in the rate of electrolyte loss between the latent and collapse periods suggests that the inducer alters and/or combines with a host metabolite which in turn has to reach a critical concentration during the latent period before host cell permeability can be drastically altered. Evidence that the host metabolism does alter during the latent period of HR was shown when the respiration rate of tobacco leaves was found to be highest just before the collapse period (Németh and Klement, 1967).

None of the other treatments gave collapse of the inoculation area, though chloramphenicol (15 μg/ml) and P. mors-prunorum did induce some electrolyte loss. From this it would appear that a certain level of electrolyte loss must be attained before collapse and necrosis of host cells occurs.

Effect of inhibitors on the susceptible reaction

The results show that rifampicin and chloramphenicol, when injected with P. phaseolicola into monofoliate leaves of CW, have different effects on SR dependent on the concentration of the inhibitor.

In the monofoliate leaf, collapse of the inoculation area following injection of P. phaseolicola normally occurs by 72 h. However, when the bacteria were injected with rifampicin or chloramphenicol at a concentration of 15 μg/ml or above the collapse was delayed, and with rifampicin at 125 μg/ml it was prevented entirely. Of the two inhibitors, rifampicin had the greatest effect in delaying the collapse of the inoculation area.

In the trifoliate leaf, following monofoliate inoculation, the effect of rifampicin and chloramphenicol was less well defined. Chloramphenicol had little effect on retarding the development of chlorosis even at the highest concentration (125 μg/ml) but rifampicin (125 μg/ml) prevented chlorosis entirely.

The monofoliate leaf

Growth of P. phaseolicola in the monofoliate leaf over 386 h followed a sigmoid pattern with numbers reaching a maximum 192 h (8 d) after inoculation though collapse of the inoculation area occurred at 72 h. Growth of P. phaseolicola with chloramphenicol was similar to that of control leaves without the inhibitor but with rifampicin at 60 and 125 μg/ml respectively, numbers had decreased by 10 and 1000 fold over control leaves after 386 h.

Diachum and Troutman (1954) reported that in inoculated tobacco leaves, numbers of P. tabaci reached a maximum at about the time of tissue collapse. Similarly, Stall and Cook (1966) reported that in susceptible pepper leaves inoculated with X. vesicatoria, visible symptoms appeared

just prior to the time when the bacterial population was greatest. Other reports have also shown that bacterial numbers in inoculated leaves decreased far sooner after the inoculation area had collapsed than the time taken (120 h) in control leaves (Klement, 1964; Ercolani and Crosse, 1966; Omer and Wood, 1969). However, a correlation between numbers of bacteria in the leaf and collapse of the inoculation area is apparent.

The results show that rifampicin has the greatest effect in delaying the collapse of the inoculation area and at the same time decreases bacterial numbers in the leaf to a level below that of control or chemical treatments. By 216 h after inoculation collapse of host tissue treated with rifampicin (60 μg/ml) occurred even though numbers of bacteria were 10 times less than in control leaves. At the same time (216 h) numbers of bacteria in the rifampicin 60 μg/ml treatment were 100 times greater than in the rifampicin 125 μg/ml treatment. This suggests that bacterial numbers have to attain a minimum level in the leaf before collapse of the inoculation area occurs. Below this level host resistance is maintained and the plant shows no visible response. As numbers in the leaf increase above this level then host response (collapse) occurs at a proportionately faster rate. Thus chloramphenicol (60 and 125 μg/ml) reduces numbers below control leaves only slightly for 48 h and cause a slight delay in collapse, but rifampicin (60 μg/ml) decreases numbers for another 48 h and so causes delay for a correspondingly longer period.

This view that a minimum number of bacteria are required to cause collapse of the inoculation area in SR contrasts with previous work (Hildebrand, 1937; Hildebrand and Lippincott, 1965; Ercolani, 1967; Thyr, 1968 and Perombelon, 1971) which suggests that a single bacterium can initiate infection in the compatible reaction (SR) to cause disease. However, Scharen (1959) working with X. phaseoli and bean leaves supports the view that an inoculum threshold is required to overcome the resistance of the host which in turn results in further bacterial multiplication and ultimate necrosis of the host.

In SR, a necrotic region develops around the inoculation area in the monofoliate leaf and enlarges radially c. 96 h after injection with P. phaseolicola. Results show that numbers of bacteria at the edge of the enlarging area did not differ significantly from numbers at the centre of the inoculation area over the period 96 - 384 h. When chloramphenicol (60 and 125 μg/ml) was injected together with P. phaseolicola, numbers again did not differ significantly from those of control leaves. This result contrasts with the work of Staub and Williams (1972) who reported that by 12 days after inoculation, numbers of X. campestris at the edge of the inoculation area on cabbage leaves were c. 10^4 cells greater/3.5 mm leaf disk than in the centre of the inoculation area.

The trifoliate leaf

Growth of P. phaseolicola in the trifoliate leaf following monofoliate inoculation, was similar in control and in chloramphenicol 60 and 125 μg/ml treatments over 384 h. However, the rifampicin 60 and 125 μg/ml treatments

decreased numbers by c. 5 and c. 15 times respectively, that of control over the same period. The development of chlorosis in trifoliate leaves was slightly delayed by chloramphenicol (60 and 125 µg/ml) and by rifampicin 60 µg/ml, but it was entirely prevented by the rifampicin 125 µg/ml treatment. The suppression of chlorosis development by rifampicin 125 µg/ml supports the result obtained in the monofoliate leaf with this treatment. This implies that development of chlorosis of trifoliate leaves is correlated to numbers of bacteria in the inoculation area in monofoliate leaves. This implication is strengthened by the fact that the trifoliate leaf disks from the rifampicin 125 µg/ml treatment had only c. 8 times fewer bacteria than control leaf disks and approximately the same numbers as the rifampicin 60 µg/ml treatment at the onset of chlorosis (192 h).

Past work has not been able to establish a clear relation between the spread of the bacterium and development of trifoliate chlorosis. Waitz and Schwartz (1956) obtained a poor correlation between the presence of the bacterium in trifoliate leaves above the inoculation point and chlorotic symptoms. However, Hildebrand and Schroth (1971) re-isolated P. phaseolicola from 39 out of 40 trifoliate leaves following inoculation of monofoliate leaves. In view of the above it is suggested that a bacterial metabolite from the monofoliate leaves is the cause of chlorosis in trifoliate leaves. This suggestion is supported by the work of Rudolph (1966) and Patil (1974) who suggested that phaseotoxin, an exotoxin produced by P. phaseolicola, was causally related to chlorosis in leaves

above the site of inoculation. It thus seems that the delay in development of chlorosis in trifoliate leaves caused by chloramphenicol and rifampicin (60 μg/ml) is due to the effects of the inhibitors on bacterial growth in the monofoliate leaf. This in turn causes less phaseotoxin to be produced and carried upwards to the trifoliate leaves.

Patil and Gnanamanickam (1976) have suggested that phaseotoxin is implicated in the initial establishment of P. phaseolicola in susceptible tissues by suppressing HR. The time difference in the collapse of the inoculation area in bean leaves between HR (30 h) and SR (72 h) when injected with P. phaseolicola may be explained by the production of phaseotoxin. A characteristic feature of plant tissue undergoing HR or SR is the increased loss of electrolytes from host cells, which occurs during their collapse and necrosis. The implication that phaseotoxin suppresses HR suggests that electrolyte loss is also retarded following injection of P. phaseolicola. This contrasts with the work of Wheeler and Black (1962) who showed that the toxin from Helminthosporium victoriae enhanced electrolyte loss from oat tissue.

In bean the rate at which electrolytes are lost from host cells, following inoculation of monofoliate leaves with P. phaseolicola, at the initial stage (0 - 16 h) after inoculation is low, but after this period electrolyte loss proceeds at a linear rate. This trend of electrolyte loss is similar to that found in susceptible pepper leaves inoculated with X. vesicatoria (Stall and Cook, 1968). When P. phaseolicola is injected into bean

leaves, collapse of the inoculation area is complete by 72 h but when P. phaseolicola and chloramphenicol at 15 and 30 µg/ml are injected collapse does not occur until 96 h. Results show that chloramphenicol delayed the time at which electrolyte loss approached a linear rate. Finally, the delay in SR caused by chloramphenicol with injection of P. phaseolicola contrasts somewhat with the work of Király et al. (1972) who showed that chloramphenicol caused a previously compatible host to respond incompatibly to Phytophthora infestans.

In summary, chloramphenicol and rifampicin retard SR by decreasing bacterial numbers in the inoculation area in monofoliate leaves which consequently affects the production of phaseotoxin and the development of chlorosis in trifoliate leaves. SR was prevented only when rifampicin, at high concentrations, decreased bacterial numbers below a level required to overcome the defenses of the plant. Electrolyte loss from host cells in the inoculation area undergoing SR correlated well with visible symptoms.

Attempts to alter the hypersensitive and susceptible reactions

Yarwood (1959) defined predisposition as the propensity of non-genetic conditions, acting before infection, to affect the susceptibility of plants to disease. However, the use of chemicals to alter host response to pathogens may not always involve predisposition since the chemicals may increase or decrease susceptibility by acting directly on the pathogen. In this respect cycloheximide has been shown to give control of wheat rust

(Hacker and Vaghn, 1957) but is known to be highly toxic to fungi. Therefore it was suggested in this case that the chemical was altering susceptibility by its effect on the fungus and not the plant.

From the results on attempt to alter HR and SR using a variety of chemical, bacterial and other treatments, no distinction was made between the effects of the chemicals on the metabolism of the host or of the pathogen. The treatments applied included the use of injected chemicals, systemic chemicals, shock treatment of plants before inoculation, and cross protection. Results showed that only some systemic chemicals (ionic solutions) and some cross protection altered host response to the pathogens P. phaseolicola races 1 and 2 and P. mors-prunorum.

Use of injected chemicals

In bacterial cells, induction of enzyme synthesis may be repressed by glucose or by biochemically related products in a process called catabolic repression (Magasanik, 1961). Additionally it has been shown that when bacteria are transferred from a glucose medium (repressive conditions) to a glucose free medium (non-repressive condition) the concentration of cyclic AMP (c. AMP) increased (Makman and Sutherland, 1965). Later it was shown that application of c. AMP can overcome the repressive effect of glucose and that this reversal of repression was dependent on DNA transcription (Perlman and Pastan, 1968).

The role of DNA histones as transcriptional regulators is well established since RNA hybridizes with a considerably larger fraction of deproteinized DNA than with chromatin (Bakhor et al., 1969).

From the results in the thesis it can be seen that injection of glucose, sucrose, c. AMP and DNA did not alter host response to P. phaseolicola race 1 when the chemicals were applied before, simultaneously with or just after the bacterium. However, with c. AMP and glucose host response was delayed. This result contrasts with the work of Panopoulos and Schroth (1971) who showed that when P. phaseolicola was suspended in 2-5 mM of glucose, glycerol or other sugars prior to injection into red kidney bean leaves, the number of lesions was reduced by 50 to 60%. Similarly Dogany (1973) showed that HR was suppressed when glucose or c. AMP (3 mM) were injected into bean leaves 12 h before injection of P. tabaci. Keen and Kennedy (1974) also showed that c. AMP (5 mM), when injected into soybean leaves simultaneously with races of P. glycinea (compatible and incompatible), prevented HR and SR. However, Panapoulos and Schroth (1971) found that c. AMP (2.5 mM) enhanced infection of bean leaves with P. phaseolicola by 2-5 fold over water controls.

A role of amino acids in inducing or suppressing enzyme systems has been reported. Induction of the synthesis of nitrate reductase has been shown to be repressed by the addition of a variety of amino acids (Joy, 1969), and addition of phenylalanine has been shown to inhibit phenylalanine-ammonia-lyase (PAL) synthesis. However, the results in the thesis show that a variety of amino acids, when applied by injection sequentially to the bacteria, did not alter host response to P. phaseolicola.

Changes in the amount or activity of several host

enzymes in bean leaves following infection by P. phaseolicola have been shown. Particularly significant was the increase in peroxidase activity found in resistant but not susceptible varieties. Catalase, a bacterial enzyme which inhibits peroxidase, was also shown to be produced in greater quantities by virulent bacteria (Rudolph and Stahmann, 1964). The relation between the peroxidase produced by the host and catalase activity of the bacterium may determine whether a plant will be resistant to a pathogen or not.

McIntyre et al. (1975) reported that when the DNA from E. amylovora was injected into pear seedlings 18 h before virulent cells of E. amylovora, protection against fireblight occurred. Specificity of the protectant was shown in that the DNA from P. tabaci did not prevent fireblight caused by later injection of E. amylovora. However, the DNA from E. herbicola and X. campestris also protected against fireblight. McIntyre et al. suggested that the protection against fireblight by the DNA of E. amylovora, E. herbicola and X. campestris was due to homology between DNA of these bacteria. This contrasts with the work of Gardner and Kado (1972) who showed that the degree of homology between the DNA of Erwinia spp. and species of other genera of the Enterobacteriaceae (Escherichia, Klebsiella) was as high or higher as between species of Erwinia.

Results in the thesis show that the DNA of P. phaseolicola, P. mors-prunorum, calf thymus and E. coli failed to prevent HR or SR caused by P. phaseolicola races 1 and 2 and P. mors-prunorum. This result is

supported by past work (Sleesman et al., 1970; Sequeira et al., 1972) which showed that protectants against HR or SR were of proteinaceous composition. Further, it has been shown by Wacek and Sequeira (1973) that the DNA of P. solanacearum failed to protect against HR caused by the same bacterium. This suggests that protection against HR or SR is caused by different substances in different systems. In support of this is the report of Howes et al. (1974) that the HR inducing fraction from wheat infected with P. graminis tritici was nucleic acid. In contrast the HR inducing fraction from E. rubrifaciens is proteinaceous (Gardner and Kado, 1972).

Use of systemic chemicals

The use of systemic chemicals to alter host response to pathogens is well reported (Cook and Stall, 1971; Longchamp et al., 1951; Lyles et al., 1957; Novacky, 1972 and Selman, 1964).

