RESISTANCE AND SUSCEPTIBILITY OF *PHASEOLUS VULGARIS* TO BACTERIA

by

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ABSTRACT

Interaction of Phaseolus vulgaris var. Red Mexican (RM) with bacteria was studied. RM leaves were resistant to Pseudomonas phaseolicola Race 1 (R1) and P. mors-prunorum (Pmp) with a hypersensitive response (HR); resistant to Xanthomonas manihotis (Xm) and Erwinia carotovora var. atroseptica (Ea) and P. fluorescens (Pf) with no macroscopically visible response; and susceptible to P. phaseolicola Race 2 (R2) with halo blight symptoms. (SR).

Electrolyte leakage from disks cut from inoculated tissue was studied and the rate of loss was found to be hyperbolic in all cases.

The nature of substances lost from disks was also investigated and levels of ions, total protein and total phenol in the ambient solutions of disks were measured. In all cases leakage was greater when there were macroscopically visible symptoms.

The use of disks floating on water to study development of HR and SR was found to give similar results to those in whole plant leaves but only if disks were inoculated in the central area with a surrounding band of healthy tissue.

A medium suitable for the study of interactions between bacteria and RM cell or protoplast suspension cultures was developed but R1 and R2 killed plant cells or protoplasts at the same rate with no sign of necrosis.

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INTRODUCTION AND LITERATURE REVIEW

The use of bacteria in the study of resistance mechanisms of plants has many practical advantages which were described by Klement in 1971. Bacteria can be grown quickly and easily on simple culture media and the inoculum concentration can be adjusted using the simple technique of nephelometry. Population levels of bacteria in plant tissues can be followed by introducing precise inocula and using plate counts. With concentrated inoculum, uniform inoculation of tissues may be achieved and with antibiotics bacterial metabolism can be altered at any time during the reaction.

In this study a range of types of plant-bacterial interactions were investigated. Plants were Phaseolus vulgaris var. Red Mexican and bacteria were Pseudomonas phaseolicola Races 1 and 2, P. mors-prunorum, P. fluorescens, Erwinia atroseptica and Xanthomonas manihotis.

In 1971, Klement set out three models of plant-bacterial interaction. These were compatible, incompatible and those involving saprophytic bacteria. The compatible model is that of virulent bacteria and susceptible plants; the incompatible model is that of avirulent bacteria and resistant plants or virulent bacteria with plants not their hosts. In both of these, bacterial multiplication takes place and visible necroses appear but not always in the incompatible system.

Saprophytes do not multiply but their survival in plant tissue tends to be long term.

Allington and Chamberlain (1949) were among the first to use population changes in bacteria in vivo as a method of characterising infection. Using Phaseolus vulgaris and Glycine max with P. glycinea and Xanthomonas phaseoli, they found that multiplication in compatible and incompatible systems was

similar until the second day. At this time numbers of bacteria recovered from susceptible plants were far greater than those recovered from resistant plants. Tissue collapse noted on the third day correlated with the decrease in numbers of bacteria.

In 1961 Klement and Lovrekovich using pseudomonads and xanthomonads on Capsicum annuum found that the reaction to pseudomonads was quicker and more intense and that multiplication of bacteria in tissues had stopped by the time of visible symptom development. The non-pathogen P. aerenguinosa did not multiply.

Cessation of growth of virulent and avirulent bacteria was correlated with tissue collapse by Klement (1964), Stall and Cook (1966), Omer and Wood (1969) and Ercolani and Crosse (1966). Tissue collapse was always earlier in resistant plants (incompatible combination).

Most bacteria gain entry to plant tissue through wounds and natural openings. Rolfs (1915) demonstrated the importance of stomatal entry sites using Xanthomonas pruni on peach leaves. Some species eg Erwinia carotovora only enter through wounds and Agrobacterium tumefaciens seems to require a substance from wounded cells before infection of plant tissue by the bacteria will occur.

The concept of inoculum threshold is important. This is the number of bacteria needed to cause a visible response in plant tissue. Meynell and Stocker (1957) introduced the hypothesis of independent and co-operative action. The first allows for plant response to be the result of invasion by only one bacterial cell; the second for the response to be the result of the combined action of several bacterial cells.

For susceptible responses (SR) independent action has been documented, for instance with Agrobacterium tumefaciens

(Hildebrand, 1942; Lipincott and Haberlein, 1965). Ercolani (1967) demonstrated single cell infection of tomato by Erwinia carotovora; Perambelon (1971) found that single cells of E. carotovora would infect potato tuber disks and Thyr (1968) showed that one cell of Corynebacterium michangense could infect tomato plants.

The co-operative effect holds good for incompatible combinations of plants and bacteria and is dealt with later.

In 1973, Ercolani introduced the term "pathogenicity factor". These were factors controlling "general metabolic activities common to all pathogenic bacteria in plant tissues". "Virulence factors" governed "the specific activities resulting in the induction of a progressive disease in the host plants".

Toxins involved in bacterial diseases have been suggested as possible virulence factors. Some of these appear to be host specific eg Erwinia amylovora produces toxin causing wilting only in rosaceous plants (Goodman et al. 1974). However the toxin produced by Pseudomonas phaseolicola is not host specific since it produces a susceptible response on application to resistant bean leaves (Rudolph, 1972). Back man and DeVay (1974) have reported that Syringomycin (the toxin of P. syringae) seems to be host specific in that peach isolates of the pathogen do not produce toxin in bean .

The presence of a common antigen as a prerequisite of a compatible combination has been suggested by DeVay (1972). The idea is that less disruption occurs in the host system of common metabolites are present and, indeed, several common antigens have been discovered in plant-bacterial systems (DeVay, 1972).

Capsular antigens of P. lachrymans and P. phaseolicola have been suggested as pathogenicity factors (Grogan et al., 1965) "Recognition fit" between bacterial cell wall polysaccharides and host lectins was thought by Dixon (1969) to be the reason

for specificity of rhizobia for their hosts. This is supported by Ercolani (1970) who suggested that during a compatible (susceptible) reaction, a virulance factor allowed for attachment of bacteria to multiplication sites. Lippincott and Lippincott (1972) made the same suggestion for Agrobacterium tumefaciens.

The protection of plants against virulent pathogens using inoculation of avirulent organisms was demonstrated by Wood and Tvent (1955); Farabee and Lockwood (1958) and Kassannis (1963). Averre and Kelman (1964) found that avirulent strains of bacteria protect against infection when mixed with Pseudomonas solanacearum in inoculations. In 1958 Farabee isolated a yellow saprophytic bacterium from fire blight cankers produced by Erwinia amylovora. The acid produced by the saprophyte was sufficient to inhibit growth of E. amylovora in vitro. When the saprophytic bacteria were introduced into apple trees at 5×10^8 cells/ml prior to E. amylovora inoculation, a significant number of shoots were protected from infection.

This work is supported by that of Baldwin and Goodman (1963) and this type of protection was observed for Xanthomonas vesicatoria by Leben (1963) and for P. mors-prunorum by Crosse (1965). Hsu and Dicky (1972) working with bean and tomato leaves pre-inoculated with incompatible bacteria (Xanthomonas phasoeli, X. vesicatoria, X. campestris and Pseudomonas fluorescens), found that on subsequent inoculation with compatible pathogens, multiplication of the compatible bacteria was much reduced.

Pretreatment by heat killed cells of P. tabaci protect tobacco leaves from live P. tabaci when inoculated later (Lovrekovich and Farkas, 1965). In apple and pear, protection from Erwinia amylovora can be given by cell-free sonicates of E. amylovora (McIntyre, Kuć, and Williams, 1973).

The effects of prior inoculations with combinations of

bacteria can be synergistic. Braithwaite and Dickey (1970), working with bean and P. phaseolicola and other pseudomonads observed that combination led to increased release of nutrients from the host. This favoured development of incompatible bacteria.

Enhancement of disease symptoms by non-pathogenic bacteria has been seen. P. syringae and species of Achromobacter increased infection by P. phaseolicola of bean probably by contributing to breakdown of cell walls (Maino et al., 1974).

Humidity can also be an important factor influencing susceptibility. Young (1974) showed that 100% relative humidity resulted in a loss of susceptibility.

In 1975, McIntyre et al. allowed pear shoots to take up DNA of Erwinia amylovora and found that this protected the plants against subsequent inoculation by virulent E. amylovora.

The susceptible response used in this study was halo blight which was first described as a disease of Phaseolus vulgaris by Burkholder in 1926. The bacteria were named Phytomonas phaseolicola (Burk.). Subsequently these were renamed Pseudomonas phaseolicola (Burk.) Dows. by Dowson(1943).

The disease is systemic and all tissues and organs except the roots are invaded. Dissemination is by infected seed which germinates producing cotyledons which bear brown spots containing the bacteria. Bacteria later invade vessels and on emergence from the soil, the growing point of the seedling may already have been killed.

Visible symptoms on foliage are small translucent water-soaked spots which arise from stomatal invasion by bacteria and are about 2mm in diameter. Spread of P. phaseolicola on shoots is by wind and water splash.

The leaf spots may be surrounded by a chlorotic ring of tissue or halo, caused by diffusion of the toxin produced by the

bacteria. Development of the halo is variable and temperature dependent. Patel and Walker (1963) found the halo large and pronounced at 16°C and 20°C but smaller at 24°C and 28°C. This temperature range corresponds closely with that obtained for toxin production in culture (Skoog, 1952), little toxin being produced from pure cultures in glucose broth at temperatures above 20°C.

The systemic nature of the disease was shown by Zaumeyer (1932) using infected seed. Bacteria rarely penetrated host cells and were to be found in intercellular spaces and xylem elements of plants. Waitz and Schwartz (1956) found a poor correlation between chlorotic symptoms and bacteria in leaves above infected lower ones. The presence of bacteria in chlorotic and apparently healthy leaves was variable and they concluded that the bacteria do not spread in large masses. A few only move around in the transpiration stream and multiply locally.

The spread of bacteria was found to be usually upwards by Omer (1966) and the failure to isolate bacteria from chlorotic leaves was a result of the method. Using a technique of infiltration followed by centrifugation Hildebrand and Schroth (1971) recovered causal organisms from 39/40 leaves. By grinding in a mortar, isolation was achieved from 16/40 leaves.

Growth responses of dwarf beans to infection by P. phaseolicola have been examined by Hale et al.(1972). At an early stage of growth, inoculation of both monofoliate leaves inhibited trifoliate production. Inoculation of one monofoliate reduced expansion of the leaf and the opposite one expanded to a size greater than controls. Inoculation of trifoliate leaves reduced primary growth of the host by as much as 30%.

If bacterial infection in very young seedlings is severe, then the apical meristem of the plant is killed. This effect

and a general dwarfing of the plant is thought to be caused by the toxin produced by P. phaseolicola. The toxin is known as phaseotoxin.

There has been conflicting evidence as to the nature of the toxin. Hointink et al.(1966) found that the halo-inducing principle present in culture filtrates gave a positive correlation with halo area but none with degree of chlososis. The toxin was thermostable, dialysable (ie of low molecular weight), not retained on resin columns and of undetermined nature.

Patil and Tam (1970) purified the halo producing toxin by gel filtration and ion exchange chromatography. The toxin was thermostable and inhibited ornithine carbamyl transferase. Patil (1972) has since characterised the toxin as being composed of three known and three unknown amino acid residues.

This evidence contrasts with the findings of Rudolph (1970) which show the toxin to be composed mostly of hexose units with a molecular weight of 2,100.

Also in 1970 Patil purified a phytotoxin from culture filtrates of P. phaseolicola and found it to be ninhydrin-positive with a molecular weight smaller than that reported by Rudolph. Chlorosis induced by the toxin is prevented by application of citrulline - the end product of the reaction inhibited in toxin-treated tissue.

More recent work by Mitchell (1976) has demonstrated that a toxin, difficult to isolate because of its extreme lability in weak acids, can be isolated and purified from liquid P. phaseolicola cultures. Mitchell named the toxin phaseolotoxin. It causes leaf chlorosis by systemic movement, suppression of leaf expansion and accummulation of ornithine. The structure of phaseolotoxin is (N^{δ}-phosphosulphamyl) ornithylalanylhomo-arginine. A serine analogue of phaseolotoxin has also been isolated by Mitchell and Parsons (1977) and it shows the same

biological effects as phaseotoxin . This toxin is named (2 - serine) phaseolotoxin.

It seems likely that phaseotoxin is not one toxin but several and that two of them are phaseolotoxin and (2 - serine) phaseolotoxin.

The study of mechanisms of resistance to phytopathogenic bacteria has, until recent years, received less attention than the study of resistance to fungal pathogens. With the realisation that bacteria provide a simpler system for investigation (Klement and Goodman, 1967) much more intensive study of the incompatible (resistant) combination has been undertaken using bacteria.

Possible combinations of bacteria and host plants and respective resistance mechanisms have been reviewed by Kelman and Sequeira in 1972.They grouped bacterial resistance mechanisms into those which were constitutive or preformed and those which were induced on or after infection.

Constitutive mechanisms may involve inhibition of bacteria by preformed compounds which are themselves toxic or which can be rapidly converted to toxic products upon cell injury or a combination of adverse conditions at present unknown.

Kelman (1972) demonstrated that a constitutive system seemed to operate in resistance of maize to soft rot Erwinia species. Maize plants highly susceptible to one isolate of E. chrysanthemi were found to be highly resistant to all other isolates and species of Erwinia. A differentially inhibitory fraction (dif) extracted from maize plants was more toxic to soft rot Erwinia species that to the corn-stalk pathogen E. chrysanthemi. Other plant pathogenic bacteria that do not attack maize and some (but not all) saprophytic bacteria were also inhibited by dif. All the bacterial maize pathogens tested were similar to corn-stalk rot pathogen in their relative

insensitivity to dif.

Induced mechanisms of resistance include the hypersensitive response (HR) which is that resistant response of the plant involving rapid death of plant cells accompanied by limitation of growth and spread of the pathogen.

The concept of HR as a form of resistance arose first in the work of Ward in 1902 and Stakman (1915) on wheat rusts. HR has been recognised as a response to bacterial pathogens since the work of Klement in 1963. He showed that pepper fruits inoculated with P. syringae var. capsici developed typical soft rot symptoms. Other species belonging to the P. syringae group caused rapidly developing brown necroses to form around the inoculation site. Other phytopathogenic bacteria tested, except those pathogenic to pepper, caused light or dark brown necroses but some soft rot Erwinia and Pseudomonas species were unable to induce necrosis.

Klement (1971) divided HR into three stages of development. The first or induction phase is the time necessary for the bacteria to initiate HR. After this time bacteria may be killed by injection of antibiotics and the reaction will still go to completion. The second (or latent) phase finds the host symptomless although respiration rate may rise (Nemeth et al., 1969). The phase of expression is characterised by tissue collapse and by changes in host cell permeability (Goodman, 1968). Permeability of cells increases a few hours after inoculation reaching a maximum at the time of tissue collapse. In tobacco the plasma membrane, tonoplast and membranes of chloroplasts and mitochondria became deranged. (Goodman and Plurad, 1971). Within 18-24 hours collapse of the host cell tissue is complete and a well demarcated border separates the inoculated area from the rest of the leaf.

Sinclair et al.(1970) have examined chemical elements leached from leaves and have found more potassium than calcium, phosphorus, nitrogen or magnesium. Both magnesium and potassium increased in the intercellular fluids of susceptible leaves.

The concentration of incompatible bacteria does not influence the formation of HR but affects the development of visible necroses. Below the inoculum threshold, microscopic lesions are produced and at or above the inoculum threshold, a visible confluent necrosis develops. HR is thus an example of the co-operative effect described by Meynell and Stocker (1957) in which several bacteria combine to produce the visible response from the plant. SR can result from only one bacterial cell but Klement (1964) found that 5×10^6 cells/ml of P. syringae are needed to cause confluent HR in tobacco. With bean plants and P. mors-prunorum, 6.8×10^7 cells/ml of bacteria were required (Klement,1967). In 1973, Stall and Cook calculated that with P. cichorii on tobacco, 100 bacterial cells per plant cell were needed for microscopic lesions. Turner and Novacky (1974) found a different ratio using P. pisi on tobacco. A 1:1 ratio was required for microscopic lesions. However the technique, staining, was different.

In 1967, Klement and Goodman found that HR in tobacco leaves was not reproducable by plant pathogenic bacteria which had been killed, sonicated, treated with antibiotics or culture filtrates. In beans, "Mickle" treated bacterial cells, bacterial cytoplasm of P. mors-prunorum, bacterial cell walls, culture filtrates all failed to produce HR in leaves (O'Brien,1973).

It appears that only living incompatible bacteria are able to induce HR. The development of HR is influenced by

age of bacterial cells (Süle and Klement, 1971), by temperature (Lozano and Sequeira, 1970a; Cook, 1971 and Süle and Klement, 1971) and by light (Lozano and Sequeira, 1970a) but there is great variation in these influences, some having no effect at all in certain combinations.

The most important phase in the development of HR is the induction phase which is measured by infiltrating antibiotics into intercellular spaces at time intervals. In the tobacco-P. syringae combination, induction phase is 25 minutes (Klement and Goodman, 1967). In bean with P. mors-prunorum it is 150 minutes; in tobacco with P. solanacearum, 180 minutes (Sequeira, 1976).

The fact that the induction phase is so short, diminishes the possibility of bacterial multiplication or nutrient levels being very important in HR development.

A role for a specific metabolite being produced has been attributed to the bacteria. Lovrekovich et al.(1969,1970) implicated ammonia evolved by bacteria in the leaf tissue as being the substance responsible for induction of HR. This was disproved by Goodman (1971, 1972); Stall et al. (1972) and O'Brien and Wood (1973). It was shown that ammonia reached toxic concentrations 16 hours after inoculation whereas cell membrane damage was detected at 6 hours. There was also a suggestion that the ammonia was being produced by the plant tissue itself.

Sequeira and Ainslie (1969) extracted from bacteria a fraction which could induce HR in tobacco when the cell free preparation was purified using chromatographic proceedures.

In 1972, Gardner and Kado extracted a high molecular weight substance from osmotic shock fluid of Erwinia rubrifaciens. It produced HR in tobacco leaves and electrolyte leakage.

"Shock protein" preparations from non-pathogenic E. herbicola did not produce HR. Normally, osmotic shock is used to produce proteins from the surface layers of enterobacteria.

Sleesman et al.(1970) produced a heat labile proteinaceous fraction from sonicated cells of P. glycinea which could induce HR. Sequeira et al.(1972) also extracted a proteinaceous compound from P. solanacearum which would protect against HR. The substance did not involve cell walls, extracellular polysaccharides or nucleic acids of P. solanacearum and it was non-specific since it reduced both number and size of TMV lesions on tobacco leaves.

In 1966, Lobenstein and Lovrekovich reported a similar protective effect with cells of P. syringae which had been heat killed. This effect was inhibited by actinomycin D and as a result RNA synthesis was concluded to be important.

The conclusion of Sequeira et al.in 1972 was that incompatible bacteria in tobacco leaves release both the HR inducing and the HR protection factors. The response to the first would be so rapid that the effect of the second would be masked.

Both factors have been observed to be proteinaceous by Gardner and Kado, (1972); Sequeira and Ainslie, (1969) and Sequeira et al.(1970). Lallyett (1977) working with selective inhibitors has demonstrated that the HR inducing fraction is formed de novo by the bacteria ie after inoculation.

Attempts to extract HR inducers in vivo have all failed (Klement, 1965; O'Brien, 1973), the one exception being the work of Howes et al.(1973) with Puccinia graminis tritici and wheat. An RNA-ase sensitive fraction elicited HR specifically.

Whatever the inducer of HR is chemically it must involve

at some point in its reaction with the plant, some crossing of the plant cell wall. Pathogenic bacteria normally inhabit the intercellular spaces in leaves and rarely penetrate the cell except in cases of extreme infection (Zaumeyer, 1932).

The importance of the plant cell wall in the development of HR was suggested by Ercolani (1970) who said that bacteria became attached to multiplication sites on the cell surface and depending on factors contained by the bacteria HRor SR results.

Agglutination of incompatible bacteria on host cell walls has been reported by Sequeira et al.(1976) and Goodman (1974). In 1976, Sequeira and Donald showed that a major difference between compatible and incompatible strains of P. solanacearum is the ability of the latter to form polysaccharide slime which apparently allows attachment of avirulent bacteria to tobacco cell walls. Virulent cells did not bind and were thus ableto multiply in intercellular spaces. Virulent cells produce and extracellular polysaccharide (EPS) which has been shown to inhibit binding (Sequeira and Graham, 1977). The addition of EPS to avirulent bacteria prevented their normal binding response. The pattern for recognition of compatible or incompatible bacteria emerging from Sequeira's work involves the interaction of three molecular constituents (a) bacterial lipopolysaccharides (b) bacterial EPS and (c) host cell wall lectins (binding sites).

Once the avirulent bacteria are attached to host cell walls , the HR inducer would be produced Klement and Goodman proposed in 1967 that the HR inducer alters the permeability of cells by denaturing the S-S bonds in protein lamellae of host cell membranes with a sulphydryl compound.

An important feature of HR is that it can be easily

suppressed. Klement and Nemeth (1966) found that high temperature ($37^{o}C$) suppressed HR in tobacco. Klement later suggested in 1972 that there was a heat sensitive period in host tissue following the induction period.

Preinoculation with low concentrations of bacteria inhibit HR in some cases if plants are subsequently inoculated with concentrations sufficient to induce HR. This was found by Turner and Novacky (1974) but not by Cook (1975).

HR has been prevented by calcium (Cook and Stall, 1971), by precipitates from bacterial sonicates (Sleesman et al., 1970), by cytokinins (Novacky, 1972), by plant extracts (Süle et al., 1973) and by heat killed bacteria (Lozano and Sequeira, 1970; Stall et al., 1974; Lovrekovich and Farkas, 1965). Sequeira et al., in 1972, and Wacek (1974) observed that suppression of HR by heat killed cells of P. solanacearum is associated with glycoproteins found in the periplastic space of the bacterial cell wall. Extracellular polysacchrides, DNA or peptiglycan failed to give a protective response (Wacek and Sequeira, 1973).

The protection factors appear to be proteinaceous since proteolytic enzymes destroy their activity (Sequeira et al., 1972). Ryan (1974) proposed that the HR protection factor causes moderate injury or irritation to the plant which in turn produces its own fraction to resist necrosis of tissue caused by the incompatible bacteria.

Intercellular fluid extracted from plants protected against HR contained bacterial growth inhibitors (Sequeira and Hill, 1974). Protection in tobacco leaves at least may be light dependent. Bacterial growth was inhibited to a greater extent by fluid from plants kept in light than by fluid from plants in darkness (Rathmell and Sequeira, 1975).

It is important to realise however that suppression of HR in the cases mentioned above does not mean conversion of HR to

SR. In none of the cases did SR develop. Plants were still resistant to the bacteria when their ability to respond hypersensitively was impaired.

A great deal of work has been carried out in order to investigate the involvement of chemical substances in induced resistance to bacteria.

The review of Walker and Stahmann (1955) on the chemical nature of disease resistance reported a few compounds inhibitory to fungi or bacteria found in healthy or infected plants but none were implicated in resistance. Since then, many compounds have been attributed with a role in resistance to fungi, the research being stimulated by the "phytoalexin hypothesis" of Müller (1958). The original definition of the term phytoalexin has subsequently been amended to those substances which are "anti-microbial components of a range of compounds produced by many plants in response to cellular damage" (Deverall, 1972).

The phenols have received much attention for their role in resistance (Farkas and Kiraly, 1962; Tomiyama, 1963; Rubin and Artsikhovskaya, 1964; Farkas and Solymosy, 1965; Cruikshank and Perrin, 1964; Rohringer and Samborski, 1967; Kosuge, 1969; Addy, 1976). From this work there is little evidence to link phenols directly with resistance. However in 1977, Sequeira and Webster found that a phenolic compound produced in bean pods inoculated with P. syringae reduced growth of the bacteria in vitro.

The resistance of pathogenic pseudomonads to phenolics has been attributed to their ability to reduce quinones to phenols and an inability to produce hydrogen peroxide which affects the oxidation of the reverse reaction (Moustaffa and Whitenby, 1970).

Phaseollin has been shown to be induced in bean tissue after bacterial infection but subsequent tests showed that it did not

inhibit growth of pathogens in vitro, (Cruikshank and Perrin, 1971; Stholasuta et al., 1971; Webster and Sequeira, 1977). However induction of hydroxy phaseollin 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 be accompanied by reduced growth of the pathogen in vitro (Keen and Kennedy, 1974; O'Brien and Wood, 1973).

Extracts of pepper leaves following inoculation with Xanthomonas vesicatoria inhibited bacterial mutiplication in vitro (Stall and Cook, 1968). Bacteriostatic compounds were detected in extracts of bean leaves inoculated with water (Rudolph and Cinar, 1971) and from potato (Zalewski and Sequeira, 1973).

Bean plants reacting hypersensitively to P. phaseolicola were found to contain isoflavanoid compounds which restricted bacterial growth in vitro (Gnanamanickam and Patil, 1977a). Further work by Gnanamanickam and Patil (1977b) suggested that phaseotoxin produced by P. phaseolicola suppressed production of these isoflavanoid compounds in the susceptible combination.

The plants used in this study were Phaseolus vulgaris var. Red Mexican. In 1932 Burkholder and Zaleski described several varieties of P. vulgaris all of which were susceptible to three strains of Pseudomonas phaseolicola. After this the situation was rather confused with some people finding bean susceptible to P. phaseolicola (Ferguson et al.,1955) and some finding bean resistant to P. phaseolicola (Jensen and Goss, 1942; Zaumeyer and Thomas, 1957).

In 1964, Patel and Walker identified two races of the pathogen. Race 1 was avirulent on Red Mexican and Race 2 was virulent on Red Mexican and other varieties previously reported resistant. Races 1 and 2 of P. phaseolicola were used extensively in this study.

The most recent screening for resistance to halo blight

(the susceptible response to P. phaseolicola) was carried out in 1965 by Patel and Walker. Species susceptible to both races included P. coccineus, P. radicatus, P. lathyroids, P. lunatus, P. bractiatus, P. acutifolius, P. polyanthus and P. polystachys. Species resistant to Race 1 in a hypersensitive way included P. calcarateus, P. mungo, P. acconitifolius, P. aureus and P. angularis. None of the species were hypersensitive to Race 2.

In 1966, Coyne et al.demonstrated that resistance in Red Mexican beans to P. phaseolicola was dominant and monogenic. This feature makes the Red Mexican - P. phaseolicola Race 1 and 2 system ideal for investigation.

Most studies of resistance to disease in plants have used intact plants or plant organs. However isolated cells or protoplasts produced from plant tissues may offer certain advantages in mode of action studies (Samadder and Scheffer, 1968; Strobel and Hess, 1974).

Isolated protoplasts have played a key role in viral research since normally the cell wall acts as a very efficient barrier to the penetration through it of virus particles. Virus particles can therefore be presented directly to the plasmalemma if protoplasts are used. Isolated plant protoplasts offer an excellent system in which to study virus infection and replication since there is the potential of obtaining efficient and synchronous infection. (For a review of the use of protoplasts in plant virology see Takebe, 1975).

Filtrates of cultures of P. phaseolicola, P. syringae and P. mors-prunorum added to callus cultures of bean stem tissue reduced growth by 77%, 41% and 10% respectively. Filtrates from P. phaseolicola produced no haloes in the callus tissue but similarities between the physiological effects of toxin-containing

filtrates of P. phaseolicola on bean callus and on green leaf tissue suggest the feasibility of using isolated cells as a means to study bacterial action (Bajaj and Saettler, 1970).

In 1975 Pelcher et al. exposed resistant and susceptible corn protoplasts to the toxin from Helminthosporium maydis race T. Susceptible protoplasts did not increase in volume and did not show the cytoplasmic streaming seen in resistant protoplasts and in controls. These effects correlated well with effects seen in whole plants.

The elicitors produced by Phytophthora megasperma var. sojae which result in phytoalexin production in soybean plants, caused soybean cell suspension cultures to produce phytoalexins in a similar way, (Ebel et al., 1976).

Because of the relative similarities, found in isolated cell or protoplast systems in the work mentioned above, with the responses of whole plants, it was thought that an isolated cell or protoplast system could be developed to study the hypersensitive reaction in a system which was simpler than that of the whole plant. As a result a large section of this study was devoted to developing a system designed to bring plant cells or protoplasts together with bacterial cells in suspension.

In this investigation several different types of plant-bacterial combinations were used. The compatible combination was Red Mexican with P. phaseolicola Race 2; incompatible combinations were Red Mexican with P. phaseolicola Race 1, P. mors-prunorum, Erwinia atroseptica and Xanthomonas manihotis. The saprophyte P. fluorescens was also used.

P. mors-prunorum is a pathogen of Prunus sp. and is motile as are P. phaseolicola Races 1 and 2. P. fluorescens is usually motile and is non-pathogenic, (Bergey's Manual of Determinative Bacteriology, 1957).

Erwinia atroseptica causes rapidly spreading soft rot of

storage tissue. The rots are characterised by the formation of a rapidly spreading water-soaked lesion and cells separate along the lines of the middle lamellae. The tissue quickly loses coherence (Stephens, 1974).