The results in the thesis show that neither the commercial or experimental systemic chemicals altered HR or SR despite, in some cases, their known effects on altering host metabolism. However, evidence from past work has shown that host response to pathogens can be altered by application of systemic chemicals. Longchamp et al. (1951) and Lyles et al. (1957) respectively showed that 2, 4-D, a systemic chemical, increased the susceptibility of wheat to Claviceps purpurea and Puccinia. Croxall et al. (1957) showed that 2:4:6-T increased the susceptibility of tomato to Didymella lycopersici.

The failure of the commercial and experimental chemicals to alter host response may be due to the inability

of the chemicals to alter the part of the metabolism of the host concerned with disease expression. Alternatively host response to P. phaseolicola may not be under the direct control of the host such that metabolic changes in the plant induced by chemical application, prove ineffective.

Past work has shown that increased levels of growth regulators in plants, derived from external application or through production by the plant and pathogen, can alter host metabolism to pathogens.

From the results in the thesis it can be seen that the growth regulators auxin, gibberellic acid and kinetin (kinin) had no effect on altering the HR or SR to P. phaseolicola. However, it is well established that the most important plant growth regulators are auxins, gibberellins and kinins and many plant pathogens are known to produce substances which are identical in their biological action to these growth regulators. Increased auxin (IAA) levels in safflower plants infected with Puccinia carthami has been reported to account for the abnormal growth of the plants (Daly and Inman, 1958). However, Corden and Edgington (1960) reported that application of high levels of auxin to infected tomato plants induced resistance to Fusarium oxysporum.

The phytopathogenic bacteria Agrobacterium tumefaciens and Pseudomonas solanacearum are known to produce IAA. In diseased solanaceous plants infected with P. solanacearum a 100 fold increase in IAA was detected (Sequeira, 1965). In most cases of abnormal plant growth caused by infection it is considered that the pathogens contribute towards the

increased level of growth regulator.

Gibberellins have been studied most extensively in relation to virus diseases. The stunting of maize plants infected with stunt virus and of tobacco plants infected with etch virus was reversed after treatment with gibberellin (Maramorosch, 1957).

Application of cytokinin has been shown to increase infection of bean to rust (Kiraly et al., 1967) and in Helminthosporium blight of oats, was shown to increase the quantity of toxin absorbed by the plant (Luke and Freeman, 1967). However, Novacky (1972) showed that HR of tobacco leaves to P. pisi could be prevented when leaves were pretreated with kinetin. Similarly, pretreating petunia and tomato leaves respectively with kinetin before inoculation with tomato spotted wilt virus has been reported to reduce the number of infections in local lesions and to reduce the multiplication of the virus in systemic infection (Selman, 1964). Kinetin has also been shown to reduce the production of the toxin of P. tabaci in infected plants (Lovrekovich and Farkas, 1963).

The failure of the growth regulators to alter host response to P. phaseolicola supports the result obtained when the systemic commercial and experimental chemicals were used. However, in view that past work has shown that host response to pathogens can be altered by application of growth regulators, it must be assumed that, in the experiments in the thesis, host metabolism was in some way modified. Therefore, failure to observe any alteration in visible host response could only be due to host expression

being controlled by metabolic pathways immune to the effects of growth regulators used.

From the results in the thesis on the effects of ions on host response it can be seen that solutions of barium, beryllium, calcium, magnesium, molybdenum and zinc all prevented HR and SR when they were taken up by the plant for 24 h before inoculation with P. phaseolicola. It was further shown that inhibition of HR and SR by the ions was not due to a reduction in bacterial numbers to a level below which visible host response does not occur. This result supports the work of Cook and Stall (1971) who showed that electrolyte loss in pepper leaves undergoing HR or SR caused by X. vesicatoria could be reduced by application of solutions of calcium and strontium. It was further shown that numbers of X. vesicatoria in leaves, following simultaneous inoculation of bacteria and solutions of magnesium, calcium and strontium, did not differ by more than two fold between the treatments. Solutions of calcium, magnesium and zinc salts have been reported to inhibit HR caused by P. tabaci in bean leaves (Dogany, 1973).

Doupnik (1968) also reported that solutions of calcium and strontium were effective in suppressing symptoms in oat leaves to victorin, the toxin of Helminthosporium victoriae. However, solutions of barium had no effect on symptoms and magnesium was reported to enhance symptoms. Bateman (1967) has shown that calcium and other divalent cations form an induced mechanism of resistance to Rhizoctonia solani by accumulating around lesions and inhibiting further maceration of the host by polygalac-

turonase.

The role of calcium and other ions as nutrients in predisposing plants against infection has also been reported. Kernkamp et al. (1952) reported that calcium suppressed infection of pea leaves by Rhizoctonia solani and Edgington and Walker (1958) have shown that tomato plants receiving excess calcium become resistant to Fusarium wilt. However, Forsyth (1957) has shown that zinc increases the susceptibility of wheat to Puccinia graminis.

The mechanism by which calcium and other ions suppress host response is unclear. However, Epstein (1961) has shown that calcium and magnesium play a part in the maintenance of cell wall and plasmalemma permeability and stability, thus reducing the rate at which materials move out of the host cells. The role of the other ions in suppressing host response is unclear.

The failure of the DNA of P. phaseolicola, P. mors-prunorum and calf thymus to alter host response when it was taken up by cut ends of hypocotyls for 24 h before inoculation of P. phaseolicola and P. mors-prunorum into the leaves supports the result obtained when DNA from similar sources was injected into leaves differentially to the bacteria. However, McIntyre et al. (1975) showed that the DNA of E. amylovora, E. hericola and X. campestris, when taken up by excised seedlings, protected against subsequent challenge by virulent E. amylovora. It thus appears, as was suggested when DNA was injected differentially to the challenging bacterium, that host response

can be altered by different metabolites or chemicals in different systems.

Use of rapid alteration of air temperature as shock treatment

Exposure of plants to high or low temperatures before inoculation of pathogens has been shown to increase or decrease the susceptibility of those plants. However, the results from the thesis showed that placing RM or CW plants, for brief periods, in temperatures which varied by c. 40°C above their normal environment to c. 30°C below before inoculation of P. phaseolicola failed to alter host response.

Past work has shown that heat treatment of plants has increased susceptibility of apple to Botrytis allii (Vasudeva, 1930); of bean to Erysiphe polygoni, Colletotrichum lindemuthianum and Uromyces phaseoli (Yarwood, 1956); of wheat to Puccinia (Straib and Noll, 1944); and of potato to Erwinia caratovora (Gregg, 1952). Kassanis (1957) has shown that if Nicotiania glutinosa plants are infected with tobacco mosaic virus (TMV) and kept at 20°C then necrotic local lesions are produced, but if the plants are kept at 30°C systemic mottling occurs. Similarly in wheat possessing the Sr 6 gene, a temperature threshold exists in that plants kept above 26°C become susceptible to Puccinia graminis tritici but below 26°C they become resistant. Nemeth and Klement (1967) also reported that in tobacco kept above 37°C no HR developed to incompatible bacteria.

In some cases preinoculation heating decreases infection (Keyworth and Dimond, 1952; Yarwood, 1956). Exposure

of plants to low temperature also affects their susceptibility to disease (Moore, 1944; Kerling, 1952; Weiss et al., 1928 and Fawcett, 1936).

Past work has shown that, in many cases, alteration of host response to pathogens has resulted through exposing plants to only brief periods in an altered environment before inoculation. In view of this, it must be assumed that any visible changes in host response result through repression or derepression of that part of the genome of the host responsible for expression to pathogens. The failure, of the experiment in the thesis, to achieve changes in host response to P. phaseolicola suggests that the metabolism of the plant was not significantly altered despite in some cases severe damage to the foliage resulting through treatment.

Use of cross protection

The results in the thesis show that inoculation of suspensions of P. fluorescens or P. solanacearum into leaves of RM or CW 24 h before inoculation of P. mors-prunorum or P. phaseolicola, into the same areas of leaves, prevented HR but not SR. Pre-inoculation of P. fluorescens or P. solanacearum as heat killed cells before inoculation of P. mors-prunorum or P. phaseolicola had no effect on HR or SR.

Averre and Kelman (1964) reported that mixed inoculation of virulent and avirulent cells of P. solanacearum protected against the susceptible reaction in tobacco. Suppression of HR in tobacco has also been reported. Novacky et al. (1973) showed, by preinoculating tobacco

leaves with low concentrations of P. pisi before inoculation with concentrations of bacteria which normally induce HR, that HR was suppressed. However, Cook (1975) obtained opposite results working with low concentrations of X. vesicatoria and pepper leaves. Protection of pear and apple against fireblight, caused by E. amylovora, by avirulent cells of E. amylovora, E. herbicola and P. tabaci has been reported (Goodman, 1967; McIntyre et al., 1973). In a fungal system, Kuć et al. (1975) showed that cucumber susceptible to Colletotrichum lagenarium race 1 could be protected against disease by the pathogen by prior inoculation with low levels of inoculum. In a viral system, Loebenstein (1962) showed that partial protection to TMV in tobacco could be attained by treating leaves with TMV protein solution 4 d before inoculation with the pathogen.

The majority of past reports show that protection against HR and SR was achieved by using heat killed cells of the pathogen. Lovrekovich and Farkas (1965) induced protection against wild fire desease in tobacco by preinoculating leaves with heat killed cells of P. tabaci prior to inoculation with live cells. Partial protection was also obtained with heat killed cells of P. syringae and C. flaccumbfaciens. Lozano and Sequeira (1970) reported that protection against HR in tobacco leaves could also be achieved by preinoculating heat killed cells of P. solanacearum before inoculation with live cells of the same bacterium. Similar protective effects were also achieved with heat killed cells of P. lachrymans and X. axonopodis.

The suppression of HR by heat killed cells of P. solanacearum has been shown to be associated with glycoproteins in the periplasmic space of the bacterial cell wall (Sequeira et al., 1972; Wacek, 1974). It is as yet uncertain as to whether the 'protectant' from live cells is also glycoprotein. The result of Sequeira et al. contrasts with that of McIntyre et al. (1975) who showed that DNA was the protectant against fireblight, when heat killed cells of E. amylovora were injected prior to virulent live cells.

Ryan (1974) suggested that the HR protecting fraction (derived from heat killed cells or otherwise) causes moderate injury to the plant which in turn produces its own fraction to resist necrotization of tissue caused by the incompatible bacteria. The antibacterial plant fraction is thought to be induced by a wound hormone which systemically migrates through the plant. This systemic protection is similar to that found in cucumber leaves infected with Colletotrichum lagenarum (Kuć et al., 1975); in tobacco with heat killed cells of P. solanacearum (Sequeira et al., 1972); and in tobacco with live cells of P. pisi (Novacky et al., 1973).

Overall, it appears that protection is dependent on the time between the treatment and challenge, and the relative concentration of protectant to live cells. The experiment in the thesis showed that heat killed cells of P. fluorescens and P. solanacearum failed to protect against HR in bean leaves. This may implicate that the protectant against P. phaseolicola and P. mors-prunorum is only produced by living cells or is denatured and inactivated by

the heat killing process. Finally the effectiveness of P. fluorescens and P. solanacearum in protecting against HR may be a reflection of their distant relation to the other bacteria used in the experiment as classified by the eighth edition of Bergey's manual.

Analysis of bacterial cell walls

From the results presented earlier based on polyacrylamide gel electrophoresis and amino acid analysis it seems that the composition of some of the proteins in the cell walls of P. phaseolicola races 1 and 2, P. morsprunorum, P. fluorescens and P. lachrymans were related to the reactions caused by the bacteria in RM and CW.

Past work has also shown that proteins within the cell walls of micro-organisms can be used for identification. Rottem and Razin (1967) showed that the electrophoretic pattern for proteins from the cell membranes of mycoplasmas were highly specific for different strains. Similarly Larsen et al. (1969) provided evidence that Streptococcus Group A could be subdivided into types, as determined by electrophoresis, based upon the presence of characteristic proteins in the cell walls. It had previously been shown that each of the types could be serologically recognized by proteins on the cell wall (Lancefield, 1962).

A role for proteins in the cell walls of pathogens, as recognition factors for hosts when challenged by the pathogens, has been suggested (Devay et al., 1972). The common antigen hypothesis suggests that common proteins (antigens) are shared by the pathogen and host in compatible reactions. In incompatible reactions, common antigens

are not found but recognition of the pathogen by the host, or vice-versa, occurs to cause a resistant reaction. In serological work with flax rust, caused by Melampsora lini, it was shown that four races of the pathogen shared an antigen with cultivars which responded compatibly. In those cultivars which reacted incompatibly there were no common antigens (Doubly et al., 1960). Similar relations have been shown in sunflower and Agrobacterium tumefaciens (Devay et al., 1970) and in cotton and Xanthomonas malvacearum (Schnathorst and Devay, 1963). However, the occurrence of immune responses in plants similar to those that occur in animals is questionable, since there is no conclusive evidence that antibodies are formed by plant cells in response to foreign antigens.

An alternative role for proteins common to cell walls of bacteria and host tissue may be based on specific multiplication or attachment sites in the host to which specific bacteria could bind and then cause a compatible reaction. Ercolani (1970) suggested that this does occur and that multiplication sites in the host are both 'sensitivity and susceptibility determinants'. Activation of the former would result in HR and activation of the latter would lead to HR. Recent evidence has shown that in HR incompatible bacteria agglutinate in clumps on the host cell surface (Goodman, 1974; Sequeira et al., 1976) and Lippincott and Lippincott (1975) have shown that this also happens when Agrobacterium tumefaciens infects plants.

No attempt was made to compare proteins of bean plants to those of bacterial cell walls. However, the presence of proteins common to the cell walls of P. phaseolicola race 1

and P. mors-prunorum (which cause HR in RM) but which are not present in cell walls of P. phaseolicola race 2 and P. fluorescens (which do not cause HR in RM) suggests that these proteins may act as recognition factors for the host. Secondly, the fact that at least a third of the complement of cell wall proteins of P. phaseolicola race 1 and P. phaseolicola race 2 are different, provides ample opportunity for similarity or dissimilarity of proteins (antigens) to occur between one race and the host but not the other. Thirdly, the substantial qualitative difference in amino acid composition between the cell walls of the bacteria examined provides evidence that the dissimilar proteins are formed by different combinations of amino acids rather than by varying proportions of the same amino acid. Serologically, this fact increases the probability that antigens in the cell walls will be different.