Xanthomonas manihotis causes blight of cassava plants, the best described and most important bacterial disease of cassava. It was first reported in Brazil (Bondar, 1912) and cassava plants in South America, Africa and Asia are affected. The symptoms are leaf spotting and blight, wilting of young stems and leaves, stem dieback, gum exudation and vascular necrosis (Lozano and Booth, 1974). Entry to the plants is via stomata and wounded tissue (Pereira and Zagatto, 1967). For a review of the work carried out using X. manihotis see Ikotun, 1975.

LIST OF ABBREVATIONS USED IN THIS THESIS

RM	*Phaseolus vulgaris* var. Red Mexican		
R1	*Pseudomonas phascolicola* Race 1		
R2	*P. phaseolicola* Race 2		
Pmp	*P. mors-prunorum*		
Pf	*P. fluorescens*		
Xm	*Xanthomonas manihotis*		
Ea	*Erwinia atroseptica* (*E. carotovora* var. *atroseptica*)		
Ia	Inoculated area	Ih	zone adjacent to Ia
c.	circa	h	hour
min	minute	ppm	parts per million
CIM	Cell incubation medium		
IIC	Initial inoculum concentration		
d	day		
SE	Standard error		
cv	cultivar		

MATERIALS AND METHODS

I Biological Material

A Plants

1 Cultivar

Phaseolus vulgaris L. cv. Red Mexican U.I. 34 (RM) grown at Imperial College Field Station, Silwood Park. The original stocks were obtained from Honey Seed Company, Twin Falls, Idaho, U.S.A.

2 Plant Culture

Plants were grown from seed soaked for ten minutes in sodium hypochlorite solution (5-7% w/v available chlorine), washed well in sterile water and placed in boxes lined with moist paper at 26°C. After two days 20 germinated seeds were placed in seed trays (20 x 34 x 6 cm^3) containing John Innes No.1 compost and incubated in a growth cabinet at 25°C. Plants were illuminated for 16 hours/day at 1,290 lux at plant level. The compost was well soaked with water once every two days.

3 Plant Inoculation

Bean plants,nine days old (ie nine days from emergence from the compost), were inoculated using a modification of the injection/infiltration technique described by Klement (1963). Injections of bacterial suspensions and other solutions or mixtures were made from a hypodermic needle (No. 30 gauge) at the junction of the main vein and the petiole of expanding monofoliate leaves into the interveinal areas to the right and left of the main vein. Leaves were washed immediately afterwards in a stream of sterile water and returned to the growth cabinet where the water-soaking produced by injection disappeared within one hour.

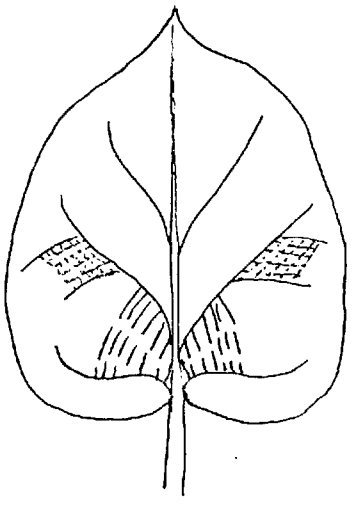
4 Plant inoculation for leaf disk experiments

A disadvantage of the inoculation technique used is that it

is difficult to control the area of inoculation. Bacterial suspension is forced into intercellular spaces and spread of the inoculum is limited only by the larger veins in the leaves.

In some experiments it was necessary that disks (1.2 cm diameter) should be cut from leaves such that the central area of the disk only was inoculated. Careful examination of the RM leaf structure revealed that on each monofoliate leaf there were two areas (see Figure 1) bounded on three sides by large veins where controlled inoculation was possible with practice.

Figure 1. Inoculation areas on RM leaf surfaces.



Usual inoculation area (Ia)

Inoculation area in disk experiments (Ih)

The inoculation area for the disk experiments was thus a small rectangle of tissue 4mm by 5mm(Ih). A high level of replication was used in these experiments but variation was not great.

Disks (1.2 cm in diameter) were cut from leaves with the inoculation area (0.4 x 0.5 cm^2) in the centre one hour after inoculation. This was to allow for the disappearance of water-soaking.

Disks were then incubated in two ways.

1 In boxes

Disks were placed on dry microscope slides lying on angled glass rods in transparent plastic boxes (16 x 10 x 5 cm^3) lined with wet tissue paper. There were three slides per box and two disks per slide. Boxes were incubated in the growth cabinet at 25°C and 16 hours light per day.

2 Floating on sterile water

Disks were floated on the surface of sterile water in petri dishes, four disks and 20 ml water per dish. Dishes were incubated in the growth cabinet as described above for boxes.

For other experiments disks one cm in diameter were used. these were cut from the usual inoculation area (Ia) shown in Figure 1 such that the full area of the disk was inoculated. Disks were cut one hour after injection of the leaves to allow for disappearance of water soaking. Disks were incubated in boxes or petri dishes as described in the paragraph.

5 Floating plant material on suspensions of bacteria

a Disks

Disks 1.2 cm diameter were cut from RM leaves which had not been inoculated. Two disks were cut from each monofoliate leaf, one to the left and one to the right of the main vein near the junction with the petiole. Disks were washed in sterile water, dried in sterile air (lamina flow bench air) and then floated on sterile solution in petri dishes. There were four disks and 18 ml solution per dish. Various solutions were used and are detailed with the results. To each dish two ml of a bacterial suspension of known concentration in sterile water were added making the total volume of suspension in each dish 20 ml. incubation was in a growth cabinet at 25°C with 16 hours per day light.

b Use of disks with parts of the lower epidermis removed

The same method as described in 5a was used except that the disks (1.2 cm diameter) had pieces of lower epidermis (c. 0.4 x 0.5 cm^2) removed. This was done using a pair of very fine forceps to tear off pieces of epidermis from slightly wilted leaves before disks were cut. Leaves were deliberately allowed to wilt to facilitate removal of epidermis. Disks were floated on bacterial suspensions (as described above) with the lower epidermis in contact with the suspension.

c Floating pieces of lower epidermis on suspensions of bacteria

In one experiment pieces of epidermis (removed from the lower surface of slightly wilted RM leaves using a pair of very fine forceps) were floated on suspensions of bacteria as described for disks in 5a above. Incubation was at 25°C. Each petri dish contained six pieces of epidermis approximately 0.4 x 1.0 cm^2 in area (each piece) and 20 ml of bacterial suspension of known concentration.

B Bacteria

1 Cultures

Pseudomonas phaseolicola

From the National Collection of Plant Pathogenic Bacteria (NCPPB).

Race 1 - isolate number 605 (R1).

Race 2 - isolate number 1321 (R2).

Pseudomonas fluorescens

From NCPPB - isolate number 1598 (Pf).

Erwinia carotovora var. atroseptica

From NCPPB -isolate number 2043 (Ea).

Xanthomonas manihotis

From NCPPB - isolate number 1159 (Xm).

Pseudomonas mors-prunorum

From Dr. Lallyett, Imperial College and originally from East

Malling Research Station.

2 Growth of bacteria

a Stock cultures were kept on storage nutrient agar slopes of the following composition:- 13g Oxoid Nutrient Broth No. 1 (CM1), 15g Standard Agar (Davis), 1 litre distilled water. Cultures were kept under oil at room temperature or were stored (without oil) at 4°C & -20°C.

b For inoculation bacteria were grown on nutrient agar slopes of the following composition:- 13g Oxoid Nutrient Broth No.2 (CM 67), 15g Standard Agar (Davis), 1 litre distilled water. Cultures were incubated at 25°C for 24 hours before use.

c For some purposes bacteria were grown in Nutrient Broth No.1 (CM 1) in conical flasks (100ml medium in 250 ml flasks or 200 ml medium in 500 ml flasks) and incubated in an orbital incubator at 25°C.

d For other purposes, a more defined liquid medium was used. The method of incubation and amounts of medium used are indicated with the results where appropriate. The medium used was of the following composition:- 4.6g casamino acids (vitamin free), 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 10 ml trace element solution* made up to 1 litre with distilled water.

* Trace element solution

$FeSO_4.7H_2O$	0.5 ppm in final solution
$ZnSO_4.7H_2O$	0.1 ppm " " "
$Na_2MoO_4.7H_2O$	0.2 ppm " " "
$CuSO_4.5H_2O$	0.1 ppm " " "
$MnCl_2.4H_2O$	0.1 ppm " " "

This medium was named Medium A.

e For measurement of bacterial growth *in vivo* and *in vitro* selective media were used as follows:-

D3 Medium (Kado and Heskett, 1970) for Erwinia carotovora var. atroseptica.

10g	sucrose	60 mg	bromothymol blue
10g	arabinose	100 mg	acid fuchsin
5g	casein hydrolysate	15 g	agar
7g	LiCl	1	litre water.
3g	glycine		
5g	NaCl		
0.3g	$MgSO_4.7H_2O$		
50mg	sodium dodecyl sulphate		

Adjust pH to 8.2 with NaOH before autoclaving. The pH of the medium should be 6.9 - 7.1 after autoclaving.

D4 Medium (Kado and Heskett, 1970) for Pseudomonas species.

10 ml	glycerol	0.6 g	sodium dodecyl sulphate
10 g	sucrose	15 g	agar
1 g	casein hydrolysate	1	litre water.
5 g	NH_4HPO_4		

D5 Medium (Kado and Heskett, 1970) for Xanthomonas manihotis

10 g	cellobiose	0.3g	$MgSO_4.7H_2O$
3 g	K_2HPO_4	15 g	agar
1 g	NaH_2PO_4	1	litre water

The degree of selectivity of these media is dealt with in the results section.

3 Standardisation of bacterial inocula

Bacteria were grown for 24 hours at 25°C on slopes of nutrient agar (c. 10 ml). Ten ml of sterile distilled water was added to each tube and bacteria were suspended by scraping the surface of the agar with a sterile inoculating loop. The suspension was centrifuged (20 minutes at 10,000g) and washed

by resuspension and centrifugation.

Washed bacteria were then resuspended in sterile distilled water and the suspension diluted until a reading on a nephelometer (Eel "Unigalvo") was obtained. Serial dilutions of the suspension were made and 0.1 ml from each dilution was transferred to a petri dish of nutrient agar and spread over the surface with a sterile glass spreader. Plates were incubated at 25°C and four replicates were made of each dilution. Numbers of bacterial colonies per plate were counted three days after inoculation and the concentration of live bacterial cells in the original suspension was calculated.

This proceedure was carried out for each species of bacteria used and throughout the work, conditions were always kept standard in preparation of inocula.

Nephelometer readings were plotted against log concentration of bacteria. From this plot a nephelometer reading of 10 corresponded to 10^8 cells/ml for all species of bacteria used.

II Measurement of Bacterial Growth

A In vivo

a Plants

After inoculation leaves were well washed with sterile distilled water to remove most surface bacteria. First samples were taken immediately, later samples at intervals over the next few days. Each sample consisted of four disks cut from leaves with a sterile cork borer (1 cm diameter). There were five samples per measurement. Disks were ground, one sample at a time, in o.2 strength Ringers solution, then made up to five ml in the same solution. After ten minutes with periodic shaking, serial ten-fold dilutions were prepared. 0.1 ml from each dilution was spread on plates of selective medium agar. (Agar surfaces were dried in sterile air for 30 minutes after pouring). Colonies were

counted after three days incubation at 25°C. Numbers of bacteria per cm^2 leaf tissue were then calculated.

b Disks (inoculated by injection with bacteria)

Disks were cut from plants one hour after inoculation and were 1 or 1.2 cm diameter depending on the experiment. First samples were taken immediately (disks being washed first in sterile water) others were placed on microscope slides in boxes or on water or solutions in petri dishes. At intervals disks were removed from incubation, washedin sterile water and each sample (four disks) was ground in 0.2 ml 0.25 strength Ringer's solution as above. There were five samples per measurement. Numbers of bacteria per cm^2 disk tissue were calculated by dilution plating as in Aa above.

c Disks (inoculated by floating on bacterial suspensions)

Disks were cut from leaves which had not been inoculated and were 1.2 cm in diameter. Measurement of numbers of bacteria per cm^2 leaf tissue was carried out as described for disks inoculated by injection of bacteria. First samples were taken immediately and others at intervals after being floated on suspensions of bacteria in petri dishes. There were four disks per sample and five samples per measurement and disks were well washed in sterile water before measurements were taken.

d On leaf surface

Plants were inoculated in areas 0.4 x 0.5 cm^2 as explained in IA4. At intervals disks (1.2 cm diameter) were cut such that the inoculated areas were in the centres of the disks and were washed in closed flasks. There were four disks and 10 ml of sterile water in each flask and flasks were gently shaken on a reciprocal shaker for 20 minutes. 0.1 ml was then taken from each flask and pipetted into 9.9 ml sterile water. Serial ten-fold dilutions and plating on selective medium agar were carried out as before.

Numbers of bacteria per ml washing water were calculated. The results gave an indication of the levels of bacteria washed from the surfaces of the lesions and from the cut edges of the disks. This method is a modification of that used by Leben et al.(1968).

B In vitro

0.1 ml samples were removed from bacterial liquid cultures and pipetted into 9.9ml sterile water. Serial ten-fold dilutions and plating on selective medium were carried out as before. Numbers of bacteria per ml culture medium were calculated.

This method was also used to measure bacterial growth in solutions in petri dishes on which leaf disks were floated.

III Measurement of electrolyte loss.

a From leaves

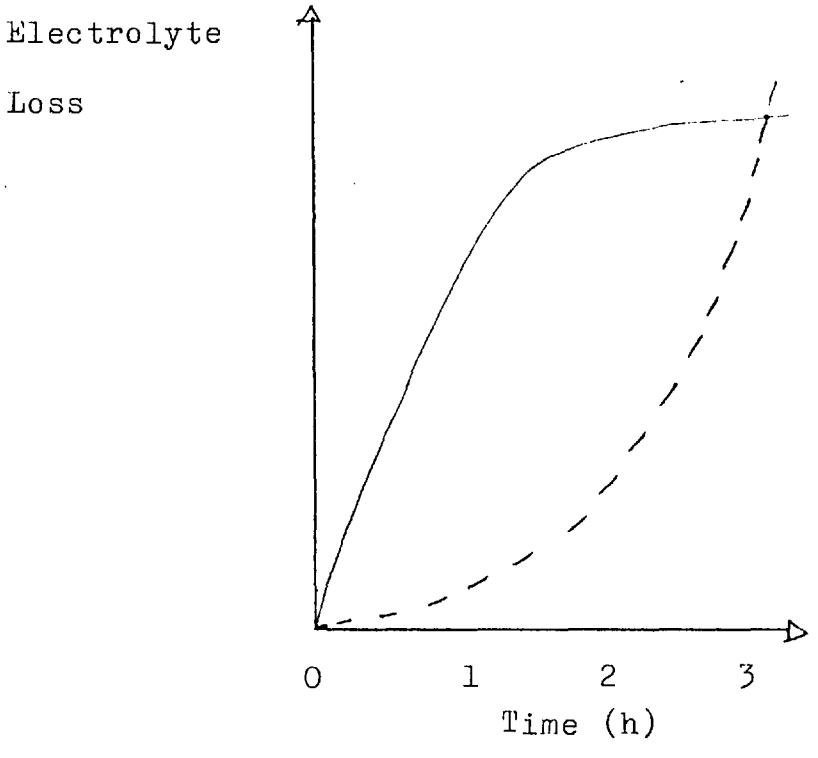
1 Twenty disks (0.3 cm diameter; c. 0.05g fresh weight) were cut from inoculated areas of RMleaves and washed quickly in distilled water. Excess water was removed with absorbent paper and disks were placed in 10 ml fresh distilled water. The conductivity of the water was measured with a "Chandos" conductivity bridge immediately and after incubation at 25°C for three hours. The increase in conductivity (in μmhos) was used as a measure of electrolyte loss.

There were three replicates (each twenty disks) per treatment.

2 The technique described above for measurement of electrolyte loss has its limitations because it does not measure rate of electrolyte loss. Thus (see Figure 2) Rate A would appear to be the same as Rate B when only two measurements are made.

In an effort to deal with this problem experiments carried

Figure 2 Taking conductivity measurements at Time 0 and Time 3 h only, Rate A would seem to be the same as Rate B.



out using the method described in a1 above were repeated taking conductivity measurements at Time 0, 3, 15, 30, 45, 60 and 120 minutes rather than o nly at Time 0 and three hours. In other respects the methods were the same.

3 The effect of temperature of incubation was also studied. The method used was basically that described in a2 above but incubation of leaking disks was at 4°C, 25°C, 37°C and 50°C.

Flasks were incubated at the relevant temperatures (before disks were added) for one hour. This allowed the water in the flasks to reach the relevant temperature before the experiment was started.

There were three replicates of each treatment.

4 In some experiments patterns of electrolyte loss following injury to RM cells caused by chemicals and autoclaving were studied in order to make comparisons with patterns of loss following injuries caused by bacteria.

a Triton X-100

Triton X-100 is a non-ionic detergent which kills plant cells by disrupting the membranes. Leaves were infiltrated with 2% Triton X-100 and measurements of electrolyte losses were made as described previously for plants infiltrated with bacteria in IIIa2. There were three replicates of each treatment.

b $ZnSO_4.7H_2O$

Leaves were infiltrated with 1% $ZnSO_4.7H_2O$ (w/v) solution. Measurements of electrolyte loss were made as described in IIIa1. but incubation time was limited to one hour at 25°C with readings every 15 minutes. There were three replicates of each treatment.

c Autoclaving

Leaves were infiltrated with bacteria or distilled water and at intervals disks were cut, washed and floated on sterile water as described in IIIa1. The Time 0 conductivity reading was made as usual and then flasks were autoclaved for 15 minutes

at 15 lb/in^2. After removal from the autoclave, flasks were allowed to cool to room temperature and a second conductivity reading was made. There were three replicates of each treatment.

5 Replacement of ambient solution

In these experiments disks were removed from the first ambient solution after a certain amount of electrolyte leakage had occurred and were then placed in fresh distilled water. The purpose of this was to find out if the patterns of conductivity loss were altered by changing the ambient solution.

Disks cut from inoculated RM leaves were placed in flasks of distilled water as described previously. After incubation for 35 minutes at 25°C (during which time several conductivity measurements were made) disks were removed from the flasks and placed in fresh distilled water and more conductivity readings were taken using the new solution.

There were three replicates of each sample.

b From disks

Measurements of electrolyte loss were made on disks floating on water in petri dishes.

Disks were 1 cm in diameter and had been inoculated over the whole disk area or were of 1.2 cm diameter and inoculated only in the central (0.4 x 0.5) cm^2 area.

There were four disks per petri dish (each containing 20 ml water) and four dishes per treatment. When conductivity measurements were made, disks were removed from the dishes.

c Calibration of Conductivity Meter.

In order to have some idea of the behaviour of the conductivity meter, measurements of the conductivity of solutions of known concentration were made.

Glassware used was thoroughly washed in distilled water and conductivity measurements were made on distilled water in the flasks used. In all cases this was shown to be normal (4.0 μmhos) for this meter.

Solutions of $CaCl_2.2H_2O$ and KCl in distilled water were made were made up ranging in concentration from 40 mg/ml to 20μg/ml and conductivity measurements were made on these. All measurements were replicated three times.

IV Flame photometry

a Ion efflux from leaf cells

Twenty disks (0.3 cm diameter; c. 0.05g fresh weight) cut from RM leaves were washed in sterile water. Excess surface water was removed with absorbent paper and the disks placed in 10 ml distilled water. Three ml of liquid was removed immediately and the levels of potassium, sodium and calcium were measured on this sample using an Eel Flame Photometer Mark II and on the remaining 7 ml after three hours incubation at 25°C. The difference between the two measurements was used as an estimate of the amounts of these ions leaking from the RM cells in the disks during three hours. There were three replicates of each treatment and adjustments in the calculations were made to allow for the first sample coming from a larger volume of ambient solution than the second.

b Rate of ion efflux from leaf cells

The technique described above for measurement of ion efflux has the same limitations as described in IIIa2 for the first method used to study electrolyte losses. In other words the rate of ion efflux is not measured. To deal with this problem experiments done by the method described in IVa1 were repeated.

This time 40 disks (0.3 cm diameter, c. 0.1g fresh weight) cut from RM leaves were washed and placed in 20 ml of distilled water. At intervals (Time 0,3, 15, 30, 45, 60 and 120 minutes) 1 ml samples were removed from each tube for measurements of ion levels. In other respects the methods were the same and adjustments were made in the calculations to allow for the changes in volume of the ambient solution during the experiment.

c The effect of temperature on ion efflux

The effect of temperature of incubation on ion efflux from disks was also studied. The method used was that described in IVa2 but temperatures of incubation over the period of measurement were 4°C, 25°C, 37°C and 50°C. There were three tubes per measurement and tubes were incubated for one hour at the relevant temperature prior to addition of disks. This was in order to allow the temperature of the ambient solution to reach the required level before the experiment began.

d Ion leakage following injection of Triton X-100.

Ion efflux from disks cut from leaves infiltrated with the detergent Triton X-100 was measured in order to compare patterns of ion leakage following chemical killing of RM cells with the patterns of ion leakage following bacterial killing of the cells.

Leaves were infiltrated with Triton X-100 at a concentration of 2% and measurements of ion efflux were made as described previously for plants infiltrated with bacteria. The influence of incubation temperatures on ion efflux after infiltration with 2% Triton X-100 was also studied (as described in IVa3).

e Ion efflux from disks.

Measurements of the levels of potassium, sodium and calcium

ions in water on which inoculated leaf disks floated were made at intervals. Disks were 1 cm in diameter and cut so that their whole surface was inoculated or were 1.2 cm in diameter and had only the central 0.4 x 0.5 cm^2 area inoculated. There were three disks per petri dish and each dish also contained 20 ml of water. There were four dishes per treatment.

Disks were either removed from the water and discarded before measurements of ion levels were made on one set of dishes over the whole period of the experiment. In this last case, 1 ml samples were removed at intervals from each petri dish using a sterile pipette and adjustments were made in the calculations to allow for changes in volume of the ambient solution during the course of the experiment.

V Measurement of Protein Levels

Eighty disks (0.3 cm diameter; c. 0.2 g fresh weight) cut from RM leaves were washed in sterile water. Excess surface water was removed with absorbent paper and the disks placed in fresh distilled water in closed flasks. For leaves inoculated with Pseudomonas phaseolicola Race 1 or Race 2 the amount of water in each flask was 10 ml. For leaves inoculated with P. fluorescens or Erwinia carotovora var. atroseptica the amount was 5 ml. The flask was then shaken vigourously by hand for three seconds and 1 ml of ambient liquid was removed. Measurement of total protein in this sample was made using the method below. After incubation at 20°C for two hours a further 1 ml sample was removed and total protein content measured again. The difference between the two measurements (Time 0 and Time two hours) was used as an estimate of total protein leaking from the RM cells in the disks in two hours.

Other experiments were carried out to ascertain the rate of protein leakage over the two hour period by incubation of flask

for periods less than two hours. In all cases there were three replicates of each treatment.

Estimation of total protein

a Reagents

A 2% w/v Na_2CO_3 (anhydrous) in 0.1 NaOH

B1 1% w/v $CuSO_4.5H_2O$ solution

B2 2% w/v potassium sodium tartrate solution

B1 and B2 are mixed in equal quantities on the day of use to form Reagent B

C Alkaline copper reagent (made up on day of use)

50 parts A to 1 part B.

D Folin-Ciocalten phenol reagent diluted to 1N (1 in 3) on day of use.

b Method

1 Add 1 ml of protein sample to 5 ml Reagent C. Mix well and keep at room temperature for 30 minutes.

2 Add 0.5 ml Reagent D. Mix and keep at room temperature for 30 minutes.

3 Estimate absorbance at 750 nm against a reagent blank.

The spectrophotometer was Beckman's DB model and was calibrated using solutions of crystalline bovine serum albumin containing between 10 and 100 μg/ml protein.

VI Measurement of total phenol

a Leaking from leaf cells

Eighty disks (0.3 cm diameter; c. 0.2g fresh weight) cut from RM leaves were washed in sterile water. Excess surface water was removed using absorbent paper and the disks placed in 5 ml distilled water in a small flask. The flask was then shaken vigourously by hand for three seconds and 1 ml of ambient

liquid was removed. Measurement of total phenol content in this sample was made using the method described below. After incubation for 24 hours at 20°C a further 1 ml sample was removed from each flask and total phenol content measured. The difference between the two measurements (Time 0 and Time 24 hours) was used as an estimate of total phenol leaking from the RM disks in 24 hours.

Other experiments were carried out to ascertain the rate of phenol leakage over the 24 hour period by incubation of flasks for periods less than 24 hours. There were three replicates of of each treatment.

Estimate of total phenol

(modified from the method of Addy, 1976)

1 Add 1 ml of phenol sample to 4 ml of absolute alcohol.
2 Centrifuge at 12,000g for 15 minutes to precipitate protein and bacteria present.
3 Filter with millipore filter (0.22 μ).
4 At this point supernatant can be stored at 4°C for subsequent estimation of total phenols.
5 Add 5 ml of aqueous sodium carbonate (2%).
6 Add 1 ml 1N Folin-Ciocalten reagent (made up on day of use).
7 Remove precipitate by centrifugation at 3,000g for 15 minutes.
8 Estimate absorbance at 750 nm against a reagent blank.

The spectrophotometer was calibrated using solutions of 0 - 300 μg/ml chlorogenic acid in 80% ethanol.

b In cultures of cells or protoplasts

At intervals, 1 ml samples were removed from culture media for estimation of phenol levels. Before carrying out the analysis samples were centrifuges at 100g for 5 minutes to precipitate

cells or protoplasts. The supernatant was decanted and centrifuged at 10,000g for 15 minutes to precipitate bacteria and cell debris. The supernatant was again decanted and filtered through 0.22μm millipore filters.

Total phenol analysis was then carried out as described above and there were five replicates per treatment.

VII Attempts to modify the hypersensitive reaction

a Leaves

1 Using bacteria

i Plants were injected in the usual way with suspension (10^8 cells/ml) of Race 1. At various times with respect to this injection , the inoculated area was given a second injection with known concentrations of either Race 1 or of other bacteria. Five plants were used per treatment.

ii Plants were injected in the usual way with suspension of 10^8 cells/ml Race 1 mixed with known concentrations of other bacteria.. Five plants were used per treatment.

iii Prior to the main injection with Race 1 at 10^8 cells/ml, plants were injected with known concentrations of Race 1 or other bacteria. Five plants were used per treatment.

2 Using chemicals

In exactly the same way as described for bacteria in section 1 above, plants were injected at various times with respect to the main injection of Race 1 (10^8 cells/ml) with known concentrations of chemical substances. Details of these are given with the results. Five plants were used per treatment.

b Disks

1 Disks of 1.2 cm diameter which had been cut from plants which had not been inoculated were placed in petri dishes on suspensions of bacteria in various chemical substances. Details of these

are given with the results and the method used was that described in I5a above.

2 Disks were cut from inoculated plants and were either 1 cm diameter with only the central 0.4 x 0.5 cm^2 area inoculated. These disks were placed in petri dishes on sterile solutions of various substances . Details of these are given with the results and the method used was that described in I4C2 above.

3 In some experiments disks of 1.2 cm diameter cut from plants which had not been inoculated were floated on suspensions of bacteria non-pathogenic to RM. The petri dishes were incubated at 25°C in the growth cabinet for various times and then disks were removed and washed. Disks were then placed on suspensions of Race 2 in fresh petri dishes. There were four disks per treatment (dish) and four dishes per sample.

VIII Production of isolated RM leaf cells.

This method was modified from that of Takebe et al.(1968). The enzyme medium used to produce isolated RM cells was as follows:-

0.5% Macerase (Calbiochem)

0.4 M Mannitol

pH adjusted to pH 5 - 9 using either 2N HCl or 0.1 N NaOH.

The medium was filter-sterilised and there was never more than 2g fresh weight of leaf tissue to 20 ml of medium.

For some experiments 0.3% potassium dextran sulphate was added.

The isolation proceedure was as follows:-

1 Plants (nine days old) were maintained for at least 24 hours at 23 - 25°C and 200 - 400 lux before use.

2 Leaves were removed and soaked in 10% sodium hypochlorite

solution for five minutes and then washed well three times in sterile water (distilled).

3 Leaves were left in a stream of sterile air in order to wilt slightly for between 15 and 30 minutes. The time is not critical but slight wilting of leaves facilitates removal of the lower epidermis.

4 As much as possible of the lower epidermis was removed from the leaves using a pair of sharp pointed forceps.

5 Leaves were cut into squares of approximately 1 cm^2 in area and placed in the enzyme solution in 50 ml flasks.

6 Flasks were incubated at 25°C in darkness with periodic shaking for four hours. After this time leaf peices were seen to be falling apart.

7 The medium was filtered through a sheet of nylon gauze (pore size 100μm) to remove debris and cells were precipitated by centrifugation at 100g for five minutes.

8 After being washed twice by centrifugation at 100g for five minutes each time using a washing medium (0.4M Mannitol with 0.1 mM $CaCl_2$), cells were resuspended in Cell Incubation Medium (CIM). There was 10 ml CIM per 50 ml flask and the medium was sterilized by autoclaving.

Cell Incubation Medium (CIM)

For convenience the medium was made up in a ten times strength concentration and diluted prior to use.

	Dissolve in 2 litres distilled water (x10 concentratio
KH_2PO_4	0.272 g
KNO_3	1.011 g
$MgSO_4.7H_2O$	2.465 g
$CaCl_2.2H_2O$	14.8 g
KI	

Make up 0.166 g in 100ml and use 1 ml

$CuSO_4.H_2O$

make up 0.00249g in 100 ml and use 1 ml.