Finally, the number of proteins (14-20) found in the cell walls of the bacterial species examined compares well with previous work by Schaitman (1970) who found c. 20 different proteins when the cell wall of E. coli was examined by polyacrylamide-gel electrophoresis.

In summary, it appears that a relationship may exist between the proteins in the cell walls of bacteria and the reactions those bacteria cause in plants. This suggests that a possible role for cell wall proteins is as recognition factors in determining the type of reaction caused by the bacteria in the plant.

Transmission electron-microscopy and analysis of bean leaves

Inoculation of monofoliate leaves with Pseudomonas phaseolicola leads to chlorosis and ultrastructural modi-

fications in trifoliate leaves. Since bacterial numbers decrease just before chlorosis becomes apparent it is suggested that chlorosis and ultrastructural modifications are due to production, accumulation and systemic spread of a bacterial metabolite or bacterial lysate and not solely to bacterial presence in trifoliate leaves. This systemic metabolite is most probably phaseotoxin as suggested by Rudolph (1966).

The modifications attributed to toxin systemicity are the appearance in the vacuole of a darkly staining material mostly associated with the tonoplast, the decrease of chlorophyll and the formation of large starch grains within the chloroplasts, the occasional appearance of granular inclusions in the middle lamella region, and the increases in endoplasmic reticulum and numbers of ribosomes.

The vacuolar inclusions appeared in three types which probably represents successive stages of formation. Histochemical tests did not establish that they were phenolic, lipid or proteinaceous in nature, but total phenolics in trifoliate leaves fourteen days after monofoliate inoculation showed an increase of c. 100 μg/g fresh wt of leaf. This increase in phenolic concentration may be sufficient to account for the widespread distribution of the inclusion within the cells.

The appearance of these inclusions as three types is similar to that found by Mueller and Beckman (1974) for banana root cells. The inclusions in that case were also osmiophilic but shown to be phenolic. Shumaway et al. (1972) provided evidence that protein also accumulates in cell vacuoles of tomato and tobacco leaves as a darkly

staining inclusion. However, the protein tests provided no evidence to support this.

The formation of phenolic and tannin compounds in the vacuole is normally associated with extra-vacuolar origin. Workers have implicated the vesicles of dictyosomes (Amelunxen and Gronau, 1969); the dilated cisternae of the endoplasmic reticulum (Chafe and Durzan, 1973; Diers et al., 1973), and modified plastids (Wardrop and Cronshaw, 1962). Mueller and Beckman (1974) could not confirm the extra vacuolar origin although they noted that the cytoplasm became granular, contained large concentrations of ribosomes and had a more extensive system of endoplasmic reticulum. A larger and more extensive endoplasmic reticulum was also found in this present work and the possibility of involvement in the production of the vacuolar inclusion seems likely.

It seems probable that the vacuolar inclusion is phenolic because of its osmophilic nature (Plate 9) and widespread distribution and appearance within cells. Both characteristics are peculiar to phenolic compounds. This is supported by an increase in concentration of total phenolics (c. 80 μg/g fresh wt) at a time (10-14 d) when chlorosis of trifoliate leaves and ultrastructural modifications became more pronounced. The increase in phenolic compounds for the first ten days was c. 30 μg/g fresh wt. The role of phenolics in the defense mechanisms of plants is well documented (Kosuge, 1969). However, the fact that the inclusions did not react positively in histochemical tests for phenolic substances suggests that they may be in some precursor form as postulated by Perera and

Gay (1976).

Previous ultrastructural studies of bean inoculated with P. phaseolicola have shown disruption of granae and membranes of the chloroplast (Bajaj et al., 1969; Sigee and Epton, 1976). No evidence of this was found in the present work. However, as in earlier work, large and numerous starch grains were found although chlorophyll levels were half those of controls. As starch production depends ultimately on photosynthesis then a substantial reduction in chlorophyll would be expected to decrease production of starch. The anomaly of excessive starch in the chloroplasts would be explained if there were reduced translocation of photosynthate from the leaves through damage to the phloem. This is known for certain virus diseases such as potato leaf roll and sugar beet yellows. Also, starch accumulation may be attributed to the inability of the plant to degrade it for translocation because of changes in the necessary enzymes or altered membrane permeability.

A relation between chlorosis of trifoliate leaves and the toxin produced by P. phaseolicola has been shown by Rudolph and Stahmann (1966). In view of the chlorophyll level in trifoliate leaves of infected plants and the normal appearance of the chloroplasts it seems probable that the integrity of the latter is not directly dependent on the former.

The granular inclusion occasionally observed in the middle lamella region appears different from that in the vacuoles; no attempt could be made to analyse the apparent difference. Phytopathogenic bacteria are usually present in intercellular spaces and it is possible that this

granular inclusion is associated with the defense mechanism of the plant.

The increase in concentration of endoplasmic reticulum and ribosomes and the possible rôle of both in the formation of the vacuolar inclusions has already been mentioned. The fact that both appeared to increase suggests enhancement of protein metabolism.

Finally, it is known that phaseotoxin is a specific inhibitor of OCT and it has been postulated that chlorosis of trifoliate leaves is causally related to this inhibition (Patil and Gnanamanickam, 1976). Thus, the decline in chlorophyll levels in the leaves may well be attributed to phaseotoxin. However, the absence of cytological degeneration, particularly in the chloroplast (Plate 8), tends to suggest that chlorosis is a side-effect of phaseotoxin action with the main effect directed at the shoot apex.

Scanning electron-microscopy of bean plant tissue undergoing the susceptible reaction

The results in the thesis show that movement of P. phaseolicola to trifoliate leaves, following inoculation of monofoliate leaves, occurs mainly in the xylem vessels. However, some bacteria were also found in phloem and in the parenchyma cells of the cortex though numbers in these two tissues always appeared (by visual assessment) to be 4-5 fold less than in the xylem. A similar result was found by Ikotun (1975) when investigating the tissues of cassava infected with X. manihotis. In this case, however, considerable degeneration of stelar tissue was caused by X. manihotis. This did not occur in bean tissue infected

with P. phaseolicola.

The increase in bacterial numbers at the junction of the monofoliate petioles and in the stem and petiole tissue above the site of inoculation is probably due to a confluence of bacteria from the two sites of inoculation.

The presence of large numbers of bacteria in tissues between the monofoliate and trifoliate leaves complements the c. 1000 fold increase in numbers of bacteria found in trifoliate leaves eight days after inoculation of monofoliate leaves. Similarly Hildebrand and Schroth (1971) recovered P. phaseolicola from 39 out of 40 trifoliate leaves. However the results contrast with those of other work in which P. phaseolicola was rarely isolated from trifoliate leaves following inoculation of monofoliate leaves (Omer and Wood, 1969; Waitz and Schwartz, 1956).

Chlorosis of trifoliate leaves, following inoculation of monofoliate leaves with P. phaseolicola has been attributed to toxin produced by the bacterium. Rudolph (1969) has isolated and purified the toxin and shown it to be polysaccharide composed predominantly of hexose units and with a molecular weight of 2100. However, purification of the toxin (phaseotoxin) by Patil (1970) has shown it to be ninhydrin positive with a molecular weight much smaller than that found by Rudolph. Both toxins have been reported to mimic disease symptoms when applied to bean in the absence of the bacterium.

Recent ultrastructure work has shown that P. phaseolicola in bean leaves produces large surface vesicles which are released into the intercellular space where they

rupture and liberate their contents (Sigee and Epton, 1975). In Escherichia coli, lipopolysaccharide has been shown to be released from cells with surface protruberances but not from cells without them (Knox et al., 1966). The toxicity in plants of lipopolysaccharides and proteins derived from bacteria has been shown (Feder and Ark, 1951; Keen and Williams, 1971). Wiebe and Chapman (1968) showed that strains of Pseudomonas produced protuberances but only at 22°C and when the medium contained a high concentration of nutrients. Patel and Walker (1963) has shown that development of the halo in monofoliate leaves and chlorosis in trifoliate leaves of bean, infected with P. phaseolicola, was also temperature dependent. At 20°C large haloes and pronounced chlorosis was evident but at 28°C these effects were rarely present. Skoog (1952) has shown that this temperature range correlated well with toxin production in vitro.

A relation between ED, toxin and vesicle production by the bacteria can be but speculative. However, the tentative inference must be that ED and the toxin produced by P. phaseolicola are one and the same. This toxin is presumably formed in the vesicles liberated from the surface layers of the bacterial cells. The vesicles, once released, rupture and liberate their contents into the lumen of the plant cells as shown by Sigee and Epton (1975). In support of this, the results in the thesis showed that ED only occurs in high concentration in cells when the bacterial population within those cells was also large. This infers that ED is, at least, a product of the bacterium. Further evidence, provided by Ikotun (1975), showed

that the lumena of cells of cassava infected with X. manihotis contained strands of polysaccharide slime which was similar in structure and electron density to ED found in bean cells infected with P. phaseolicola.

Results from the previous section of the thesis showed that the onset of chlorosis of trifoliate leaves in bean was concomitant with a decline in bacterial numbers in the trifoliate leaves. This suggests that the toxin is probably translocated from other regions in the plant to the trifoliate leaves. Evidence from this section of the results to support the hypothesis that the toxin is translocated is poor since the amount of ED found in cells in the absence of bacteria was considerably less than when the bacteria were present. However, the chlorotic halo formed around the inoculation area in monofoliate leaves does enlarge radially over 16 days thus providing tentative evidence of toxin translocation.

The anomaly regarding translocation of phaseotoxin may be resolved if it is considered that a source-sink relationship exists between the site of production and site of action of phaseotoxin. In this hypothesis small amounts of phaseotoxin are translocated from the bacteria in the monofoliate leaves and other tissues of the plant (source) to regions of active growth, namely the shoot apex and developing trifoliate leaves (sink). Phaseotoxin, having reached the sink, accumulates in increasing concentrations to stunt the growth of the plant and induce chlorosis of trifoliate leaves.

Microautoradiography of bacterial cells

The results in the thesis showed that the method used to label the RNA of bacterial cells was successful. This is particularly shown in the thin section autoradiograms where the label is clearly incorporated within the cell of the bacterium. In the whole cell autoradiograms silver grains could have been caused by contamination of the surface of the bacterium (despite precautions taken to reduce this error) from tritium in the growth medium. This method of autoradiography was designed for use on a flexible model basis such that on a subsequent occasion it could be applied to labelling other metabolites such as DNA or protein within the bacterial cell. The results also showed that bacteria with labelled RNA could cause HR or SR when injected into bean leaves. Thus, in future, experiments, by injecting P. mors-prunorum or P. phaseolicola (which have specific metabolites labelled) into bean leaves it should be possible to assess which metabolites or types of metabolites are passing from the bacterium to the host cells in HR or SR. Movement of labelled metabolites from the bacterium to host cells will be recorded by silver grains appearing directly above the host cells. From the results it was shown that thin section autoradiography gave a resolution of c. 0.2 μ which is sufficient to eliminate errors which may be involved in determining the point source of the label within host cells.

Comparison of the results for resolution in whole cell and thin section autoradiography reveal that in the former a figure of c. 0.75 μ was achieved whereas in the latter a resolution of c. 0.2 μ was achieved. These fig-

ures are supported by the results obtained by van Tubergen (1961) and Caro (1962) for whole cell and thin section autoradiography respectively. Thus, as resolution is the main criterion in autoradiography it is evident that of the two techniques, thin section is the better despite the long incubation period needed because of the thinness of the section. However whole cell autoradiography could be applied to preliminary experiments by taking sections c. 1.0 μ thick of plant tissue (which has previously been injected with bacteria) to determine the optima required to achieve the best results.

Finally, it was shown by Caro (1962) that when cells of Escherichia coli were fully labelled with tritiated leucine an average 2-4 silver grains per bacterial cell were recorded. The results in the thesis show that on average 1.0 grain per bacterial cell is present. In the work by Caro (1962), E. coli was fully labelled by growing it for 7 generations in a medium containing the tritium source. However when the RNA of P. mors-prunorum was labelled, the bacterium was grown in a medium for an optimal period of $2\frac{1}{2}$ h. Assuming the divisional time of P. mors-prunorum to be c. 40 min at 25°C then the bacterium will have divided only 4 times at the end of the incubation period. Thus, it appears that in the labelling of P. mors-prunorum an unknown factor is involved which prevents the maximum amount of tritium from being incorporated in the bacterial cell. Elimination of this factor will obviously enhance the profitability of future experiments.

REFERENCES

ALLINGTON, W. B. and CHAMBERLAIN, W. D. (1949). Trends in population of pathogenic bacteria within leaf tissues of susceptible and immune plant species. Phytopathology 39, 657-660.

AMELUNXEN, F. and GRONAU, G. (1969). Unterschungen an den Gerbstoffzellen der Niederblätter von Acorus calarius L. Cytobiologie 1, 58-69.

ARONSON, A. I. (1961). The effect of 5-fluorouracil on bacterial protein and ribonucleic acid synthesis. Biochem. Biophys. Acta 49, 98-107.

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

BACKMAN, P. A. and DEVAY, J. E. (1971). Studies on the mode of action and biogenesis of the phytotoxin syringomycin. Physiological Plant Pathology 1, 215-233.

BAJAJ, Y. P. S., SPINK, G. C. and SAETTLER, A. W. (1969). Ultrastructure of chloroplasts in bean leaves affected by Pseudomonas phaseolicola. Phytopathology 59, 1017 (Abstr.).

BAKHOR, I., KUNG, G. M. and BONNER, J. (1969). Sequence-specific interaction of DNA and chromosonal protein. J. Mol. Biol. 39, 351.

BALDWIN, C. H. and GOODMAN, R. N. (1963). Prevalence of E. amylovora in apple buds as detected by phage typing. Phytopathology 53, 1299-1303.

BARBARA, D. J. and WOOD, K. R. (1974). The influence of actinomycin D on cucumber mosaic virus (strain w) multiplication in cucumber cultivars. Physiological Plant Pathology 4, 45-50.