Mannitol added and p H adjustments are mede prior to use ie after dilution to normal strength.

Mannitol	0.4 M
pH	5 - 9 (using 0.1 N NaOH)

9 Flasks were incubated in darkness at 25°C and cells survived for at least four days.

Various experiments were carried out using methods described previously and detailed with the results.

IX Method of production of isolated RM leaf protoplasts

This method was modified from that of Pelcher et al. (1974). The enzyme media used to produce isolated protoplasts from RM leaves were as follows:-

1 0.25% Macerase (Calbiochem)
0.25% Driselase (Kyowa Hakko Kogyo Co. Ltd, Japan)
0.3 % potassium dextran sulphate (Calbiochem)
0.4 M Mannitol
Made up in distilled H_2O and pH adjusted to pH 5 - 9 using 0.1 N NaOH.

2 As Medium 1 but using 0.25% Onozuka SS (All Japan Biochemical Company Ltd.) instead of Driselase.

3 As Medium 1 but without potassium dextran sulphate.

4 As Medium 2 without potassium dextran sulphate.

5 0.45% Macerase
0.45% Driselase
0.3 % potassium dextran sulphate
Made up in CIM and pH adjusted to pH 5 - 9 using 0.1 N NaOH.

6 As Medium 5 using 0.45% Onozuka SS as the cellulase instead of Driselase.

7 As Medium 5 using 0.4M sucrose in the CIM instead of Mannitol.

8 As Medium 6 using 0.4M sucrose in the CIM instead of Mannitol.

All media were filter-sterilized prior to use.

The isolation proceedure was as follows:-

1 Plants (9 days old) were maintained for at least 24 hours at 23 - 25°C and 200 - 400 lux before use.

2 Leaves were removed and washed in 10% chloros (sodium hypochlorite solution) followed by three washes in sterile distilled water.

3 Leaves were then left for about 30 minutes in a stream of sterile air. This wilted them and facilitated the removal of lower epidermis.

4 As much as possible of the lower epidermis was removed from leaves using a pair of very fine forceps.

5 Leaves were cut into squares of approximately 1 cm^2 area and placed in the enzyme solution in 50 ml flasks. There was never more than 2 g fresh weight of leaf tissue to 20 ml enzyme solution.

6 Flasks were then incubated at 25°C in darkness for 18 hours and by this time leaf pieces were seen to be falling apart.

7 Protoplasts were then filtered through a sheet of nylon gauze (pore size 100 μm) and precipitated by centrifugation at 100g for five minutes.

8 Protoplasts were then washed twice (by centrifugation at 100g for five minutes using a washing medium of CIM. Finally the protoplasts were resuspended in CIM.

9 Flasks were incubated at 25°C in darkness and protoplasts appeared to survive for at least three days. No examinations were made after three days.

Various experiments were carried out using methods previously described and are detailed with the results.

X Phenoloxidase assay

A 0.05 M solution of catechol (BDH Chemicals Ltd.) and 0.2 M sodium phosphate buffer at pH 7.0 were prepared. Reaction mixtures were prepared in spectrophotometer cuvettes as follows:-

	Reference Cuvette	Sample Cuvette
Catechol	2.0 ml	2.0 ml
Water	0.5 ml	0.3 ml
Buffer	0.5 ml	0.5 ml

After adjusting to zero absorbance at 490 nm, 0.2 ml enzyme sample was added to the sample cuvette and mixed. Absorbance readings were taken every 15 seconds for two minutes. Corrections were made for colour in the enzyme samples by measuring absorbance at 495 nm of the 0.2 ml enzyme sample in water and buffe against water and buffer. Phenoloxidase activity was expressed as change in absorbance at 495 nm in one minute. There were three reading per sample.

XI Controls

Unless otherwise stated controls consisted of the following:-

a Plant material not treated in any way (Blank Controls).

b Plant material injected with sterile water (Water Controls).

c Plants or disks not treated in any way other than by being

injured by the hypodermic syringe needles used for normal injections. The injuries were more extensive than would be expected from normal injection (Injured Controls).

Mechanical injury is an integral part of the injection-infiltration technique and these controls were carried out in order to find any effect that mechanical injury might have.

d Plant material injected with bacterial suspensions which has been previously autoclaved at 15 lb/in^2 for 10 minutes (Dead Cell Controls).

In none of the results reported here was there any significant difference amongst controls. In all cases where control values are given, they are those from Water Controls.

XII Chemicals

All chemicals used were of Analar grade and supplied by BDH with the following exceptions:-

Sodium dodecyl sulphate	Sigma
Thiamine hydrochloride	Sigma
Dioctyl sulphosuccinate	Sigma
Potassium dextran sulphate Macerase Protamine sulphate	Calbiochem, San Diego, California 92112.
Absolute Alcohol	James Burrough Ltd.
Sodium polypectate	Sunkist Growers
Casein hydrolysate	Oxoid
Onozuka SS	All Japan Biochemicals Co. Ltd
Driselase	Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan

EXPERIMENTAL RESULTS

I Preliminary Experiments

1 Characterization of the hypersensitive response(HR)

a Grading of host response

The following system was adopted for describing observations (macroscopic) of host responses. It is slightly modified from the system devised by O'Brien (1973).

Hypersensitive reaction grades in the inoculated area (Ia)

0 No macroscopically visible response.

1 Shiny patches and pale green colour.

2 Pale bronze.

3 Deep bronze.

4 Dark brown.

5 Desiccated.

Susceptible reaction grades

0 No macroscopically visible response.

1 Shiny patches in the Ia.

2 Pale bronze in the Ia.

3 Deep bronze in the Ia.

6 Dark brown in the Ia, with beginnings of chlorotic halo around the Ia.

7 Ia surrounded by halo.

8 Monofoliate leaf death.

9 First trifoliate leaves showing slight interveinal chlorosis

10 First trifoliate leaves showing marked interveinal chlorosis.

11 Subsequent trifoliates green but monofoliates chlorotic.

12 Subsequent trifoliates chlorotic and monofoliates chlorotic.

13 Plants stunted but continue to grow.

14 Plants die.

b Development of the hypersensitive and susceptible responses.

Red Mexican (RM) leaves were injected with suspensions of Pseudomonas phaseolicola Race 1 (R1) and Race 2 (R2) and with P. mors-prunorum (Pmp) at concentrations of 10^8 cells per ml estimated by nephelometry. Plants (five per treatment) were then incubated at 25°C in the growth cabinet and examined at intervals for 15 days. Using the host reaction grades described above, the responses of the plants were noted (Table 1). RM plants were found to be resistant to R1 and Pmp in the form of RM and to be susceptible to R2.

Table 1 Characterization of host response to R1, R2 and Pmp.

Time (d) after inoculation	Bacterium R1	R2	Pmp	Water Control
1	1*	0	1	0
2	2	1	2	0
3	2	2	2	0
4	3	3	3	0
5	4	6	4	0
8	4	7	4	0
15	5(HR)	8(SR)	5(HR)	0

HR Hypersensitive response

SR Susceptible response

* each value is the meanof five results

2 Responses of RM leaves to several species of bacteria at a range of concentrations.

RM leaves were injected with the following bacteria.

a R1

b R2

c Pmp

d P. fluorescens (Pf)

e *Xanthomonas manihotis* (Xm)

f *Erwinia carotovora* var. *atroseptica* (Ea)

Five were used per treatment and incubation was at 25°C in the growth cabinet. The concentrations of the bacterial suspensions were 1.0×10^5; 1.0×10^6; 5.0×10^6; 1.0×10^7; 5.0×10^7; 1.0×10^8; 2.5×10^8; 5.0×10^8; 1.0×10^9 and 1.0×10^{10}, cells/ml as estimated by nephelometry.

a R1

The time before HR appeared was shorter with increase of inoculum concentration between 5.0×10^6 cells/ml and 1.0×10^8 cells/ml with a minimum of 18h. Below 5.0×10^6 cells/ml there was no response visible to the naked eye. Above 1.0×10^8 cells/ml, increasing concentration of bacteria gave no increase in the rapidity of the initial response (18h).

b R2

At all concentrations typical halo blight symptoms appeared. Time before initial symptom expression was shorter with increase of inoculum concentration up to c. 5.0×10^8 cells/ml (48h). Above this there was no increase in rapidity of reaction.

c Pmp

Time of initial response development was shorter with increase of inoculum concentration until 1.0×10^8 cells/ml. Greater concentrations caused no increase in rapidity of reaction (18h). At concentrations below 5.0×10^6 cells/ml there was no macroscopically visible response.

d Pf

Inoculated areas showed no macroscopically visible response at any inoculum concentration.

e Xm

Inoculated areas showed no macroscopically visible response at any inoculum concentration.

f Ea

Inoculated areas showed no macroscopically visible response at inoculum concentrations up to 2.5 x 10^8 cells/ml. At concentrations of 5.0 x 10^8 cells/ml and above some yellowing of the Ia was noted about six days after inoculation. This yellowing was not seen to increase over the remainder of the 15 day observation period and plants otherwise remained healthy.

This experiment was repeated with similar results. From the results of this work it was decided that in subsequent experiments, bacterial suspensions need not exceed concentrations of 10^8 cells/ml. The results are summarised in Table 2.

Table 2. Responses of RM leaves to bacteria at 10^8 cells/ml.

Bacterium	Race 1	Race 2	Pmp	Pf	Xm	Ea
Response	HR	SR	HR	NR	NR	NR

NR no macroscopically visible response.

3 Possible effects on RM plants due to the method of inoculation.

A criticism of the method of inoculation used in this work is that it causes a certain amount of machanical injury to the plant and that this may influence the responses of the plants to the bacteria.

In order to find out more about this, an experiment was carried out to ascertain whether HR or SR was modified by prior injections of sterile water.

RM plants were injected with R1 or R2 at concentrations of 10^8cells/ml after having been previously injected once, twice or three times with sterile water in the same area. After each injection a period of 60 minutes was allowed to elapse to let water-soaking disappear before the next injection was carried out.

Controls were injected once, twice or three times with sterile water before either being injected again with sterile water or with with 10^8 cells/ml dead R1 cells.

There were five plants per treatment and incubation was in the growth cabinet as usual. The experiment was carried out twice with the same results.

With cultures of R2 (24h and 7d old) development of SR was not altered in any way visible to the naked eye by repeated prior injection with water.

With 24h old cultures of R1 prior injection with water once, twice or three times before injection with R1, there was no difference in rate of development of HR from that considered normal and described in 1b above.

With 7d old cultures of R1 onset of HR was delayed by 6h by prior injection with water but only in plants which had been injected three times previously in the same area.

Water and Dead Cell controls showed no macroscopically visible response.

Re-injection became progressively more difficult to carry out and although it may be that some damage is done to the leaf cells by this method or that it induces some form of resistance to HR (but not to SR), the injection-infiltration method did not affect the development of normal HR produced in response to 24h old cultures of R1. All bacterial suspensions used subsequently in this work were from cultures 24h old unless otherwise stated.

4 Effects on bacteria of suspension in sterile water for inoculation.

There are criticisms of using water for suspension of bacteria from nutrient agar slopes because of damage caused by osmoti shock is made at this stage.

In one experiment tubes of nutrient agar in slope form were prepared with 10 ml agar per tube. Each slope had 0.1 ml of 10^8 cells/ml R1 suspension streaked over the surface and tubes wereincubated for 24h at 25°C.

Ten ml of the following were then used to suspend the bacteria in each tube (five tubes per suspension medium):- distilled water, nutrient broth, liquid D4 medium, 0.4 M sucrose, 0.4 M Mannitol. All media were sterilised by autoclaving at 15 lb/in^2 for 15 minutes prior to use.

One ml was removed from each tube and by dilution plating on nutrient agar, the number of viable bacteria per ml of original suspension was calculated. In all cases this was found to be 1.0×10^8 cells/ml.

This process was repeated using Ea and Xm with the same rate of multiplication in 24h and with no effect of suspending medium.

When injected into RM leaves, water, nutrient broth , D4 medium and 0.4M sucrose suspensions produced normal responses from the plants but suspensions in mannitol were found to be extremely toxic to the leaves (as was 0.4 M Mannitol alone).

It was decided to use sterile water for suspensions as it was the most convenient, least toxic and least complex.medium.

5 Use of nutrient agar for growth of bacteria.

The use of nutrient agar for the culture of bacteria pathogenic on plants was thought to be perhaps inappropriate.

For comparison bacteria (all species used) were grown on V8 juice agar (200 ml V8 juice, 25 g Davis Standard Agar, 800 ml water) at pH 7.0 with calcium carbonate added in excess. V8 juice is a mixture of vegetable juices and is often used for growth of plant pathogenic fungi. No differences were found

in responses of RM plants to bacteria grown on V8 agar from those to bacteria grown on nutrient agar. Rate of growth of bacteria on the two media was also the same.

Over a longer period of culture ie more than the normal 24h, bacteria began to lose pathogenicitiy or ability to produce HR. This was also found for R1 and Pmp on RM plants by O'Brien in 1973. On both V8 and nutrient agar bacteria were not used after 3 days of culturing.

It was decided that the use of nutrient agar did not influence development of responses of RM plants to the bacteria and so this medium continued to be used for cultures grown for 24h before use.

6 Specificity of media used in population assays.

During the course of this work, it was found that the media used as selective were not quite so selective as was hoped. This has already been found by other workers for instance Stead (1973) who found that D3 medium was not as specific as Kado and Heskett (1970) claimed. Erwinia spp. on D3 medium produce a red colouration which depends on the species of Erwinia. Ea produces a more intense colour than does for instance E. amylovora. Some saprophytes (eg Escherischia spp.) will grow on D3 but do not produce the red colour; Xanthomonas spp. will also grow on D3 but produce a dark blue colour easily distinguished from the Erwinia red.

In this work it was found that Pseudomonas spp. would not grow on D3 medium. Xm would do so also but the distinctive dark blue colour was easily seen.

D4 medium (supposedly selective for the growth of Pseudomonas spp.) supported the growth of all the bacteria used. However Pseudomonas spp. could easily be identified by the small, round,

opaque white colonies produced in 2 - 3 days of incubation at 25°C. The other bacteria (Eh and Xm) appeared much more slowly.

D5 medium supported the growth of Pseudomonas spp. but not of Erwinia spp. Xanthomonas spp. could be identified because the colonies only became visible after 3 days of incubation at 25°C and were small, round and nearly transparent.

The only real problem arose with D4 medium where it was not possible to tell apart R1,R2,Pmp or Pf. However during the work frequent subcultures were made of colonies on the media and after 24h growth on nutrient agar slopes, the bacteria were suspended in sterile water and injected into RM plants. Responses were noted and helped greatly in identification.