BATEMAN, D. F. (1967). Alteration of cell wall components during pathogenisis by Rhizoctonia solani. In "The dynamic role of molecular constituents in plant parasite interaction" (Ed. Mirocha, C. J. and Uritani, I.) Bruce, St. Paul, Minnesota.

BRAITHWAITE, C. W. D. and DICKEY, R. S. (1970). Synergism between Pseudomonas caryophylli and a species of Corynebacterium. Phytopathology 60, 1046-1051.

BROCK, T. D. and BROCK, M. L. (1959). Similarity in mode of action of chloramphenicol and erythromycin. Biochem. Biophys. Acta 33, 274-275.

BURKHOLDER, W. H. (1926). A new bacterial disease of the bean. Phytopathology 16, 915.

BURKHOLDER, W. H. and ZALESKI, K. (1932). Varietal susceptibility of beans to an American and European strain of Phytomonas medicaginis var. phaseolicola and a comparison of the strain in culture. Phytopathology 22, 85-94.

BYTHER, R. S. and STEINER, G. W. (1975). Heat induced resistance of sugar cane to Helminthosporium sacchari and Helminthosporide. Plant Physiology 56, 415-419.

CARO, L. G. (1962). High resolution autoradiography. J. Cell Biol. 15, 189-199.

CHAFE, S. C. and DURZAN, D. J. (1973). Tannin inclusions in cell suspension cultures of white spruce. Planta 113, 251-262.

CHATTERJEE, A. K., GIBBINS, L. N. and CARPENTER, J. A. (1969). Some observations on the physiology of Erwinia herbicola and its possible implications as a factor antagonistic to Erwinia amylovora in the "fire-blight" syndrome. Can. J. Microbiol. 15, 640-642.

COHEN, S. S., FLAKS, J. G., BARNER, H. D., LOEB, M. R. and LICHTENSEIN, J. (1958). The mode of action of 5-fluorouracil and its derivatives. Proc. Natl. Acad.

Sci. 44, 1004-1012.

COOK, A. A. and STALL, R. E. (1968). Effect of Xanthomonas vesicatoria on the loss of electrolytes from leaves of Capsicum annum. Phytopathology 58, 617-619.

COOK, A. A. and STALL, R. E. (1971). Calcium suppression of electrolyte loss from pepper leaves inoculated with Xanthomonas vesicatoria. Phytopathology 61, 484-487.

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

CORDEN, M. E. and EDINGTON, L. V. (1960). A calcium requirement for growth regulator induced resistance to Fusarium wilt of tomato. Phytopathology 50, 625-626.

COYNE, D. P., SCHUSTER, M. L. and SHAUGHNESSY, L. (1966). Inheritance of reaction to halo blight and common blight bacteria in a Phaseolus vulgaris variety cross. Plant Dis. Reptr. 50, 29-32.

CROSSE, J. E. (1965). Bacterial canker of stone fruit VI. Inhibition of leaf scar infection of cherry by a saprophytic bacterium from leaf surfaces. Annals of Applied Biology 56, 449-460.

CROXALL, H. E., NORMAN, T. M. and GWYNE, D. C. (1957). Effect of 2:4:6 trichlorophenoxyacetic acid on the susceptibility of tomato plants to Didymella lycopersici. Plant Pathology 6, 27-31.

CRUICKSHANK, I. A. M. and PERRIN, D. R. (1964). Pathological function of phenolic compounds in plants. In Biochemistry of phenolic compounds. (Ed. J. B. Harborne) Academic Press, New York.

CRUICKSHANK, I. A. M. and PERRIN, D. R. (1971). Studies on phytoalexins XI. The induction, antimicrobiol spectrum and chemical assay of phaseollin. Phytopath. Z. 70, 209-229.

CUNLIFFE E. and McQUILLEN, K. (1967). Bacterial Protein Synthesis : The effects of antibiotics. J. Mol. Biol. 30, 137-146.

DALY, J. M. and INMAN, R. E. (1958). Changes in auxin levels in sunflower hypocotyls infected with Puccinia carthami. Phytopathology 48, 91-97.

DAVIES, J., ANDERSON, P. and DAVIS, B. D. (1965). Inhibition of protein synthesis by spectinomycin. Science 149, 1096-1098.

DEVAY, J. E., ROMANI, R. J., MONADJEM, A. M. and ETZLER, M. (1970). Induction of phytoprecipitins in sunflower glass tissue in response to infection by Agrobacterium tumefaciens. Phytopathology 60, 1289 (Abstr.).

DEVAY, J. E., CHARUDATTAN, R. and WIMALAJEEWA, D. L. S. (1972). Common antigenic determinants as a possible regulator of host pathogen compatibility. American Nature 106, 185-194.

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

DIERS, L., SCHÖTZ, F. and MEYER, B. (1973). Über die Ausbildung von Gerbstoffvakuolen bei Oenothoren. Cytobiologie 7, 10-19.

DIXON, R. O. D. (1969). Rhizobia, with particular reference to relationship with host plants. Ann. Rev. Microbiol. 23, 137-158.

DOGANY, Z. U. (1973). Bacterial motility and plant hypersensitivity. Ph.D. Thesis. University of Delaware, U.S.A.

DOUBLY, J. A., FLOR, H. H. and CLAGETT, C. O. (1960). Relation of antigens of Melampsora lini and Linum usitatissium to resistance and susceptibility. Science 131, 229.

DOUPNIK, B. (1968). The suppression of victorin induced disease by calcium. Phytopathology 58, 215-218.

DOWSON, W. J. (1943). On the generic names Pseudomonas, Xanthomonas and Bacterium for certain bacterial plant pathogens. Bri. Mycol. Soc. Trans. 26, 4.

EDGINGTON, L. V. and WALKER, J. C. (1958). Influence of calcium and boron nutrition on development of Fusarium wilt of tomato. Phytopathology 48, 324-326.

ENNIS, H. L. and LUBIN, M. (1964). Cycloheximide : Aspects of inhibition of protein synthesis in mamallian cells. Science 146, 1474-1476.

EPSTEIN, E. (1961). The essential role of calcium in selective cation transport by plant cells. Plant Physiology 36, 437-444.

ERCOLANI, G. L. and CROSSE, J. E. (1966). Growth of Pseudomonas phaseolicola and related plant pathogens in vivo. J. Gen. Microbiol. 45, 429-39.

ERCOLANI, G. L. (1967). Bacterial canker of tomato. I. Analysis of some factors affecting the response of tomato to Corynebacterium michiganense. Phytopathologia Mediterranea 6, 19-29.

ERCOLANI, G. L. (1970 a). Bacterial canker of Tomato. III. The effect of auxotrophic mutation on the virulence of Corynebacterium michiganense. (E. F. Sm.) Jens. Phytopathologia Mediterranea 9, 145-150.

ERCOLANI, G. L. (1970 b). Bacterial canker of tomato. IV. The interaction between virulent and avirulent strains of Corynebacterium michiganense (E. F. Sm.) Jens. Phytopathologia Mediterranea 9, 151-159.

ERCOLANI, G. L. (1973). Two hypotheses on the aetiology of response of plants to phytopathogenic bacteria. J. Gen. Microbiol. 75, 83-95.

ERIKSON, D. (1945). Certain aspects of resistance of plum trees to bacterial canker. Part II. On the nature of bacterial invasion of Prunus spp. by Pseudomonas

mors-prunorum Wormald. Annals of Applied Biology 32, 112-117.

ÉRSEK, T., BARNA, B. and KIRÁLY, Z. (1973). Hypersensitivity and the resistance of potato tuber tissues to Phytophthora infestans. Acta Phytopath. Acad. Sci. Hung. 8, 3-12.

FARABEE, G. J. and LOCKWOOD, J. L. (1958). Inhibition of E. amylovora by bacterium species isolated from fire blight cankers. Phytopathology 43, 209-211.

FARKAS, G. L. and KIRALY, Z. (1962). Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopath. Z. 44, 105-150.

FAWCETT, H. S. (1936). Citrus diseases and their control. McGraw-Hill, New York.

FEDER, W. A. and ARK, P. A. (1951). Wilt inducing polysaccharides derived from crown-gall, bean-blight, and soft-rot bacteria. Phytopathology 41, 804-808.

FERGUSON, W., LYALL, L. H. and JASMIN, J. J. (1956). Progress report, Horticulture Division, Central Experimental Farm, Ottawa. Rev. Applied Mycol. 35, 419-422.

FLOR, H. H. (1956). The complementary genetic systems in flax and flax rust. Advances in Genetics 8, 29-54.

FORSYTH, F. R. (1957). Effect of ions of certain metals on the development of stem rust in the wheat plant. Nature 179, 217-218.

FOX, R. T. V., MANNERS, J. G. and MYERS, A. (1972). Ultrastructure of tissue disintegration and host reactions in potato tubers by Erwinia caratovora var. atroseptica. Potato Res. 15, 130-145.

GARDNER, J. M. and KADO, C. I. (1972 a). Induction of the hypersensitive reaction in tobacco with specific high molecular weight substances derived from the osmotic shock fluid of Erwinia rubrifaciens. Phytopathology 62, 759 (Abstr.).

GARDNER, J. M. and KADO, C. I. (1972 b). Deoxyribonucleic acid and homologies between Erwinia species and between other members of the Enterobacteriaceae as determined by molecular hybridisation and renaturation techniques. Phytopathology 62, 759 (Abstr.).

GAÜMANN, E. (1950). Principles of plant infection. Crosely Lockwood and Son Ltd., London.

GAYLER, K. R. and GLASZIOU, K. T. (1968). Plant enzyme synthesis : Decay of messenger RNA for peroxidase in sugar cane stem tissue. Phytochemistry 7, 1247-1251.

GHABRIAL, S. A. and PIRONE, T. P. (1967). Physiology of tobacco etch virus-induced wilt of Tobasco peppers. Virology 31, 154-162.

GLASZIOU, K. T. (1969). Control of enzyme formation and inactivation in plants. Ann. Rev. Plant Physiology 20, 63-88.

GOODMAN, R. N. (1967). The protection of apple stem tissue against Erwinia amylovora infection by avirulent strains and three other bacterial species. Phytopathology 57, 22-24.

GOODMAN, R. N. (1968). The hypersensitive reaction in tobacco : a reflection of changes in host permeability. Phytopathology 58, 872-873.

GOODMAN, R. N. (1971). Re-evaluation of the role of NH_3 as the cause of the hypersensitive reaction. Phytopathology 61, 893 (Abstr.).

GOODMAN, R. N. and PLURAD, S. R. (1971). Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacterial. Physiological Plant Pathology 1, 11-16.

GOODMAN, R. N. (1972). Electrolyte leakage and membrane damage in relation to bacterial population, PH and ammonia production in tobacco leaf tissue inoculated with Pseudomonas pisi. Phytopathology 62, 1327-1331.

GOODMAN, R. N. (1972). Phytotoxin induced ultrastructural modifications of plant cells. In Phytotoxins in Plant Disease (Ed. Wood, R. K. S., Graniti, A. and Ballio, A.). Academic Press, London.

GOODMAN, R. N. (1974). Induced resistance and hypersensitive reaction to bacterial infection. In Proc. of Post Congress Osaka Symposium on Phytopathology.

GROGAN, R. G., LUCAS, L. T. and KIMBLE, K. A. (1965). The correlation of pathogenicity in Pseudomonas lachrymans and P. phaseolicola with specific capsular antigens. Phytopathology 55, 1060.

GOODMAN, R. N., HUANG, S. and HUANG, P. Y. (1974). A host specific phytotoxic polysaccharide from apple tissue infected by Erwinia amylovora. Science 183, 1081-1082.

GREGG, M. (1952). Studies in the physiology of parasitism. XVII. Annals of Botany 16, 235-250.

HACKER, R. G. and VAUGHN, J. R. (1957). Cycloheximide analogues cause preinfection resistance to Puccinia graminis var. tritici in spring wheat. Phytopathology 47, 14. (Abstr.).

HALE, C. N., WHITBREAD, R., JANSSEN, B. and MARSHALL, C. (1972). Growth response of dwarf bean to infection by P. phaseolicola (Burk.) Dows. Annals of Botany 36, 135-143.

HANCHEY, P., WHEELER, H. and LUKE, H. H. (1968). Pathological changes in ultrastructure. Effects of victorin on oat roots. American Journal of Botany 55, 53-61.

HEBERLEIN, G. T. and LIPPINCOTT, J. A. (1965). Ultraviolet induced changes in the infectivity of Agrobacterium tumefaciens. J. Bacteriology 89, 1511-1514.

HEIDELBERGER, C. (1957). Fluorinated Pyrimidines, a new class of tumour inhibitory compounds. Nature 179, 663-667.

HILDEBRAND, E. M. and MACDANIELS, L. H. (1935). Modes of entry of Erwinia amylovora into the flowers of the principal pome fruits. Phytopathology 25, 20 (Abstr.).

HILDEBRAND, E. M. (1937). Infectivity of the fire blight organism. Phytopathology 27, 850-852.

HILDEBRAND, E. M. (1942). A micrurgical study of crown gall infection in tomato. J. Agr. Res. 65, 45-59.

HILDEBRAND, D. C. (1970). Fireblight resistance in Pyrus : hydroquinone formation as related to arbutin activity. Can. J. Botany 48, 177-181.

HILDEBRAND, D. C. and RIDDLE, B. (1971). Influence of environmental conditions on reactions induced by infiltration of bacteria into plant leaves. Hilgardia 41, 33-43.

HILDEBRAND, D. C. and SCHROTH, M. N. (1971). Isolation of P. phaseolicola from bean leaves exhibiting systemic symptoms. Phytopathology 61, 580-581.

HILIE, B. M., MILLER, M. J., IWASAKI, K. and WAHBA, A. J. (1967). Translation of the genetic message, VI. The role of ribosomal units in binding of formyl-methionyl f-RNA and its reaction with puromycin. Proc. Nat. Acad. Sci. 58, 1652-1654.

HOITINK, H. A. J., PELLETIER, R. L. and COULSON, J. G. (1966). Toxemia of halo blight of beans. Phytopathology 56, 1062-1065.