In this way and also by checking colony formation on nutrient agar and by the distinctive smells of the different cultures, it was possible to be reasonably sure of the identity of the bacteria on the selective media plates.

7 Estimation of bacterial populations of leaf surfaces.

In order to estimate the numbers of bacteria washed from leaf surfaces before population assays were carried out, the following experiment was done.

Disks of 1.2cm diameter were cut from RM leaves such that their central area (0.4 x 0.5 cm^2) only was inoculated. Disks were washed in closed flasks on a reciprocal shaker for 20 minutes. There were four disks and 10 ml sterile water per flasks and three flasks per treatment. 0.1 ml was removed from each flask and numbers of bacteria in the original suspension estimated by dilution plating on D4 agar.

This process was carried out at various times after leaf inoculation with 10^8 cells/ml R1 or R2 and gave an indication of the numbers of bacteria on lesion surfaces as the lesions developed.

Control levels (washings of disks cut from plants which were Blank, Injured, injected with Water or Dead Cells) always remained at around 2.5×10^2 bacterial cells per cm^2 leaf disk surface. Water controls were subtracted from the values from inoculated plants to obtain the values in Table 3.

This experiment was repeated with similar results. Levels of both R1 and R2 recovered increased until browning of the inoculated area occurred. In the case of R1 this was at two days and with R2 , three days. After this levels of R1 fell and levels of R2 remained high.

The results indicated well the need to wash leaf disks prior to grinding them up in order to estimate bacterial populatio within the leaves and agreed with the findings of Leben et al., who in 1968 found that HR inducing bacteria on soybean leaf sufaces remained low for 29 days. Bacteria inducing susceptible reaction (blight) were present at much higher levels on the leaf surfaces.

Table 3. Surface colony counts made on disks cut from RM leaves inoculated with R1 or R2 at 10^8 cells/ml (25°C).

Time after inoculation	Log no. bacterial cells/ cm^2 leaf surfac	
	R1	R2
0	* 3.9	3.6
1	4.2	5.9
2	3.6	6.5
3	2.9	5.9
4	2.8	5.2
5	2.9	5.4
6	2.4	5.4
7	2.1	5.4

* Each value is the mean of three results (SE = 2.3) with control value subtracted (mean control value 2.45).

II Growth of bacteria in bean leaves

Using the method described in Materials and Methods IIAa, the growth of R1, R2, Pmp, Pf, Xm and Ea in RM leaves was estimated. Bacteria were injected in suspensions of concentrations 10^6, 10^7 and 10^8 cells/ml prepared by use of nephelometry. For each measurement the numbers of bacteria recovered from control leaves (injected with sterile distilled water) were subtracted from the numbers recovered from inoculated leaves to give the results detailed in Table 4. Levels of bacteria recovered from Control leaves were of the order of 10^3 /cm^2 leaf tissue over the whole period of the experiment. Selective media were used in all cases including controls.

As can be seen from Table 4, R1 bacteria began to mutiply within 24h of inoculation. At initial inoculum concentration (IIC) 10^8 cells/ml, maximum populations were found at 1 - 2 days after inoculation; at IIC 10^7 cells/ml, 2 - 3 days and at IIC 10^6 cells/ml at 3 - 4 days. After this, populations fell slightly and remained stationary until 10 days when the experiment ended.

R2 bacteria also began to multiply rapidly but continued to do so for a longer period. At IIC 10^8 and 10^7 cells/ml the logarithmic phase of growth reached its maximum level four days after inoculation and then a stationary phase developed. At IIC 10^6 cells/ml the maximum population level was reached slightly later (ie 6 - 8 days).

With Pmp at IIC 10^8 and 10^7 cells/ml very little difference in population levels was seen. Levels remained fairly constant over the period of the experiment. At IIC 10^6 cells/ml after an initial fall in population levels, numbers of bacteria recovered increased reaching a peak at three days and then falling again remaining stationary from 5 - 10 days.

With Pf at all IIC's populations fell within the first 24h and then remained stationary over ten days.

Table 4 Growth of bacteria in RM leaves at 25°C.

Time (h)	Bacterium	Initial Inoculum Concentration 10^6 cells/ml	10^7 cells/ml	10^8 cells/ml
0	R1	*3.7	4.3	5.8
24		5.5	5.3	6.8
48		5.0	6.3	7.0
72		6.0	6.3	6.6
144		5.3	5.6	5.3
240		4.9	5.3	5.5
0	R2	4.8	5.5	6.5
10		5.6	6.6	6.9
14		6.4	7.7	7.9
24		7.5	7.7	7.0
48		8.8	8.1	8.6
96		9.2	9.7	9.8
144		9.4	9.4	9.5
192		9.2	8.8	9.2
0	Pmp	5.3	6.1	6.6
24		3.6	6.0	7.0
48		4.3	5.8	6.7
72		6.0	6.0	6.9
120		5.2	-	-
144		4.6	6.0	7.3
240		4.35	6.3	6.6
0	Pf	3.5	4.9	5.6
24		2.6	2.9	4.3
48		3.6	4.3	4.4
144		3.4	4.1	3.9
240		3.9	3.6	4.6
0	Xm	3.9	4.9	5.9
24		3.2	4.8	5.9
48		3.3	5.5	6.4
72		3.4	5.0	5.8
96		3.1	4.5	4.0
144		3.1	4.5	5.7
240		3.0	4.2	5.6

Table 4 Growth of bacteria in RM leaves at 25°C. (contd.)

Time (h)	Bacterium	Initial Inoculum Concentration		
		10^6cells/ml	10^7cells/ml	10^8cells/ml
0	Ea	*4.0	4.9	5.8
24		3.9	4.8	6.0
48		3.8	4.6	6.4
72		3.5	4.8	5.9
96		3.2	4.2	6.0
144		3.2	4.5	5.9
240		3.4	4.4	5.8

* Each value is the mean of three results (SE = 2.3) with control value subtracted (mean control value 3.1).

In the case of Xm at IIC 10^8 cells/ml after a rise in levels of recovered bacteria at two days (48h) the levels fell over the following two days and then began to rise again remaining fairly constant until the experiment ended. At IIC's 10^7 and 10^6 cells per ml recovered bacteria remained fairly constant although in general over the ten day period, numbers fell.

In the case of Ea levels of bacteria remained steady over the sampling period at all IIC's.

This experiment was repeated with similar results.

Populations of bacteria recovered from inoculated leaves reflect the visible responses of the leaves. Inhibition of growth of R1, R2 and P mp occurred at the same time as tissue browning. With R1 and Pmp, IIC 10^6 cells/ml was not sufficient to cause a visible HR but populations of the bacteria increased to levels sufficient (in the cases of IIC 10^7 and 10^8 cells/ml) to cause HR. The difference is in the time at which these levels are reached.

The drop in levels of bacteria from IIC 10^8 cells/ml Xm inoculated plants could not be explained by visible symptoms and although in the case of Ea quite high levels of bacteria could be recovered during the course of the experiment, no symptoms were visible in the Ia.

III Responses of RM disks to bacteria

1 In an effort to simplify the system being studied the responses of leaf disks to the bacteria were studied in (a) humid boxes and (b) disks floating on water.

a Leaves were injected with known concentrations of bacterial suspensions in areas 0.4 x 0.5 cm^2 as described in Materials and Methods IIAb. After one hour of incubation at 25°C to allow water soaking to disappear, disks of 1.2 cm diameter were

cut such that the inoculated area was in the centre. Nine disks were used per treatment and were placed on dry microscope slides in closed transparent plastic boxes lined with wet tissue paper and incubated at 25°C in the growth cabinet.

1 Response to Pmp

Suspensions of 10^6, 10^7, 10^8 and 10^9 cells/ml Pmp prepared using the nephelometer were used. Disks were examined over a period of 25 days and the responses compared with those of whole plants. Controls (Water, Blank, Injured, Dead Cell) showed no response visible to the naked eye, remaining green and turgid until 18 days when chlorosis and senescence in general began to be visible. By 25 days controls were yellow with some brow patches but did not show visible signs of infection.

Because of the long period of survival of the disks it was felt that it would be possible to use this system for experimentation.

Disks inoculated with 10^6 cells/ml Pmp responded as controls. Disks inoculated with 10^7 cells/ml Pmp responded visibly first at 17h when shiny patches were seen on the lower surfaces of the disks, directly under the Ia. At 48h lower surfaces appeared slightly bronzed and over the next few days this gradually darkened but the desiccation and confluent necrosis seen in the whole plant controls did not occur and was perhaps not to be expected because of the humidity. (Young, 1974 showed that responses of plants could be altered by use of high humidity)

At concentrations 10^8 and 10^9 cells/ml the responses were very similar. At 14h shiny patches were seen on the lower surface of the Ia. At 17h bronzing was seen of this area and this darkened gradually until at 48h the first response on the upper disk surface was seen. The upper Ia was dark brown in colour.

and remained so over the whole of the rest of the experiment with no change. None of the desiccation and confluent necrosis of the whole plant controls was seen and fungal infection was always visible by 25 days.

When compared with the responses of whole leaves, it was noted that the times of initial responses in disks and leaves were similar but development of the response was different.

2 Assessment of the importance of surrounding healthy tissue.

Disks were either 1.0 cm in diameter and cut such that their whole area had been inoculated or were 1.2 cm diameter and cut such that only the central 0.4 x 0.5 cm^2 area had been inoculated. Plants were first inoculated with either Pmp or R1 at 10^8 cells/ml and disks were cut after one hour of incubation at 25°C to allow watersoaking to disappear. Conditions were as described above with nine disks per treatment. Controls all remained green and healthy with no sign of senescence until 18 days and were Water, Blank, Injured or Dead Cell as before.

With fully inoculated disks in the case of both R1 and Pmp the tissue became soft and watersoaked and could be easily pulled apart within three days. There were always signs of fungal infection at this stage and it was not clear whether tissue degradation was due to the action of the bacteria or to the action of the secondary infecting fungi.

With disks inoculated only in the central 0.4 x 0.5 cm^2 area, the responses to R1 and Pmp were as described for 10^8 and 10^9 cells/ml Pmp above (IIIa1). These responses were much more comparable with HR in whole plant controls than was the response of disks which were inoculated throughout their whole volume.

This work seems to indicate an involvement or surrounding healthy tissue in the browning response seen following injection

with R1 and Pmp.

When the experiment was repeated disks of 1.2cm diameter inoculated throughout their whole volume were used in addition to disks of 1 cm diameter. The results with the larger disks were the same as those recorded above for the smaller disks ie when the disks were fully inoculated, no browning of the tissue developed.

3 Response to R2

When disks were cut such that their whole volume was inoculated with 10^8 cells/ml R2, tissue degradation occurred by three days (72h) and fungal infection was also noted. Controls(Water, Blank, Injured and Dead Cell) were not affected in this way when incubated in the same conditions. Such disks remained green and healthy for 18 days.

If disks of 1.2cm diameter were cut such that only the central 0.4 x 0.5 cm^2 area was inoculated, responses were similar to those in whole plant controls. The Ia became (at 72h) darker green than the surrounding tissue and remained so for the whole of the experiment. At 96h the beginning of a chlorotic halo around the Ia was visible and this gradually extended until reaching the disk edges. Although similar to the response of whole leaves to R2, the response in disks developed slightly more slowly. Again there was the implication of the importance of surrounding healthy (uninoculated) tissue.

b) Floating on water

In this series of experiments, leaf disks were floated on sterile water in petri dishes, 20 ml water and five disks per dish. There were three dishes per treatment. All disks were cut one hour after inoculation of the leaves and some disks (1 cm in diameter) were cut such that the whole of their

volume was inoculated ; others (1.2 cm in diameter) were cut such that only the central (0.4 x 0.5 cm^2) was inoculated. Incubation at 25°C was in the growth cabinet with 16h light per day.

Half of the number of disks were floated adaxial surface facing upwards and half were floated abaxial surface uppermost. Controls were injected with sterile water or dead cells or were injured or not treated at all.

1 Plants were injected with R1, R2, Pmp or Pf at concentrations of 10^6, 10^7, 10^8 or 10^9 cells/ml. Disks were cut as described above and floated on sterile water. Examination took place over a period of 10 days.

Controls all remained green and turgid for six days. Slight chlorosis was generally observed on day 7 but at day 10 disks were still mainly green with the cut edges showing slight browning Because of the length of time that control disks appeared to remain healthy, it was thought that it would be reasonable to set up a series of experiments based on this system.

A Disks completely inoculated.

With R1 and Pmp disks (1 cm diameter) remained green and turgid until 7 days when some slight chlorosis was seen at all concentrations of suspension used. By 10 days all disks were soft and showed signs of secondary infection by fungi.

In the case of R2, disks remained green and turgid until day 3. From then on disks progressively became softer and more water-soaked although still being green in colour at day 10. This applied to all concentrations of suspension used.

With Pf at all inoculum concentrations disks responded in a similar way to controls. They remained green and turgid until day 7 when slight chlorosis became noticeable. At day 10 disks were still mainly green with the cut edges only showing

some browning.

B Disks inoculated only in the central area.

Disk diameter was 1.2 cm with the inoculated area (Ia) being 0.4 x 0.5 cm^2 in the disk centre.

With R1 and Pmp at 10^8 and 10^9 cells/ml the Ia's could be distinguished as being slightly darker green than the rest of the disk at 22h. By 48h Ia's were slightly bronzed and in the following three days this darkened and the Ia became very soft. The uninoculated tissue did not show any response, remaining green and turgid. The cut edges of the disks showed slight browning as seen in controls and probably due to injuries to the cells at the time of disk cutting.

The soft dark brown tissue in the Ia at this point closely resembled grade 5 HR tissue (from attached leaves) which had been floated on water for 24h. Because of this, the disk Ia tissue was referred to as being "wet-necrotic" rather than the desiccated necrotic tissue seen in attached leaves.

At concentrations of R1 of Pmp below 10^7 cells/ml no response was seen. At concentrations below 10^8 cells/ml no wet-necrosis was seen, the response being limited to a slight bronzing of the Ia.

In the case of R2 at concentrations of 10^8 and 10^9 cells/ml a response in the Ia was seen at three days. At inoculum concentrations of 10^7 and 10^6 cells/ml the time of development of the first response was four and five days respectively. In all cases the Ia became dark green and soft. A halo of chlorotic tissue noted in the Ia began to spread outwards but did not in any case reach the disk edges by ten days. The soft Ia tissue resembled grade 7 SR tissue (taken from leaves inoculated with R2) which had been floated on water for 24h.

In all cases at all concentrations of inoculum, leaf disks showed no macroscopically visible responses to Pf. Disks responded exactly as controls.

In no case did the side on which leaf disks were floated make any difference to the response seen. As a result in later experiments all disks were floated upper surface upwards.

In disks in which there is some uninoculated tissue around the Ia, responses to R1, Pmp, R2 and Pf are largely similar to the responses of whole plants although disiccation never occurs probably due to the tissues of the disks being in contact with water.

In disks which are fully inoculated the responses to R1, Pmp and R2 are different from responses of whole plants. Injection with these bacteria over the whole disk area seems to facilitate the degradation and infection of the disks by fungi and possibly other bacteria. The softening of the disks does not seem to be a response normally seen in leaf tissue to these bacteria but maybe under the experimental conditions used here, the bacteria could produce this effect. In the case of Pf no disk softening or invasion by other organisms was seen. Perhaps the effect is limited to pathogenic bacteria.

These experiments again suggest that there is a necessity for the presence of uninoculated tissue around the Ia for HR to develop in the Ia.

2 A further experiment was carried using R1 and Pmp at 10^9 cells per ml to find out if responses were affected by the time of disk cutting with respect to inoculation. Disks were floated on water in petri dishes as described previously.

a Disks fully inoculated

Disks were 1 cm in diameter and cut from inoculated leaves such that their whole volume was inoculated. If the disks were

cut before water soaking had disappeared, both control disks and those inoculated with bacteria sank below the surface of the water and remained dark green and water-soaked for 15 days. The tissue became very soft.

If the disks were cut from leaves before signs of HR were visible but after disappearance of water-soaking the responses were as described previously in b1A above.

If disks were cut after bronzing had been seen (around 48h following leaf inoculation) the disks remained bronzed for five days on the water and showed signs of fungal infection after this. Even at this stage the development of wet-necrosis seems to require the presence of uninoculated tissue around the Ia.

b Disks inoculated in central area only.

Disks were 1.2 cm in diameter and were cut such that the central area(0.4 x 0.5 cm^2) only was inoculated.

If the disks were cut before watersoaking had disappeared the Ia remained water-soaked and no symptoms developed that were visible to the naked eye. Control and inoculated disks responded similarly.

If disks were cut before signs of HR were seen but after the disappearance of water-soaking, wet-necrosis developed normally in the Ia in response to R1 and Pmp. Control disks showed no symptoms.

If disks were cut after bronzing had been seen, wet-necrosis of the Ia in response to R1 and Pmp developed normally with no sign of response in control disks.

Time of cutting of disks inoculated in the central area only made little difference to responses to R1 and Pmp as long as disks were cut after water-soaking had disappeared.

3 The effect of light on disk response.

Disks inoculated fully (1 cm diameter) or in the central area only (1.2 cm diameter) with R1 or R2 at 10^9 cells/ml were incubated in boxes or floating on water as described previously.

Some treatments were incubated with 16L:8D light regime in the growth cabinet at 25°C ; other treatments were incubated at 25°C in the growth cabinet but boxes or dishes were wrapped in aluminium foil. Controls were inoculated with water or dead R1 or R2 cells, were injured or not treated at all.

Disks were examined at intervals and care was taken to ensure that exposure to light of dark-treated disks at these times was as little as possible.

In all cases disks incubated in darkness (including control disks) were much more liable to secondary infection by fungi. With disks floated on water, no results were available at all because of this but disks in boxes did not show signs of infection until five days after the experiment began. From these it was possible to see that responses in light - treated disks and dark - treated disks were very similar over a four day period following inoculation.

Light would seem therefore to have little effect on macroscopic responses of RM leaf disks to R1 and R2 although lack of light facilitates secondary infection of the disks.

These experiments suggested that the disk method could probably be used as a simplified system to investigate HR and SR and that it would be worth further investigation.

The experiments in this section have all been repeated with similar results.

IV Growth of bacteria in RM leaf disks

a Disks in boxes

Disks were cut from leaves one hour after inoculation with 10^6, 10^7 or 10^8 cells/ml Pmp. The disks were 1.2 cm in diameter and were cut such that the central (0.4 x 0.5 cm^2) area only was inoculated.

Growth of Pmp in the disks which were incubated in boxes as described previously was estimated by grinding up the disks in 0.25 strength Ringer's solution followed by dilution plating on D4 medium. This process is described fully in Materials and Methods IIAb. Each sample consisted of nine disks and there were four replicates of each sample.

Control population numbers (never more than 10^3 bacteria per cm^2 leaf tissue were subtracted from the numbers obtained from inoculated disks to give the figures in Table 5.

In all cases multiplication of bacteria increased until a stationary phase 3 - 5 days after inoculation. This is unlike the pattern of growth of Pmp in attached leaves where very little difference in population levels was seen over a seven day period. However it is again noticeable that although in disks inoculated with 10^6 cells/ml Pmp, populations of Pmp reached levels high enough to produce a browning response with disks inoculated with 10^7 or 10^8 cells/ml Pmp, no response was obtained. The stationary phase noted with disks inoculated with 10^7 or 10^8 cells/ml P mp began at the onset of the browning response.

b Disks floating on water

Disks were cut from RM leaves one hour after inoculation with R1 or R2 at 10^8 cells/ml. The disks were 1 cm in diameter and cut so that their whole area was inoculated or were 1.2 cm in diameter and cut such that only the central area (0.4 x 0.5 cm^2) was inoculated. There were five disks and 20 ml sterile water per dish and four dishes per treatment. Petri dishes

Table 5 Growth of Pmp in disks (1.2 cm diameter) incubated in boxes at 25°C.

Time (d) after inoculation	Log concentration bacteria/cm^2 leaf disk tissue		
	Original inoculum concentration		
	10^6cells/ml	10^7cells/ml	10^8cells/ml
0	*3.1	4.5	5.2
1	5.5	5.6	5.0
2	5.6	6.3**	6.2**
3	5.8	6.2	6.7
5	5.8	6.5	6.8
7	5.8	6.8	6.6

* Each value is the mean of four results (SE = 2.1) with control values subtracted (mean control = 2.9).

** Time of appearance of browning response in Ia.

were incubated at 25°C in the growth cabinet and bacterial populations were estimated at intervals over a seven day period. This was done by dilution plating on D4 medium as described before. Control disks were Blank, Water, Injured or Dead Cell as in previous experiments.

Bacterial populations in the water on which the leaf disks floated were also estimated and results are presented in Table 6. These are however rather misleading. It would appear from Table 6 that numbers of Pseudomonas spp. in control disks are not very different from those in inoculated disks. This needs some clarification. In dilution plates made from disks inoculated with R1 or R2, colonies on D4 agar in almost all cases appeared identical and subculturing from the plates followed by injection at 10^8 cells/ml into RM leaves resulted in HR and halo blight symptoms respectively. In dilution plates from control disks colonies were of at least six different types. The numbers of those resembling R1 or R2 were always low (around $10^3/cm^2$). Six different colony type bacteria and one colony of yeast-like organisms were isolated from control plates and injected into RM leaves at 10^8cells/ml. In no case was there any response from the tissue. It was concluded that these bacteria were saprophytic or at least non-pathogenic on RM leaves. The bacteria may have originated from the phyllosphere and the general senescence of the disks may have enabled them to multiply. It would also seem from these results that the presence of R1 and R2 to a large extent limits the ability of any other bacteria present in small numbers to multiply. Colonies isolated from dilution plates made from disks inoculated with R1 or R2 induced HR or SR respectively in RM leaves.

The previous notes can also be applied to the population levels of bacteria in the water on which the disks floated. In

the case of control plates most colonies seen on D4 agar were unlike R1 or R2 in appearance and did not produce HR or SR when injected into RM leaves. The colonies resembling R1 or R2 gave no response either. In the case of plates made from the water on which floated disks inoculated with bacteria (R1 or R2) the colonies gave the correct response in RM leaves(HR or SR).

A Disks fully inoculated (Table 6)

In these disks (1 cm diameter) levels of R1 increased initially more rapidly than R2 but R2 reached higher levels. Stationary growth phases were reached by R1 at day 2 and by R2 at day 3.

This is similar to the situation in attached leaves where R1 reaches a maximum level before R2 but R2 reaches a higher maximum level. However in attached leaves R2 multiplies to a much greater extent (10^9 cells/cm^2) than in disks ($10^7/cm^2$). Numbers of R1 are approximately similar but it must be remembered that the responses are visually very different. In disks fully inoculated/ R1 has no effect above controls and R2 produces a softening response quite different from the response of whole leaves.

Numbers of bacteria in the water on which disks floated seemed more or less to reflect the situation in the disks. Levels of R1 and R2 reached maxima at day 3 and then remained stationary.

B Disks inoculated only in the central area (Table 6)

In these disks (1.2 cm diameter with la 0.4 x 0.5 cm^2) R1 again began to multiply more rapidly than R2 and reached a stationary phase at day 3 - slightly later than in (A). R2 continued to multiply until day 5 when numbers dropped slightly. This is again very similar to the situation in attached leaves. However the stationary phase in disks was reached later than in leaves.

Table 6 Growth of Pseudomonas spp. in leaf disks and in the water on which the disks floated.

A 1 cm diameter disks (fully *inoculated)

Time (d) after inoculation	Water control		R1 inoculated		R2 inoculated	
	Disk	Water	Disk	Water	Disk	Water
0	0	0	3.3	0	3.3	0
1	a 4.0	b 2.0	5.3	3.7	4.1	1.7
2	4.0	3.0	6.6	4.7	5.9	4.6
3	5.8	5.6	6.3	5.6	6.9	6.1
4	5.3	5.3	5.5	4.9	6.2	5.8
5	5.1	5.4	6.7	5.4	6.8	6.4
6	-	-	-	-	7.2	6.1
7	4.6	5.1	6.6	5.8	5.3	4.6

B 1.2 cm diameter disks (inoculated*in central area only)

Time (d) after inoculation	Water control		R1 inoculated		R2 inoculated	
	Disk	Water	Disk	Water	Disk	Water
0	a 2.1	0	3.4	1.1	2.9	0
1	2.6	0	5.4	4.5	4.4	4.3
2	5.3	b 5.0	5.8	5.1	5.5	5.8
3	5.3	5.7	6.3	5.9	5.9	5.4
4	5.0	4.4	6.3	5.6	6.3	5.1
5	3.9	3.0	6.3	5.1	7.4	5.8
6	4.9	4.2	5.9	5.4	6.3	-
7	5.6	4.6	6.8	5.8	6.6	6.8

* inoculation of disks was at 10^9 cells/ml.

a Each figure is the log concentration of the number of bacteria per cm^2 leaf disk tissue. SE = 1.9 Four replicates.

b Each figure is the log concentration of the number of bacteria per ml water. SE = 2.1 Four replicates.

Bacterial numbers in water on which floated disks inoculated with R1 or R2 increased rapidly in the first day of incubation reaching stationary phases at 2-3 days - very little difference from the patterns observed in water in (A).

From these results it can be concluded that as in attached leaves, mutiplication of R1 ceases in partly inoculated disks at the onset of surface browning of the disk cells; multiplication of R2 in partly inoculated disks ceases around the time of initial halo development and softening of the Ia and multiplication of R2 in fully inoculated disks ceases around the onset of tissue softening.

The cessation of multiplication of R1 in fully inoculated disks cannot be related to visible symptoms. There is no confluent necrosis of the HR type nor is there any tissue softening . In disks, therefore, confluent necrosis or tissue browning are not the only factors preventing further increase in numbers of R1.

These experiments have all been repeated with similar results.

V Floating of plant material on suspensions of bacteria.

1 Responses of RM leaf disks floating on suspensions of bacteria.

Disks of 1.2 cm diameter were cut from RM leaves as described in Materials and Methods IA5 and floated on 18 ml sterile water in petri dishes, four disks per dish. To the water in each dish was then added 2 ml of bacterial suspension (in sterile water) of known concentration such that the final bacterial suspension concentration in each dish was 10^8 cells/ml. Bacteria used were R1, R2, Pmp, Xm, Ea and Pf. There were five dishes per treatment and incubation was in the growth cabinet at 25°C. Controls consisted of disks floating on sterile water or on suspensions of

dead bacterial cells.

In these conditions control disks remained green and healthy until day 7 when slight chlorosis was seen. By day 11 disks were still mostly green with browning at the damaged cut edges but showed no signs of infection.

Disks floating on suspensions of R1, Pmp, Xm, Ea and Pf responded exactly as the controls did.

In the case of R2 at day 3 the edges of the disks were showing chlorosis. That is to say there was a band of chlorotic tissue approximately 2mm wide running around the circumference of each disk. By day 5 the band had become wider extending towards the disk centre and softening of the tissue at the disk edges was obvious. By day 11 the whole of each disk was soft and yellow in colour. It was not clear whether the softening was due to R2 or some other organism as this is not a typical symptom of R2 infection of bean leaves. However it was thought that the movement of the ring of chlorosis inwards towards the centre of the disks was very similar to the movement of the chlorotic halo outwards from the Ia in attached leaves. The softening of the tissue could have been the result of the extreme conditions of the experiment and caused by R2 or could have been the result of secondary infection of the chlorotic tissue by other organisms.

This experiment has been repeated with similar results.

2 The effect of epidermis removal

The experimental proceedure used here was exactly as in V1 above except that pieces of lower epidermis (approximate area 0.4 x 0.5 cm^2) were torn from RM leaves using a pair of fine forceps before disks were cut. The exposed tissue was in the centre of the disks which were floated lower side downwards on the bacterial suspensions.

The removal of pieces of epidermis had no effect on the responses of control disks or of disks floating on suspensions of R1, Pmp, Xm, Ea or Pf. Disks remained green and turgid for about ten days with no sign of infection. Disk edges and tissue exposed by removal of epidermis became slightly brown in all cases in five days.

In the case of disks floating on suspensions of R2 the removal of epidermis seemed to facilitate the degradation of the disks. Chlorosis by day 5 had covered the whole of the disk area, this time originating from both the cut edges and from the area above the site of epidermis removal.

This experiment was repeated three times with similar results.

3 Influence of RM leaf age on the response of leaf disks to R1 and R2.

In this experiment disks were cut from plants of ages 3, 6, 9 or 12 days (ie number of days after emergence from the soil) and from the first trifoliate leaves (14 days after emergence from the soil).

Disks were 1.2 cm in diameter and were floated on suspensions (in sterile water) of 10^8 cells/ml R1 or R2 in petri dishes with four disks and 20 ml suspension per dish. There were five dishes per treatment.

Control disks cut from plants of the same ages as above were floated on 20 ml of 10^8 cells/ml of dead R1 or R2 cells. Dishes were incubated at 25°C in the growth cabinet and were examined over a period of 15 days.

All control disks began to show slight chlorosis at about ten days the first time the experiment was carried and at about eight days during the repeat experiment. There was no obvious reason for this difference.

As regards disks floating on suspensions of R1 or R2 the age of the leaf from which the disk was cut and whether the leaf was monofoliate or trifoliate had no noticeable influence or the disk responses to the bacteria. Disks floating on R1 remained green and healthy in appearance until around 10 days when signs of senescence were seen. Disks floating on R2 showed the development of a chlorotic band around the disk edges at around the disk edges at around five days.