HOITINK, H. A. J. and SINDEN, S. L. (1970). Partial purification and properties of chlorosis inducing toxins of Pseudomonas phaseolicola and Pseudomonas glycinea. Phytopathology 60, 1236-1237.

HOWES, N. K., KIM, W. K., ROHRINGER, R. and SAMBORSKI, D. J. (1973). Evidence for a gene specific RNA determining resistance in wheat to stem rust. Nature 249, 585-588.

HSU, S. T. and DICKEY, R. S. (1972). Interaction between Xanthomonas phaseoli, Xanthomonas vesicatoria,

Xanthomonas campestris and Pseudomonas fluorescens in bean and tomato leaves. Phytopathology 62, 1120-1125.

HURWITZ, J., FURTH, J. J., MALAMY, M. and ALEXANDER, M. (1962). The role of DNA in RNA synthesis III. The inhibition of the enzymatic synthesis of RNA and DNA by actinomycin D and proflavin. Proc. Natl. Acad. Sci. 48, 1222-1230.

IKOTUN, B. (1975). Cassava bacterial blight disease caused by Xanthomonas manihotis (CBB). Ph.D. Thesis. University of London.

JACOB, F. and MONOD, J. (1961). On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol. 26, 193-211.

JENSEN, J. H. and GOSS, R. W. (1942). Physiological resistance to halo blight in beans. Phytopathology 32, 246-253.

JENSEN, J. H. and LIVINGSTONE, J. E. (1944). Variation in symptoms produced by isolates of Phytomonas medicaginis var. phaseolicola. Phytopathology 34, 471-480.

JENSEN, W. A. (1962). Botanical histochemistry : principles and practice. Freeman, San Fransisco.

JENSEN, W. A., POLLOCK, E. G., HEALEY, P. and ASHTON, M. (1964). Kinetin and the nucleic acid content of onion root tips. Exp. Cell Res. 33, 523-530.

JOY, K. W. (1969). Nitrogen metabolism of Lemma minor. 1. Growth, nitrogen sources and amino acid inhibition. Plant Physiology 44, 845-848.

KALINENKO, V. O. (1936). The inoculation of phytopathogenic microbes into rubber bearing plants by nematodes. Phytopath. Z. 9, 407-416.

KASSANIS, B. (1957). Effects of changing temperature on plant virus diseases. Advan. Virus Res. 4, 222-41.

KASSANIS, B. (1963). Interactions of viruses in plants. Advan. Virus Res. 10, 219-55.

KEEN, N. T. and WILLIAMS, P. H. (1971). Chemical and biological properties of a lipomucopolysaccharide from Pseudomonas lachrymans. Physiological Plant Pathology 1, 247-264.

KEEN, N. T. and KENNEDY, B. W. (1974). Hydroxyphaseollin and related isoflavanoids in the hypersensitive resistance reaction of soybeans to Pseudomonas glycinea. Physiological Plant Pathology 4, 173-185.

KEEN, N. (1975). Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? Science 187, 74-75.

KELMAN, A. (1969). How bacteria damage crops. Bull. Cam. Agric. Expt. Sta. No. 780.

KELMAN, A. and SEQUIERA, L. (1972). Resistance in plants to bacteria. Proc. R. Soc. Lond. B. 181, 247-266.

KENNEDY, R. W., MEW, T. W. and OLSON, L. (1971). Reaction of soybean tissue culture to pathogenic and saprophytic bacteria. Proc. Third Int. Conf. Plant Pathogenic Bacteria, Wageningen.

KERLING, L. C. P. (1952). Damage and fungal attack on peas as consequences of night frost. Rev. Applied Mycol. 32, 163 (Abstr.).

KERNKAMP, M. F., de ZEEUW, D. J., CAHEN, S. M., ORTEGA, B. C., TSIANG, C. T. and KHAN, A. M. (1952). Investigations on physiologic specialization and parasitism of Rhizoctani solani. Univ. Minn. Agr. Expt. Sta. Tech. Bull. 200, 1-36.

KEYWORTH, W. G. and DIMOND, A. E. (1952). Root injury as a factor in the assessment of chemotherapeutics. Phytopathology 42, 311-315.

KIRK, J. M. (1960). The mode of action of actinomycin D. Biochem. Biophys. Acta 42, 167-169.

KIRÁLY, Z., EL HAMMADY, M. and POZSÁR, B. I. (1967). Increased cytokinin activity of rust infected bean and broad bean leaves. Phytopathology 57, 93-94.

KIRÁLY, Z. (1972). Hypersensitivity as a consequence and not the cause of plant resistance to infection. Nature 239, 456-458.

KLEMENT, Z. and LOVREKOVICH, L. (1961). Defence reactions induced by phytopathogenic bacteria in bean pods. Phytopath. Z. 41, 217-227.

KLEMENT, Z. and LOVREKOVICH, L. (1962). Studies on host parasite relations in bean pods infected with bacteria. Phytopath. Z. 45, 81-88.

KLEMENT, Z., FARKAS, G. L. and LOVREKOVICH, L. (1964). Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54, 474-477.

KLEMENT, Z. (1965). Method of obtaining fluid from the intercellular spaces of foliage and the fluids' merit as substrate for phytobacterial pathogens. Phytopathology 55, 1033-34.

KLEMENT, Z. and GOODMAN, R. N. (1967). The role of the living cell and induction time in the hypersensitive response of tobacco plants. Phytopathology 57, 322-323.

KLEMENT, Z. and GOODMAN, R. N. (1967). The hypersensitive reaction to infection by bacterial plant pathogens. Ann. Rev. Phytopath. 5, 17-44.

KLEMENT, Z. (1971). The hypersensitive reaction of plants to bacterial infections. Acta Phytopath. Acad. Sci. Hung. 6, 115-118.

KLEMENT, J. (1972). Development of the hypersensitive reaction induced by plant pathogenic bacteria. In Proc. Third Int. Conf. Plant Pathogenic Bacteria, Wageningen, The Netherlands.

KNOX, K. W., VESK, M. and WORK, E. (1966). Relation between excreted polysaccharide complexes and surface structures of a lysine limited culture of Escherichia coli. J. Bacteriology 92, 1206-1217.

KOSUGE, T. (1969). The role of phenolics in host response to infection. Ann. Rev. Phytopath. 7, 194-222.

KUĆ, J., SHOCKLEY, G. and KEARNEY, K. (1975). Protection of cucumber against Colletotrichum lagenarium by Colletotrichum lagenarium. Physiological Plant Pathology 7, 195-199.

LAKSO, J. U. and STARR, M. P. (1970). Comparative injuriousness to plants of Erwinia spp. and other enterobacteria from plants and animals. J. Applied Bacteriology 33, 692-707.

LANCEFIELD, R. C. (1962). Current knowledge of type-specific M antigens of group A. streptococci. J. Immunol. 89, 307-313.

LANGE, L. and MOHR, H. (1965). Die hemmung der phytochrom-induzierten authocyansynthese durch actinomycin D und puromycin. Planta 67, 107-121.

LARSEN, S. A., WEBB, C. D. and MOODY, M. D. (1969). Acrylamide gel electrophoresis of group A streptococcal cell walls. Applied Microbiol. 17, 31-33.

LEBEN, C. (1963). Multiplication of Xanthomonas vesicatoria on tomato seedlings. Phytopathology 53, 778-781.

LELLIOT, R. A., BILLING, E. and HAYWARD, A. C. (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriology 293, 470-489.

LESEMAN, D. and RUDOLPH, K. (1969). Die bildung von Ribosomen - helices unter den Einfluss des Toxins Pseudomonas phaseolicola in Blattern von Mangold (B.U.L.). Z. Pflanzenphysiol. 62, 108-115.

LEWIN, B. M. (1970). The molecular basis of gene express-

ion. Wiley-Interscience, New York.

LEWIS, S. and GOODMAN, R. N. (1965). Mode of penetration and movement of the fire blight bacteria in apple leaf stem tissue. Phytopathology 55, 719-723.

LIPPINCOTT, J. K. and HABERLEIN, G. T. (1965). The quantitative determination of the infectivity of Agrobacterium tumefaciens. American Journal Botany 52, 856-863.

LIPPINCOTT, J. A. and LIPPINCOTT, B. B. (1975)*. The genus Agrobacterium and plant tumorigenesis. Ann. Rev. Microbiol. 29, 377-405.

LOEBENSTEIN, G. (1962). Inducing partial protection in the host plant with native virus protein. Virology 17, 574-581.

LOEBENSTEIN, G. and 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.

LOENING, U. E. (1967). The fractionation of high molecular-weight ribonucleic acid by polyacrylamide-gel-electrophoresis. Biochem. J. 102, 251-257.

LOGAN, C. (1960). Host specificity on two Xanthomonas species. Nature 188, 479-480.

LONGCHAMP, R., ROY, M., and GAUTHERET (1951). Action de l'ester ethylique du 2,4-D sur le développement de Claviceps purpuma dans le champs de blé. Compt. Rend. 233, 888-890.

LOVREKOVICH, L. and FARKAS, G. L. (1963). Kinetin as an antagonist of toxin effect of Pseudomonas tabaci. Nature 198, 710.

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

LOVREKOVICH, L., LOVREKOVICH, H. and GOODMAN, R. N. (1969). Role of ammonia in wildfire disease of tobacco caused by P. tabaci. Phytopathology 59, 1713-1716.

LOVREKOVICH, L., LOVREKOVICH, H. and GOODMAN, R. N. (1970). Ammonia as a necrotoxin in HR caused by bacteria in tobacco. Can. J. Botany 48, 167-171.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1950). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193, 265-275.

LOZANO, J. C. and SEQUEIRA, L. (1970 a). Differentiation of races of P. solanacearum by leaf infiltration. Phytopathology 60, 833-838.

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

LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytology 9, 409-414.

LUKE, H. H., WARMKE, H. E. and HANCHEY, P. (1966). The effects of the pathotoxin victorin on ultrastructure of root and leaf tissue of Avena species. Phyto-pathology 56, 1178-1183.

LUKE, H. H. and FREEMAN, T. E. (1967). Relationship between the molecular structure of cytokinins and victorin activity. Phytopathology 57, 820 (Abstr.).

LYLES, W. E., FUTRELL, M. C. and ATKINS, I. M. (1957). The effect of plant growth regulators on the response of wheat varieties to leaf rust. Proc. Standford Meeting Am. Soc. Plant Physiol..

MACKINNEY, G. (1941). Absorption of light by chlorophyll solutions. J. Biol. Chem. 140, 315-322.

MAGASANIK, B. (1961). Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. 26, 249-256.

MAKMAN, R. S. and SUTHERLAND, E. W. (1965). Adenosine 3′.5′-phosphate in Escherichia coli. J. Biol. Chem. 240, 1309-1313.

MAINO, A. L., SCHROTH, M. R. and VITANZA, V. B. (1974). Synergy between Achromobacter sp. and Pseudomonas phaseolicola resulting in increased disease. Phytopathology 64, 277-283.

MARAMOROSCH, K. (1957). Reversal of virus caused stunting in plants by gibberellic acid. Science 126, 651-652.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3, 208-218.

McINTYRE, J. L., KUĆ, J. and WILLIAMS, E. B. (1973). Protection of pear against fire blight by bacteria and bacterial sonicates. Phytopathology 63, 672-877.

McINTYRE, J. L., KUĆ, J. and WILLIAMS, E. B. (1975). Protection of Bartlett pear against fireblight with deoxyribonucleic acid from virulent and avirulent Erwinia amylovora. Physiological Plant Pathology 7, 153-170.

MEYNELL, G. G. and STOCKER, B. A. D. (1957). Some hypotheses on the actiology of fatal infections of partially resistant hosts and their application to mice challenged with Salmonella paratyphi - β or Salmonella typhimurium by intraperitoneal injection. J. Gen. Microbiol. 16, 38-58.

MOORE, W. C. (1944). Chocolate spot of beans. Agriculture (Engl.) 51, 266-269.

MOUSTAFA, D. F. A. and WHITTENBURY, R. (1970). Properties which appear to allow phytopathogenic pseudomonads to counteract plant defence mechanisms. Phytopath. Z. 67, 214-224.

MUELLER, W. C. and BECKMAN, C. H. (1974). Ultrastructure of the phenol storing cells in the roots of banana.

Physiological Plant Pathology 4, 187-190.

MULLER, K. O. (1959). Hypersensitivity. In Plant Pathology. 1. 469-519. (Horsfall, J. G., Dimond, A. E., Eds., Academic Press, New York.

NEMETH, J. and KLEMENT, Z. (1967). Changes in the respiration rate of tobacco leaves infected with bacteria in relation to the hypersensitive reaction. Acta Phytopath. Acad. Sci. Hung. 2, 303-308.

NEMETH, J., KLEMENT, Z. and FARKAS, G. L. (1969). An enzymological study of the hypersensitive reaction induced by Pseudomonas syringae in tobacco leaf tissue. Phytopath. Z. 65, 267-278.

NICKELL, L. G. (1959). Antimicrobial activity of vascular plants. Econ. Bot. 13, 281-318.

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

NOVACKY, A., ACEDO, G. and GOODMAN, R. N. (1973). Prevention of bacterially induced hypersensitive reaction by living bacteria. Physiological Plant Pathology 3, 133-136.

O'BRIEN, F. M. (1973). Resistance of french bean leaves to bacteria. Ph.D. Thesis. University of London.

O'BRIEN, F. M. and WOOD, R. K. S. (1973). Antibacterial substances in hypersensitive responses induced by bacteria. Nature 242, 532-533.

OMER, M. E. H. (1966). A study of halo blight of beans. Ph.D. Thesis. University of London.

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

PANOPOULOS, N. J. and SCHROTH, M. N. (1971). Enhancement of Pseudomonas phaseolicola infection of beans by

3′:5′-cyclic AMP. Phytopathology 61, 905 (Abstr.).

PATEL, P. N. and WALKER, J. C. (1963). Relation of air temperature and age and nutrition of the host to the development of halo and common bacterial blights of bean. Phytopathology 53, 407-411.

PATEL, P. N. and WALKER, J. C. (1964). Reactions of Phaseolus spp. to two races of Pseudomonas phaseolicola and a brown spot organism. Phytopathology 54, 903 (Abstr.).

PATEL, P. N. and WALKER, J. C. (1965). Resistance in Phaseolus to halo blight. Phytopathology 55, 889-894.

PATIL, S. S. and TAM, L. Q. (1970). Mode of action of P. phaseolicola toxin : Mechanism of ornithine accumulation. Phytopathology 60, 1536 (Abstr.).

PATIL, S. S. (1972). Purification of the phytotoxin from P. phaseolicola. Phytopathology 62, 782 (Abstr.).

PATIL, S. S. (1974). Mode of action of the toxin from Pseudomonas phaseolicola. In Proc. of Post Congress Osaka Symposium on Phytopathology.

PATIL, S. S. and GNANAMANICKAM, S. S. (1976). Suppression of bacterially induced hypersensitive reaction and phytoalexin accumulation in bean by phaseotoxin. Nature 209, 486-487.

PERERA, R. and GAY, J. L. (1976). The ultrastructure of haustoria of Spaerotheca pannosa and changes in infected and associated cells of rose. Physiological Plant Pathology 9, 57-65.

PERLMAN, R. L. and PASTAN, I. (1968). Regulation of β-galactosidase synthesis in Escherichia coli by cyclic adenosine 3′,5′-monophosphate. J. Biol. Chem. 243, 5420-5427.

PEROMBELON, M. C. M. (1971). A quantitative method for assessing virulence of Erwinia caratovora var caratovora and E. caratovora var atroseptica and susceptibility

to rotting of potato tuber tissue. In Proc. Third Int. Conf. Plant Pathogenic Bacteria, Wageningen.

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

RATHMELL, W. G. and SEQUEIRA, L. (1975). Induced resistance in tobacco leaves: the role of inhibitors of bacterial growth in the intercellular fluid. Plant Physiology 56, 65-73.

REEVE, R. M. (1950). Histochemical tests for polyphenols in plant tissues. Stain Techniques 26, 91-96.

REICH, E. and GOLDBERGER, I. H. (1964). Actinomycin and nucleic acid function. Progress in Nucleic Acid Res. and Molecular Biol. 3, 183-234.

RISSLAND and MOHR (1967). Phytochrom induzierte enzymbildung (phenylalanine-disaminase), ein schnell ablaufender prozess. Planta 77, 239-249.

ROHRINGER, R. and SAMBORSKI, D. J. (1967). Aromatic compounds in the host-parasite interaction. Ann. Rev. Phytopath. 5, 77-86.

ROHRINGER, R., HOWES, N. K., KIM, W. K. and SAMBORSKI, D. J. (1974). Evidence for a gene-specific RNA determining resistance in wheat to stem rust. Nature 249, 585-588.

ROLFS, F. M. (1915). A bacterial disease of stone fruits. Cornell Univ. Agr. Exp. Sta. Mem. 8, 377-436.

ROTTEM, S. and RAZIN, S. (1967). Electrophoretic patterns of membrane proteins of Mycoplasma. J. Bacteriology 94, 359-364.

RUDOLPH, K. and STAHMANN, M. A. (1964). Interactions of peroxidases and catalases between phaseolus vulgaris and Pseudomonas phaseolicola (halo blight of bean). Nature 204, 474-475.

RUDOLPH, K. and STAHMANN, M. A. (1966). The accumulation of L-ornithine in halo blight infected bean plants (Phaseolus vulgaris L.) induced by the toxin of the pathogen Pseudomonas phaseolicola (Burk.). Phytopath. Z. 57, 29-46.

RUDOLPH, K. (1969). Ein phytotoxisches Polysaccharid von Pseudomonas phaseolicola. Naturwissenschaften 56, 569-570.

RUDOLPH, K. and CIMAR, O. (1971). Bacteriostatic compounds from bean leaves. Acta Phytopath. Sci. Hung. 6, 105-113.

RUDOLPH, K. (1972). The halo-blight toxin of Pseudomonas phaseolicola : Influence on host parasite relationship and counter effect on metabolites. In Phytotoxins plant diseases. (Ed. Wood, R. K. S., Ballio, A. and Graniti, A.). Academic Press, London and New York.

RYAN, C. A. (1974). Assay and biochemical properties of the proteinase inhibitor - inducing factor, a wound hormone. Plant Physiology 54, 328-332.

SADASIVAN, T. S. (1959). In vivo changes in Fusarium wilted cotton. Recent Advance. Bot. 2, 1021-1024.

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

SCHNAITMAN, C. A. (1970). Examination of the protein composition of the cell envelope of Escherichia coli by polyacrylamide gel electrophoresis. J. Bacteriology 104, 882-889.

SCHNATHORST, W. C. and DEVAY, J. E. (1963). Common antigens in Xanthomonas malvacearum and Gossypium hirsutum and their possible relationships to host specificity and disease resistance. Phytopathology 53, 1142 (Abstr.).

SCHUSTER, M. L., COYNE, D. P. and KERR, E. D. (1965). New virulent strain of halo blight bacterium orcmonters in the field. Phytopathology 55, 1075 (Abstr.).

SELMAN, I. W. (1964). The effects of kinetin on infection of petunia and tomato leaves with tomato spotted-wilt virus. Ann. Appl. Biol. 53, 67-76.

SEQUEIRA, L. (1965). Origin of indoleacetic acid in tobacco plants infected with Pseudomonas solanacearum. Phytopathology 55, 1232-1236.

SEQUEIRA, L. and AINSLIE, V. (1969). Bacterial cell-free preparations that induce or prevent the hypersensitive reaction in tobacco. Eleventh Int. Bot. Congr. (Abstr.), 195.

SEQUEIRA, L., AIST, S. and AINSLIE, V. (1972). Prevention of the hypersensitive reaction in tobacco by proteinaceous constituents of Pseudomonas solanacearum. Phytopathology 62, 536-542.

SEQUEIRA, L. and HILL, L. M. (1974). Induced resistance in tobacco leaves: the growth of Pseudomonas solanacearum in protected tissues. Physiological Plant Pathology 4, 447-455.

SEQUEIRA, L. (1976). Induction and suppression of the hypersensitive reaction caused by phytopathogenic bacteria: specific and non specific components. In specificity in plant diseases. (Ed. R. K. S. Wood and Graniti, A.) Plenum Press, New York and London.

SEQUEIRA, L. and DONALD, B. (1976). Induction and suppression of hypersensitive response. In Specificity in Plant Diseases. (Ed. R. K. S. Wood and Graniti, A.) Plenum Press, New York and London.

SHAW, L. (1935). Intercellular humidity in relation to fire blight susceptibility in apple and pear. Cornell Univ. Agr. Exp. Sta. Mem. 181, 1-40.

SHAW, M. and SAMBORSKI, D. J. (1956). The physiology of the host-parasite relations. I. The accumulation of

radioactive substances at infections of facultative and obligate parasites including tobacco mosaic virus. Can. J. Botany 34, 389-405.

SHUMAWAY, L. K., CHENG, V. and RYAN, C. A. (1972). Vacuolar protein in apical and flower petal cells. Planta 106, 279-290.

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

SIGEE, D. C. and EPTON, H. A. S. (1976). Ultrastructural changes in resistant and susceptible varieties of Phaseolus vulgaris following artificial inoculation with Pseudomonas phaseolicola. Physiological Plant Pathology 9, 1-8.

SIPPEL, A. and HARTMANN, S. (1968). Mode of action of rifampicin on the RNA polymerase reaction. Biochem. Biophys. Acta 157, 218-219.

SKOOG, H. A. (1952). Studies on host-parasite relations of bean varieties resistant and susceptible to P. phaseolicola and toxin production by the parasite. Phytopathology 42, 475 (Abstr.).

SLEESMAN, H. C., PERLEY, J. E. and HOITINK, H. A. J. (1970). Susceptibility and hypersensitivity in tobacco and their prevention by cell-free extracts of P. tabaci and P. glycinea. Phytopathology 60, 1314 (Abstr.).

SMITH, E. F. (1897). Description of Bacillus phaseoli. Bot. Gaz. 24, 192.

STALL, R. E. and COOK, A. A. (1966). Multiplication of Xanthomonas vesicatoria and lesion development in resistant and susceptible pepper. Phytopathology 56, 1152-54.

STALL, R. E. and COOK, A. A. (1968). Inhibition of Xanthomonas vesicatoria in extracts from hypersensitive and

susceptible pepper leaves. Phytopathology 58, 1584-1587.

STALL, R. E., BARTZ, J. A. and COOK, A. A. (1972). Induced susceptibility in pepper to Xanthomonas vesicatoria. Phytopathology 62, 791 (Abstr.).

STALL, R. E. and COOK, S. A. (1973). Hypersensitivity as a defense mechanism against natural infection. Proc. Fourth Int. Congr. Plant Pathol. (Abstr.).

STALL, R. E., BARTZ, J. A. and COOK, A. A. (1974). Decreased hypersensitivity to xanthomonads in pepper after inoculations with virulent cells of Xanthomonas vesicatoria. Phytopathology 64, 731-735.

STAUB, T. and WILLIAMS, P. H. (1972). Fluorescing materials associated with vein blackening and necrosis in leaves of black rot-resistant cabbage. Phytopathology 62, 858-866.

STEWART, R. N. and SCHINDLER, A. F. (1956). The effect of some endoparasitic nematodes on the expression of bacterial wilt in carnations. Phytopathology 46, 219-222.

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

STRAIB, W. and NOLL, A. (1944). Untersuchungen über den Einfluss der Hitze auf den Hostparasitismus. Zentr. Bakteriol. Parasitenk, Abt. II 106, 257-277.

STROBEL, G. A. and HESS, W. M. (1968). Biological activity of a phytotoxin glycopeptide produced by Corynebacterium sepedonicum. Plant Physiology 43, 1673-1688.

STROBEL, G. A. (1974). Phytotoxins produced by plant parasites. Ann. Rev. Plant Physiology 25, 541-566.

SÜLE, S. and KLEMENT, Z. (1971). Effect of high temperature and the age of bacteria on the hypersensitive reaction of tobacco. Acta Phytopath. Acad. Sci. Hung. 6, 119-122.

SÜLE, S., COLENO, A. and LE NORMAND, M. (1973). Inhibition de la reaction hypersensible par des extraits de feuilles. Acta Phytopath. Acad. Sci. Hung. 8, 71-75.

TAKAYAMA, K., MACLENNON, D. H., TZAGOLOFF, A. and STONER, C. D. (1966). Studies on the electron transfer system LXVII. Polyacrylamide gel electrophoresis of the mitochondrial electron transfer complexes. Arch. Biochem. Biophys. 114, 223-230.

TANI, T., YOSHIKAWA, M. and NAITO, N. (1973). Template activity of ribonucleic acid extracted from oat leaves infected by Puccinia coronata. Ann. Phytopath. Soc. Japan 39, 7-13.

THYR, B. D. (1968). Bacterial canker of tomato: inoculum level needed for infection. Plant Dis. Reptr. 52, 741-743.

TONGUR, V. S., WLADYTCHENSKAYA, N. S. and KOTCHINA, W. M. (1968). A natural RNA-DNA complex in bacterial cells. J. Mol. Biol. 33, 451-464.

TRAUT, R. R. and MONRO, R. E. (1964). The puromycin reaction and its relation to protein synthesis. J. Mol. Biol. 10, 63-72.

TSCHESCHE, R. and WULFF, G. (1965). Über die antimikrobielle Wirksamkeit von Saponinen. Z. Naturf. 206, 543-546.

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

VAN TUBERGEN, R. P. (1961). The use of autoradiography and electron microscopy for the localisation of tritium label in bacteria. J. Biophys. Biochem. Cytol. 9, 219-222.

VARNER, J. G., RAM CHANDRA, S. and CHRISPEELS, M. J. (1965). Gibberellic-acid controlled synthesis of α-amylase in barley endosperm. J. Cell Comp. Physiol. 66, 55-68.

VASQUEZ, D. (1966). Mode of action of chloramphenicol and related antibiotics. Symp. Soc. Gen. Microbiol. 16, 169-189.

VASUDEVA, R. S. (1930). Studies in the physiology of parasitism. Annals of Botany 44, 469-493.

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

WACEK, T. J. (1974). Isolation and characterization of bacterial fractions that induce resistance in tobacco leaves. Ph.D. Thesis, University of Wisconsin, U.S.A.

WAITE, M. B. (1896). The cause and prevention of pear blight. U.S. Dept. Agr. Yearbook 1895, 295-300.

WAITZ, L. and SCHWARTZ, W. (1956). Untersuchungen über die von Pseudomonas phaseolicola (Burkh) hervorgenifene Fettfleckrankheit der Bohme II Untersuchungen zur pathogenese. Phytopath. Z. 26, 297-312.

WALKER, J. C. and STAHMANN, M. (1955). Chemical nature of disease resistance in plants. Ann. Rev. Plant Physiology 6, 351-366.

WARDROP, A. B. and CRONSHAW, J. (1962). Formation of phenolic substances in the ray parenchyma of Angiosperms. Nature 193, 90-92.

WEHRIL, W. and STAEHELIN, M. (1971). Action of rifampicins. Bacteriology Rev. 35, 290-309.

WEISBLUM, B. and DAVIES, J. (1968). Antibiotic inhibitors of the bacterial ribosome. Bacteriology Rev. 32, 493-528.

WEISS, F., LAUMTZEN, J. I. and BRIERLEY, G. (1928). Factors in the inception and development of Fusarium rot in stored potatoes. U.S. Dept. Agr. Tech. Bull. 62, 1-58.

WHEELER, H. and BLACK, A. S. (1962). Changes in permeability induced by victorin. Science 137, 983-984.

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

WIEBE, W. J. and CHAPMAN, G. B. (1968). Variation in the fine structure of a marine achromobacter and a marine pseudomonad grown under selected nutritional and temperature regimes. J. of Bacteriology 95, 1874-1886.

WOOD, R. K. S. and TVEIT, M. (1955). Control of plant diseases by use of antagonistic organisms. Bot. Rev. 21, 441-492.

YARWOOD, C. E. (1956). Heat induced susceptibility of beans to some viruses and fungi. Phytopathology 46, 523-525.

YARWOOD, C. E. (1959). Predisposition. In "Plant Pathology" (J. G. Horsfall and A. E. Dimond, Eds), Vol 1, 521-562. Academic Press, New York.

YOUNG, J. M. (1974 a). Development of bacterial populations in vivo in relation to plant pathogenicity. N.Z. J. Agric. Res. 17, 105-113.

YOUNG, J. M. (1974 b). Effect of water on bacterial multiplication in plant tissue. N.Z. J. Agric. Res. 17, 115-119.

ZALEWSKI, J. C. and SEQUEIRA, L. (1973). Inhibition of bacterial growth by extracts from potato tissues. Phytopathology 63, 942-944.

ZAUMEYER, W. J. (1932). Comparative pathogenical histology of three bacterial diseases of bean. J. Agr. Res. 44, 605-632.

ZAUMEYER, W. J. and THOMAS, H. R. (1957). A monographic study of bean diseases and methods of their control. U.S. Dept. Agr. Tech. Bull.

ZELLER, W. and RUDOLPH, K. (1972). Einfluss des Toxins von Pseudomonas phaseolicola auf die Permeabilität und den osmotischen Wert von Mangoldblättern (Beta vulgaris L.). Z. Pflanzenphysiol. 67, 183-187.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people all of whom contributed in some way or other to making this thesis possible.

My supervisor, Professor R. K. S. Wood, for his advice and valuable criticism in preparation of the manuscript and for his attentiveness and ability to help at a moment's notice.

Professor F. J. Schwinn of Ciba-Geigy for his patience and understanding during the most difficult of times and for making possible my two visits to Ciba-Geigy in Basel during February, 1974 and April, 1975.

Drs. T. Egli and Th. Staub of Ciba-Geigy for their advice, criticism and hospitality.

Drs. A. H. Dadd, J. L. Gay, and R. J. Threlfall for their help and advice during various sections of the research.

Miss Marion Martin for her technical assistance in the electron microscopic and photographic aspects of the work.

Mrs. Sandra Coward for typing this manuscript and her unfailing patience.

Other members of the secretarial staff, notably Mrs. June Cheston for her cheerfulness and ability to find time to type reports and letters associated with the work involved in the thesis.

And finally but not least to all my colleagues and

friends who made the impossible times bearable and the difficult times surmountable.

These studies were made possible by a bursary from Ciba-Geigy, Agrochemicals Division, Basel, Switzerland.

Physiological Plant Pathology (1977) **10**, [illegible]

Ultrastructural changes in trifoliate leaves of bean (*Phaseolus vulgaris*) following inoculation of monofoliate leaves with *Pseudomonas phaseolicola*

C. I. K. Lallyett

Botany Department, Imperial College, London SW7, U.K.

(*Accepted for publication January 1977*)

Monofoliate leaves of French bean cv. Canadian Wonder were inoculated by injection with suspensions of *Pseudomonas phaseolicola* (*c.* $3{\cdot}0 \times 10^8$ cells/ml). Trifoliate leaf tissue was examined by light and electron microscopy 3, 10 and 14 days later. A darkly staining inclusion which occurs in three forms appeared in cell vacuoles after 10 days and increased in concentration 4 days later. Large starch grains occurred in chloroplasts and despite chlorophyll levels being about half those of controls, chloroplast integrity appeared normal. Other organelles also appeared normal, though there were increases in endoplasmic reticulum and numbers of ribosomes.

INTRODUCTION

Halo blight of beans (*Phaseolus vulgaris*) is characterized by the appearance on leaves of watersoaked translucent spots, usually with a chlorotic halo, by systemic interveinal chlorosis of developing leaves and by the general dwarfing of plants.

There is some evidence that chlorosis in upper leaves is caused by phaseotoxin, an exotoxin produced by the causal organism *Pseudomonas phaseolicola* in lower leaves. Treatment of leaves with culture filtrates mimics disease symptoms [*18*]. Phaseotoxin specifically inhibits activity of ornithine carbamoyltransferase (OCT) and this leads to an accumulation of ornithine which is thought to be causally related to chlorosis [*14*].

Past work has not established a clear relation betwen the spread of the bacterium and development of trifoliate chlorosis [*21*]. However, Hildebrand & Schroth [*7*] recovered the bacterium from 39 out of 40 trifoliate leaves following inoculation of monofoliate leaves.

There are only a few studies of ultrastructural modifications of plant tissues induced by toxic substances produced by plant pathogens. Victorin, from *Helminthosporium victoriae*, causes breakages in plasma membranes, disruption of chloroplast lamellae and abnormally large vesicles to develop in Golgi apparatus [*6, 11*]. Toxin from *Corynebacterium sepedonicum* damaged membranes of chloroplasts, mitochondria the plasma membrane and the structural integrity of the cell wall of tomato plants [*20*]. Leseman & Rudolph [*9*] reported that the toxin from *P. phaseolicola* caused a helix-like aggregation of ribosomes but that other organelles were hardly affected. Other work showed that infection of tobacco leaves with *Pseudomonas tabaci* causes a

swelling and then a decrease in the stroma of the chloroplasts with no starch accumulation. Also groundplasm of the mitochondria decreased and the plasma membrane separated from cell walls [*5*]. Infection of bean leaves with *P. phaseolicola* showed disruption of chloroplast grana and the accumulation of large starch grains, although membranes remained intact. Other cellular organelles appeared normal [*2*]. Sigee & Epton [*19*] reported that in monofoliate bean leaves, infection by *P. phaseolicola* caused breakdown of the chloroplast and an increase in the cytoplasmic ribosomes. This work was undertaken to try and determine how *P. phaseolicola* and subsequent phaseotoxin production by the bacterium causes halo blight.

MATERIALS AND METHODS

Plants

Plants of *Phaseolus vulgaris* L. cv. Canadian Wonder were grown from seed soaked and germinated at 25 °C for 48 h and then grown in John Innes no. 2 potting compost in a growth cabinet at 23 ± 1 °C supplemented with fluorescent lighting for a 16 h/day to an intensity of 4200 lx at the level of the leaves.

Inoculation of plants

Bacteria were injected through main veins from a hypodermic syringe (no. 30 needle) into intercellular spaces of interveinal areas of expanding monofoliate bean leaves of 10- to 13-day-old plants. Injection started at the junction of lamina and petiole and continued until a prescribed area became watersoaked. Leaves were then washed with sterile water and plants were replaced in the growth cabinet. Leaves appeared normal about 30 min later after watersoaking had disappeared.

Bacteria

Pseudomonas phaseolicola race 1 isolate 605 and race 2 isolate 1321 and *Pseudomonas mors-prunorum* isolate 560 were obtained from the National Collection of Plant Pathogenic Bacteria (M.A.A.F., Hatching Green, Harpenden).

Bacteria were grown for 18 to 24 h at 27 °C on plates of nutrient agar containing 13 g Oxoid Nutrient Broth no. 2 (CM 67), 15 g Davis Standard Agar at pH 7·3. Cells were suspended in sterile water (10 ml/9 cm plate), centrifuged (10 min at 6000 *g*) and washed again and resuspended to the required concentration for inoculation of *c.* $3{\cdot}0 \times 10^8$ cells/ml.

Measurement of bacterial growth

At various intervals following monofoliate inoculation, monofoliate and trifoliate leaves were removed, washed in sterile water to remove most surface bacteria and four discs (4·5 mm diam.) were punched out using a sterile cork borer. For monofoliate leaves the discs were taken from the inoculation zone; in trifoliate leaves they were removed from the junction of the petiole and lamina, with one disc per lateral and two per terminal leaflet. Discs were ground with a pestle in 1 ml sterile water. The homogenate was serially diluted and then 1 ml transferred to Petri dishes (8·5 cm diam.) immediately before adding 10 ml of nutrient agar at 43 °C.

After mixing, the plates were incubated at 27 °C for 4 days with three replicates each of the last two dilutions. After counting bacterial colonies on the plate the concentration of bacteria in the original suspensions was calculated.

Extraction of chlorophyll

Chlorophyll from trifoliate leaves was extracted by the method of MacKinney [*12*]. Results are expressed as mg chlorophyll/g fresh wt leaf.

Extraction of phenolics

Total phenolics were extracted by homogenizing 1·0 to 1·5 g fresh wt trifoliate leaves in 20 ml of 80% (v/v) ETOH for 10 min. The homogenate was filtered and the residue washed with 10 ml ETOH. The filtrate was shaken with petroleum ether (2 vol.) for 10 min and partitioned by allowing to stand for 10 min. The alcohol phase was removed and shaken 3 times more with petroleum ether. The final straw-coloured solution was evaporated in a Buchi rotary evaporator and the residue dissolved in 5 ml water. Total phenolics were estimated by the Folin method and results expressed as catechol equivalents in μg/g fresh wt.

Light microscopy

Trifoliate leaves were examined as fresh material and after fixation at room temperature in 5% buffered glutaraldehyde (phosphate buffer 0·1 M pH 7·2) for 6 h.

Staining for phenolics. Nitrose stain. The nitrous acid reagent [*16*] stains specifically for orthodihydroxy phenols and was applied to 5 μm hand-cut sections. Some leaf material was also embedded in Epon after fixation and 5 μm sections taken before application of stain.

Ferric chloride stain. Ferric chloride (2% w/v in 90% ETOH) for 5 min was used as a general stain for phenols.

Ammonia vapour. This was applied to sections for 2 min and was used as a general stain for phenols.

Staining for protein. Coumassie Brilliant Blue. Sections were immersed in this stain (0·5% w/v in 7% acetic acid v/v) for 2 min and then rinsed in water.

Bromophenol blue. Sections were immersed in this reagent (0·5% w/v in 7% acetic acid v/v) for 2 min and rinsed in water.

Staining for lipids. Sudan IV reagent. Sections were placed in 50% alcohol for 2 min and then transferred to a saturated filtered solution of Sudan IV in 70% alcohol.

Electron microscopy

Trifoliate leaf tissue was sampled 3, 10 and 14 days after inoculation of monofoliate leaves and cut to *c.* 1 mm^3 under 2% buffered glutaraldehyde fixative at room temperature (phosphate buffer pH 7·2, 0·1 M). Segments were transferred to fresh fixative for a further 1½ h, washed twice in buffer for 10 min and post-fixed in 1% buffered osmium tetroxide at room temperature (phosphate buffer pH 7·2, 0·1 M) for 1 h. Dehydration was carried out in an ethanol series with absolute alcohol

being exchanged for 2 × 10 min changes in epoxy propane, before embedding in Epon 1A : 1B resin [*10*].

Sections were cut on a Reichert ultramicrotome and placed on 100 mesh formvar-coated copper grids and stained for 30 min in uranyl acetate at 60 °C followed by 15 min lead citrate at room temperature. Sections were viewed in an AEI 6B electron microscope at 60 keV.

RESULTS

Following inoculation of monofoliate leaves, bacterial numbers in the site of inoculation and in trifoliate leaves were determined (Table 1). Bacterial numbers in monofoliate and trifoliate leaves appear to increase at a similar rate until about 8 days and then decrease when chlorosis normally begins to appear. In general, numbers in the monofoliate leaves were at least a thousand times greater/disc than in the trifoliate leaves over the 14-day period.

TABLE 1

Multiplication of P. phaseolicola *race 2 in bean leaves following inoculation of monofoliate leaves*

Time after inoculation (days)	Bacterial numbers/leaf disc[a] Monofoliate leaf	Trifoliate leaf
0	$5{\cdot}2 \times 10^{5}$[b]	$8{\cdot}2 \times 10^{1}$[b]
0·5	$4{\cdot}5 \times 10^{5}$	$2{\cdot}2 \times 10^{2}$
1	$3{\cdot}0 \times 10^{6}$	$6{\cdot}5 \times 10^{2}$
2	$1{\cdot}5 \times 10^{7}$	$5{\cdot}5 \times 10^{2}$
4	$1{\cdot}5 \times 10^{8}$	$5{\cdot}0 \times 10^{3}$
8	$3{\cdot}0 \times 10^{7}$	$2{\cdot}5 \times 10^{4}$
12	$1{\cdot}0 \times 10^{7}$	$1{\cdot}3 \times 10^{4}$
16	$6{\cdot}5 \times 10^{6}$	$4{\cdot}5 \times 10^{3}$

[a] Leaf disc 4·5 mm diameter.
[b] Mean of six replicates.

Three days after injection the inoculation area of monofoliate leaves had collapsed and was pale bronze in colour. At this stage no effects were observed in trifoliate leaves. After 10 days monofoliate (m.f.) leaves had a watersoaked margin, usually with a chlorotic halo, around the inoculated area and a mild interveinal chlorosis appeared in trifoliate (t.f.) leaves. Ultrastructurally a marked change in t.f. leaves had now occurred as shown in Plate 2. Chloroplasts now contained large starch grains [Plate 2(c)] and the vacuoles contained one or more of three types of darkly staining inclusions. Type 1 [Plates 2(a) and 3] appeared as a darkly staining, agglutinated amorphous matrix within the vacuole. This was different from the contents normally present in the vacuole (NS), which were less darkly stained and more fibrous [Plates 1(b) and 3]. Type 2 occurred as a densely fibrous matrix lining the tonoplast membrane (Plates 3 to 5), and Type 3 was a globular coalescent deposit lying on the tonoplast [Plates 2(b) and 5). In Plate 5, Type 3 seems to be directly adjoining the tonoplast membrane suggesting a lipoidal content.

Additional to the vacuolar inclusions, a granular inclusion (GI) occasionally occurred within the middle lamella region of cell walls [Plate 2(a) and (b)]. Other organelles in cells containing one or more of these inclusions appeared normal.

After 14 days m.f. leaves had died or become very chlorotic and desiccated, and the t.f. leaves exhibited extreme interveinal chlorosis. Ultrastructurally the chloroplasts of t.f. leaves were now so full of starch grains (which had increased in size and number) as to displace the grana, although structurally the chloroplast appeared normal (Plate 4). Vacuolar inclusions (Types 1 to 3) were now more common and membranes became less distinct as the groundplasm stained more intensely. Other organelles appeared normal except for a higher incidence of endoplasmic reticulum and increased ribosomal concentration. In both cases the amount appeared to be at least double that of the controls.

The effects of inoculation of virulent bacteria on chlorophyll levels in t.f. leaves are shown in Fig. 1. After 14 days the levels were approximately half those of leaves in plants injected with water control or suspensions of incompatible bacteria (*Pseudomonas mors-prunorum*). In all cases there is a steady decline in chlorophyll levels but the rate is twice as fast with virulent bacteria.

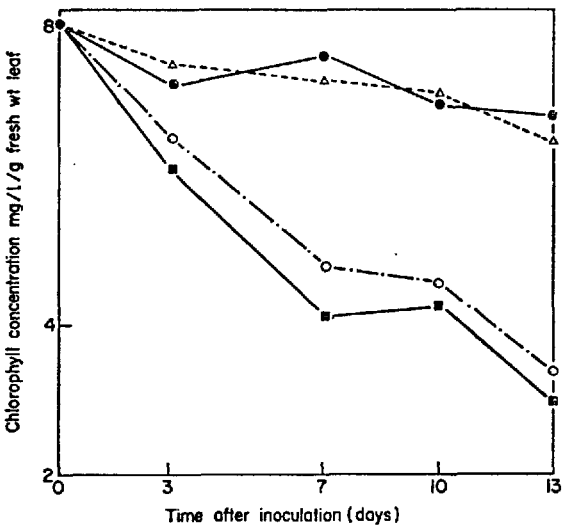


FIG. 1. Chlorophyll content of trifoliate leaves following monofoliate inoculation by *Pseudomonas* spp. (●——●) water control; (△ - - - - △) *P. mors-prunorum*; (○ · — · — · ○) *P. phaseolicola* race 1; (■——■) *P. phaseolicola* race 2. Bacterial suspensions $3{\cdot}0 \times 10^8$ cell/ml. All readings are mean of three replicates.

Histochemical tests on tissue from chlorotic t.f. leaves to determine the nature of the vacuolar inclusion (Types 1 to 3) were carried out. Tests for phenolic compounds gave positive reactions only in the phloem parenchyma. Reactions for lipids (Sudan IV) and proteinaceous material (Coumassie and Bromophenol blue) were no different from those in the controls.

The amount of total phenolic in t.f. leaves over 14 days after inoculation of m.f. was determined and results expressed as μg phenolic/g fresh wt of trifoliate leaf in

Table 2. No increase in phenolic occurs until 10 days after inoculation when *c.* 30 μg is noted, but by 14 days an increase of *c.* 100 μg over the original concentration has occurred.

TABLE 2

Total phenolic compounds in trifoliate leaves following inoculation of monofoliate leaves by P. phaseolicola *race 2*

Time after inoculation (days)	Total phenolic content μg/g fresh wt leaf	
	Water	*P. phaseolicola*[2]
0	401[a] (388 414)	416[a] (380 432)
3	412 (405 419)	408 (430 386)
10	397 (364 430)	452 (475 429)
14	428 (399 457)	539 (566 512)

[a] Mean for two replicates in brackets.

DISCUSSION

Inoculation of m.f. leaves with *Pseudomonas phaseolicola* leads to chlorosis and ultrastructural modifications in t.f. leaves. Since bacterial numbers decrease just before chlorosis becomes apparent it is suggested that chlorosis and ultrastructural modifications are due to production, accumulation and systemic spread of a bacterial metabolite or bacterial lysate and not solely to bacterial presence in t.f. leaves. This systemic metabolite is most probably phaseotoxin as suggested by Rudolph [*17*] and Patil & Gnanamanickam [*14*].

The modifications attributed to toxin systemicity are the appearance in the vacuole of a darkly staining material mostly associated with the tonoplast, the decrease of chlorophyll and the formation of large starch grains within the chloroplasts, the occasional appearance of granular inclusions in the middle lamella region, and the increases in endoplasmic reticulum and numbers of ribosomes.

The vacuolar inclusions appeared in three types which probably represent successive stages of formation. Histochemical tests did not establish that they were phenolic, lipid or proteinaceous in nature, but total phenolics in t.f. leaves 14 days after m.f. inoculation showed an increase of *c.* 100 μg/g fresh wt of leaf. This increase in phenolic concentration may be sufficient to account for the widespread distribution of the inclusion within the cells.

The appearance of these inclusions as three types is similar to that found by Mueller & Beckman [*13*] for banana root cells. The inclusion in that case was also osmophilic but shown to be phenolic. Shumaway *et al.* [*18*] provided evidence that protein also accumulates in cell vacuoles of tomato and tobacco leaves as a darkly staining inclusion. The protein tests provided no evidence to support this.

The formation of phenolic and tannin compounds in the vacuole is normally associated with extra-vacuolar origin. Workers have implicated the vesicles of dictyosomes [*1*]; the dilated cisternae of the endoplasmic reticulum [*3, 4*], and modified plastids [*22*]. Mueller & Beckman [*14*] could not confirm the extra-vacuolar origin although they noted that the cytoplasm became granular, contained

large concentrations of ribosomes and had a more extensive system of endoplasmic reticulum. The appearance of a larger and more extensive endoplasmic reticulum was also found in this present work and the possibility of involvement in the production of the vacuolar inclusion seems likely.

It is probable that the vacuolar inclusion is phenolic because of its osmophilic nature (Plate 5) and widespread distribution and appearance within cells. Both characteristics are peculiar to phenolic compounds. This is supported by an increase in concentration of total phenolics (*c.* 80 μg/g fresh wt) at a time (10 to 14 days) when chlorosis of trifoliate leaves and ultrastructural modification became more pronounced. The increase in phenolic compounds for the first 10 days was *c.* 30 μg/g fresh wt. The rôle of phenolics in the defence mechanisms of plants is well illustrated in the literature [*8*]. However, the fact that the inclusions did not react positively in histochemical tests for phenolic substances suggests that it may be in some precursor form as postulated by Perera & Gay [*15*].

Previous ultrastructural studies of bean inoculated with *P. phaseolicola* have shown disruption of grana and membranes of the chloroplast [*2*, *19*]. No evidence of this was found in the present work. However, as in earlier work, large and numerous starch grains were found even though chlorophyll levels were half those of controls. As starch production depends ultimately on photosynthesis then a substantial reduction in chlorophyll would be expected to decrease production of starch. The anomaly of excessive starch in the chloroplasts would be explained if there were reduced translocation of photosynthate from the leaves through damage to the phloem. This is known for certain virus diseases such as potato leaf roll and sugar beet yellows. Also, starch accumulation may be attributed to the inability of the plant to degrade it for translocation because of changes in the necessary enzymes or altered membrane permeability.

A relation between chlorosis of trifoliate leaves and the toxin produced by *P. phaseolicola* has been shown by Rudolph [*17*]. In view of the chlorophyll level in trifoliate leaves of infected plants and the normal appearance of the chloroplasts it seems probable that the integrity of the latter is not directly dependent on the former.

The granular inclusion occasionally observed in the middle lamella region appears different from that in the vacuoles; no attempt could be made to analyse the apparent difference. Phytopathogenic bacteria are usually present in intercellular spaces and it is possible that this granular inclusion is associated with the defence mechanism of the plant. However, not many bacteria were seen in the intercellular space of t.f. leaves in close proximity to GI.

The increase in concentration of endoplasmic reticulum and ribosomes and the possible rôle of both in the formation of vacuolar inclusions has already been mentioned. The fact that both appeared to increase suggests enhancement of protein metabolism.

Finally, it is known that phaseotoxin is a specific inhibitor of OCT and it has been postulated that chlorosis of t.f. leaves is causally related to this inhibition [*14*]. Thus, the decline in chlorophyll levels in the leaves may well be attributed to phaseotoxin. However, the absence of cytological degeneration, particularly in the chloroplast (Plate 4), tends to suggest that chlorosis is a side-effect of phascotoxin action with the main effect directed at the shoot apex.

The author is grateful for the helpful advice and criticism of Dr J. L. Gay and Professor R. K. S. Wood. The work was supported by a bursary from Ciba-Geigy (Agrochemical Division), Basel, Switzerland.

REFERENCES

1. Amelunxen, F. & Gronau, G. (1969). Untersuchungen an den Gerbstoffzellen der Niederblätter von *Acorus calarius* L. *Cytobiologie* **1**, 58–69.
2. Bajaj, Y. P. S., Spink, G. C. & Saettler, A. W. (1969). Ultrastructure of chloroplasts in bean leaves affected by *Pseudomonas phaseolicola*. *Phytopathology* **59**, 1077 (Abstr.).
3. Chafe, S. C. & Durzan, D. J. (1973). Tannin inclusions in cell suspension cultures of white spruce. *Planta* **113**, 251–262.
4. Diers, L., Schötz, F. & Meyer, B. (1973). Über die Ausbildung von Gerbstoffvakuolen bei Oenothoren. *Cytobiologie* **7**, 10–19.
5. Goodman, R. N. & Plurad, S. B. (1971). Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. *Physiological Plant Pathology* **1**, 11–15.
6. Hanchey, P., Wheeler, H. & Luke, H. H. (1968). Pathological changes in ultrastructure. Effects of victorin on oat roots. *American Journal of Botany* **55**, 53–61.
7. Hildebrand, D. C. & Schroth, M. N. (1971). Isolation of *Pseudomonas phaseolicola* from bean leaves exhibiting systemic symptoms. *Phytopathology* **61**, 580–581.
8. Kosuge, T. (1969). The rôle of phenolics in host response to infection. *Annual Review of Phytopathology* **7**, 195–222.
9. Leseman, D. & Rudolph, K. (1969). Die Bildung von Ribosomen Helices unter den Einfluss des Toxins *Pseudomonas phaseolicola* in Blättern von Mangold (B.U.L.). *Zeitschrift für Pflanzenphysiologie* **62**, 108–115.
10. Luft, J. H. (1961). Improvements in epoxy resin embedding methods. *Journal of Biophysical and Biochemical Cytology* **9**, 409–414.
11. Luke, H. H., Warmke, H. E. & Hanchey, P. (1966). The effects of the pathotoxin victorin on ultrastructure of root and leaf tissue of *Avena* species. *Phytopathology* **56**, 1178–1183.
12. Mackinney, G. (1941). Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry* **140**, 315–322.
13. Mueller, W. C. & Beckman, C. H. (1974). Ultrastructure of the phenol storing cells in the roots of banana. *Physiological Plant Pathology* **4**, 187–190.
14. Patil, S. S. & Gnanamanickam, S. S. (1976). Suppression of the bacterially induced hypersensitive reaction and phytoalexin accumulation in bean by phaseotoxin. *Nature* **259**, 486–487.
15. Perera, R. & Gay, J. L. (1976). The ultrastructure of haustoria of *Spaerotheca pannosa* and changes in infected and associated cells of rose. *Physiological Plant Pathology* **9**, 57–65.
16. Reeve, R. M. (1950). Histochemical tests for polyphenols in plant tissues. *Stain Techniques* **26**, 91–96.
17. Rudolph, K. & Stahmann, M. A. (1966). The accumulation of L-ornithine in halo blight infected bean plants (*Phaseolus vulgaris* L.) induced by the toxin of the pathogen *Pseudomonas phaseolicola* (Burk.). *Phytopathologische Zeitschrift* **57**, 29–46.
18. Shumaway, L. K., Cheng, V. & Ryan, C. A. (1972). Vacuolar protein in apical and flower petal cells. *Planta* **106**, 279–290.
19. Sigee, D. C. & Epton, H. A. S. (1976). Ultrastructural changes in resistant and susceptible varieties of *Phaseolus vulgaris* following artificial inoculation with *Pseudomonas phaseolicola*. *Physiological Plant Pathology* **9**, 1–8.
20. Strobel, G. A. & Hess, W. M. (1968). Biological activity of a phytotoxin glycopeptide produced by *Corynebacterium sepedonicum*. *Plant Physiology* **43**, 1673–1688.
21. Waitz, L. & Schwartz, W. (1956). Untersuchungen über die von *Pseudomonas phaseolicola* (Burk.) hervorgerufene Fettfleckrankheit der Bohne. II. Untersuchungen zur Pathogenese. *Phytopathologische Zeitschrift* **26**. 297–312.
22. Wardrop, A. B. & Cronshaw, J. (1962). Formation of phenolic substances in the ray parenchyma of Angiosperms. *Nature* **193**, 90–92.

Abbreviations used in plates

CHL, chloroplast; CW, cell wall; ER, endoplasmic reticulum; G, golgi body; GI, granular inclusion; M, mitochondrion; NS, normal vacuolar inclusion; T1 to 3, three types of vacuolar inclusions; SG, starch grain; VAC, vacuole. (All plates show mesophyll cells.)

PLATE 1(a). Trifoliate chloroplasts 14 days after inoculation of monofoliate leaves with water. × 12 300. (b) As (a) but showing the normal valuolar inclusion (NS). × 35 000.

PLATE 2(a). Trifoliate cells 10 days after inoculation of monofoliate with *P. phaseolicola* showing granular inclusions in the middle lamella (GI) and vacuolar inclusion (T1). × 20 190. (b) As (a) showing high power of granular inclusion (GI) and vacuolar inclusions (T3). × 41 700. (c) As (a) showing chloroplast containing numerous starch grains (SG) but with chloroplast membranes and grana intact. × 27 100.

PLATE 3. Trifoliate leaf 14 days after inoculation of monofoliate leaves with *P. phaseolicola.* Note large and numerous starch grains in chloroplasts, vacuolar inclusions T1 and NS in some vacuoles; also T2 inclusion in different vacuoles. × 12 300.

PLATE 4. As Plate 3, showing high power of chloroplasts with no degeneration of membrane or granae, golgi body (G) and mitochondrion (M) and vacuolar inclusion on tonoplast (T2 to T3). × 85 800.

PLATE 5. As Plate 3, showing high power of two cells. Note osmiophiolic properties of T3, the high incidence of ER and ribosomes, the intact tonoplast and plasma membrane. × 106 800.

[*facing page 666*]