The experiment was repeated with similar results.

4 Influence of light on RM leaf disks floating on suspensions of bacteria.

Disks (1.2 cm diameter) were cut from RM leaves and floated on 20 ml 10^8 cells/ml R1 or R2 in petri dishes, four disks per dish. Some dishes were left uncovered, some were covered with aluminium foil such that halfof the lid area was exposed to light; others were covered such that quarter of the lid area was exposed to light and still others were completely covered.

Controls were disks floating on 20 ml sterile water or 20 ml 10^8 cells/ml dead R1 or R2 cells and control dishes were covered as above.

Petri dishes were incubated in the growth cabinet at 25°C and 16L:8D light regime. There were five replicates of each treatment and disks were examined at intervals. Disks in light deprived conditions were exposed to light for as little time as possible during examination.

It was found that in complete darkness and with quarter exposed conditions all disks (including controls) were liable to become infected with bacteria or fungi. Results from these dishes could not be considered.

In dishes half exposed to light, control disks over a period

of twelve days did not become infected obviously. Senescence was detected at 2 - 3 days earlier than in fully exposed dishes (seven days as opposed to twelve). It was thought reasonable to consider the results from these dishes.

It was found that the slight deprivation of light in half exposed dishes had little effect on responses of disks to R1 and R2 beyond the earlier onset of senescence already noted.

5 Growth of bacteria in disks floating on suspensions of bacteria and in the suspensions themselves.

Disks of 1.2 cm diameter were cut from RM leaves, well washed in sterile water and dried in a stream of sterile air before floating on suspensions of bacteria, of dead bacteria (control), or of sterile water (control). If disks are not surface dried properly before floating , then they sink below the surface of the liquid almost immediately. Bacteria (R1 and R2) were at concentrations of (a) 10^6 cells/ml, (b) 10^7 cells/ml and (c) 10^8 cells/ml There were four disks and 20 ml liquid per petri dish and five dishes per sample. At intervals dishes were removed from incubation (in the growth cabinet at 25°C) and 1 ml of bacterial suspension or water removed from each dish. By dilution plating the number of bacteria per ml was estimated. At the same time the disks were removed, washed in sterile water, ground in 0.25 strength Ringer's solution and by dilution plating the number of bacteria per cm^2 leaf tissue estimated. D4 agar was used for this purpose.

The results are presented in Table 7 but they are perhaps rather misleading. It appears from Table 7, especially the sections involving inoculum concentration 10^6 cells/ml, that levels of Pseudomonas spp. in control disks and in water are not very much different from levels in disks floating on bacterial

suspensions. This situation is very similar to that described in IVb above. In dilution plates made from disks floating on R1 or R2 colonies on D4 agar appeared identical in almost all cases and subculturing from the plates followed by injection at 10^8 cells per ml into RM leaves resulted in HR and halo blight symptoms respectively. In dilution plates from control disks, 14 different colony types in all were observed. Levels of those resembling R1 or R2 were very low (c. $10^3/cm^2$ in each case). The 14 different colony type bacteria were isolated and injected into RM leaves. In no case was there any response from the leaves. It was concluded that these bacteria were saprophytic or at least non pathogenic on RM leaves. They may have originated from the leaf surfaces or intercellular spaces and the general senescence of the disks may have enabled them to multiply. It would also seem from these results that the presence of R1 or R2 to a large extent limits the increase in population of any other bacteria present in small numbers.

This can also be applied to the bacterial suspensions or water on which the disks floated. In the case of controls, most colonies were unlike R1 or R2 in appearance and did not produce any visible response on injection into RM leaves. Colonies resembling R1 or R2 gave no visible responses either.

a) Inoculum level 10^6 cells/ml

1 disks

Levels of R1 and R2 obtained from disks were from day one consistently high, R2 levels remaining higher than R1.

2 suspensions

Levels of R2 recovered dropped between day 2 and day 4 not rising to levels above controls until 5 - 8 days. Levels recovered then remained fairly steady. Numbers of R1 remained fairly steady over the period of the experiment.

Table 7 Growth of bacteria in RM disks floating on suspensions of bacteria and in the suspensions themselves.

a) disks

Time (d) after inoculation	Control (water)*	$^{a}10^{6}$ cells/ml		$^{a}10^{7}$ cells/ml		$^{a}10^{8}$ cells/ml	
		R1	R2	R1	R2	R1	R2
0	**3.0	3.1	2.9	3.0	3.3	3.5	2.9
1	5.6	6.5	6.9	6.8	7.1	6.9	7.0
3	5.8	5.8	6.5	7.0	7.1	7.1	7.3
5	5.8	6.5	6.9	6.7	7.2	7.0	7.3
8	5.3	6.5	7.3	6.9	7.3	7.2	7.6
10	6.0	6.8	7.3	7.2	7.3	7.3	7.0
14	6.5	7.0	7.6	7.6	7.3	7.6	7.0

b) suspensions

Time (d) after inoculation	Control (water)*	$^{a}10^{6}$ cells/ml		$^{a}10^{7}$ cells/ml		$^{a}10^{8}$ cells/ml	
		R1	R2	R1	R2	R1	R2
0	**0.1	6.2	6.2	7.3	7.3	8.1	8.2
1	6.0	6.3	6.4	7.1	7.3	8.3	8.3
3	6.2	6.3	5.0	7.2	7.3	8.3	8.4
5	6.0	6.5	5.0	7.2	7.4	8.5	8.6
8	6.2	-	6.2	7.5	7.5	8.2	8.6
10	6.2	6.8	6.3	7.6	7.5	8.6	8.8
14	6.5	6.3	6.5	7.9	7.9	8.8	8.9

a Initial bacterial concentration in suspension.

** Each value is the mean of five results (SE = 2.5) and is the log concentration of bacteria per cm^2 leaf tissue (in a) and per ml suspension (in b).

* Water control results were not significantly different from Dead Cell control results

b) Inoculum level 10^7 cells/ml

1 disks

Over the four to ten day period, levels of R2 recovered from the disks, remained slightly higher then levels of R1 recovered. At day 14 numbers of R2 dropped below those of R1 perhaps because disks inoculated with R2 had almost completely disintegrated by this time. In other words the general level of nutrients may have fallen off.

Levels of R1 recovered increased consistently over the 5 - 14 day period possibly due to the slow senescence of the disks increasing the general level of nutrients available and allowing R1 bacteria to grow in the disks.

2 suspensions

In the bacterial suspension, numbers of R1 and R2 recovered remained fairly similar (with R1 levels slightly lower) over the 14 day period. The drop in levels seen in (a) of R2 recovered was not noted here.

It is interesting that despite the different responses of disks to R1 and R2, there was very little difference in populations in the suspensions over 14 days.

c) Inoculum level 10^8 cells/ml

In disks and suspensions, numbers of bacteria recovered took the same pattern as in (b) above.

These experiments have all been repeated with similar results.

VI Physiological changes in the host as measured by electrolyte losses.

a Leaves

A Electrolyte losses

1 RM plants were inoculated in the usual Ia zone with 10^8 cells /ml R1 or R2 and disks were cut from the Ia or Ih zones (see Figure 1, p 22). Measurements of electrolyte loss were made as described in Materials and Methods IIIa over a period of 8 days. The results are presented in Table 8. All control results (Blank, Water, Injured and Dead Cell) were similar and did not vary from 20 - 40μ mhos over the course of the experiment. Control results are not presented in Table 8 but the results from the water controls were subtracted in each case from the "inoculated" results to give the final results.

With R1 treated plants in the Ia zone there was an early rise in electrolyte losses at 2h but with R2 there was no rise until 7h. Another peak in electrolyte loss occurred in R1 and R2 Ia's at 72h, R2 treated plants showing higher levels of electrolyte loss than R1 treated leaves.

In the Ih zone of leaves inoculated with R1 there was very little difference from control values over 192h although very small peaks were seen at 2h and 48 - 72h.

With R2 the elctrolyte loss from disks cut from the Ih zone was very little different from the control results until the chlorotic halo began to spread into this zone. This happened at 120h and a rapid increase in electrolyte loss began then becoming even greater over the next few days as the tissue in zone Ih became yellow, softened and then brown but not desiccated.

The development of HR, therefore seemed to have little effect on the leakage of electrolytes from the disks cut from the healthy tissue of zone Ih. However as the susceptible

Table 8 Measurements of electrolyte loss from disks cut from RM leaves inoculated with R1 or R2 at 25°C

Time(h) after inoculation	Ia zone		Ih zone	
	R1	R2	R1	R2
0.25	*2.0μmhos	6.5μmhos	5.0μmhos	15.0μmhos
1.5	0.5	-	6.0	-
2.0	21.0	3.5	11.5	-
3.0	15.0	0	1.5	3.0
5.0	19.5	4.0	4.0	3.0
7.0	25.0	24.0	1.3	2.9
13.0	69.0	31.5	5.5	4.0
16.0	47.5	-	2.5	4.3
17.5	59.0	36.3	[a] -5.0	4.5
24.0	54.5	61.5	-4.0	6.0
48.0	60.0	79.3	12.0	3.5
72.0	81.3	104.3	12.0	0.5
96.0	-	75.0	-	8.5
120.0	-	85.0	-	24.0
144.0	55.0	65.0	1.8	81.5
168.0	-	67.5	-	121.0
192.0	60.3	72.5	12.5	205.0

* Each value is the mean of three replicates (SE = 5μmhos for Ia results; SE = 5.5 mhos for Ih results) with control results (mean 30μmhos) subtracted after three hours incubation.

[a] Negative results had values less than controls.

Table 9 Measurements of electrolyte leakage from disks (in three hours) cut from Rm plants inoculated with R1 at 25°C.

Time (h) after inoculation	Inoculum concentration of R1 suspension		
	10^6 cells/ml	10^7 cells/ml	10^8 cells/ml
2	*22.0μmhos	[a] -2.0μmhos	-8.5μmhos
12	68.0	-	-
17.5	56.0	4.0	-2.0
24	52.0	53.0	-8.0
48	60.0	47.5	-6.0
72	80.0	71.5	-
120	-	45.5	-0.5

(contd)

Table 9 (contd).

Time(h) after inoculation	Inoculum concentration of R1 suspension 10^6cells/ml	10^7cells/ml	10^8cells/ml
144	*55.0 μmhos	-	-
168	-	-	[a]-0.5
192	59.5	53.0	-

* Each value is the mean of three replicates (SE = 6.5 for 10^8 cells/ml; 5.5 for 10^7 cells/ml and 5.0 for 10^6 cells/ml) with control results subtracted. Mean control value was 30μmhos.

[a] Negative results had values less than controls.

Table 10 Measurements of electrolyte losses from disks (in three hours) cut from RM plants inoculated with Pmp, Pf, Xm or Ea at 10^8 cells/ml at 25°C.

Time (h) after inoculation	Bacterium	Pmp	Pf	Xm	Ea
		All values in μmhos			
1		*[a]-10.0	-	8.0	8.0
2		-18.0	-4.0	-	-
24		40.0	-19.5	19.0	0.5
48		35.0	-8.0	18.5	9.5
72		46.5	8.0	21.3	11.3
96		-	0.5	30.0	15.5
120		49.5	-	30.0	22.5
144		-	-	29.5	22.0
168		42.0	-6.5	11.3	11.3
192		47.0	-	-	-
216		27.5	-	-	-
SE		8.5	9.0	6.5	6.0

* All values are means of three results with water control results subtracted (mean control = 30μmhos).

[a] Negative values were less than control values.

reaction developed there was a marked and rapid increase in electrolyte loss from zone Ib disks. This experiment was repeated with similar results.

2 As part of the same experiment, RM plants inoculated with R1 at 10^7 and 10^6 cells/ml were examined for electrolyte loss patterns. Results are presented as before with water control results (mean 30μmhos) subtracted in Table 9, page 79 - 80.

No early peak (as in R1 at 10^8 cells/ml at 2h) was detected in electrolyte leakage from disks cut from plants inoculated with R1 at 10^7 cells/ml. A rapid rise between 20 and 24h was found. It can also be seen from Table 9 that with inoculum concentration at 10^6 cells/ml there were no significant differences from control values.

With 10^7 cells/ml, the period of rapid release of electrolytes was also the period of tissue collapse. With 10^6 cells/ml there was no tissue collapse and no rises in electrolyte loss.

3 RM plants were inoculated with 10^8 cells/ml of Pmp, Pf, Xm and Ea and at intervals disks were cut from the Ia and measurements of electrolyte leakage made over three hours incubation at 25°C. Results are presented in Table 10, page 80, with control (water) results subtracted as before. Control results did not vary much over the period of the experiment and were always 20 - 40 μmhos.

With Pmp there was a rise in electrolyte losses within 24h of inoculation. There was another increase at 72h when losses became more or less stationary. This is broadly similar to the pattern observed with disks cut from leaves inoculated with R1.

With Pf very little difference was noted in electrolyte losses over 168h compared with controls. However at 24h in this experiment there was a drop in losses to levels well below control

values. When the experiment was repeated, however, this drop in losses was not so great and was around 10μmhos.

With disks cut from plants inoculated with Xm there was a rise in electrolyte losses within 24h. This increased again to a plateau at 96 - 120h and after 144h the losses decreased again.

In the case of Ea after a slight drop in electrolyte loss in the first 24h following inoculation, a gradual rise in losses was noted until 120h when the levels began to fall again.

This experiment was repeated with similar results.

It can be seen from the results of this and previous experiments that large, rapid increases in electrolyte losses can be linked with visible symptoms ie tissue collapse in the case of R1, Pmp and R2 and the spread of chlorosis into the Ih zone of leaves inoculated with R2. Smaller electrolyte loss increases and decreases could not be related to macroscopic observations but the early increases (at 2h with R1 and 7 - 8h with R2) are probably are important in the development of HR and SR. No explanation was obvious for the rises noted with the incompatible Xm and Ea and although in each case no symptoms developed, the electrolyte loss pattern was very different from that seen in response to Pf, the saprophyte.

B Measurement of rate of electrolyte losses from disks cut from inoculated leaves.

As described in Materials and Methods IIIa2, the experiments in section A above have limitations in interpretation because the rate of electrolyte loss from the disks is not measured. As Figure 2 (Materials and Methods page 22) shows, Rate A and Rate B, very different from each other, would appear to be the same with measurements only taken at Time 0 and Time 3h.

The following experiments were done to investigate rates of electrolyte losses from the disks cut from inoculated RM leaves. Conductivity measurements were made during incubation at 15, 30, 45, 60 and 120 minutes but otherwise the experimental method was as before.

P lants were inoculated with R1, R2, Pmp, Pf, Xm or Ea at 10^8 cells/ml and disks were cut from both zone Ia and zone Ih. A selection of the results are presented in Table 11 and for ease of interpretation, the conductivity measurement at 120 minutes has, in each case, been made equal to 100μmhos in value. The values are only given for incubation times of 15, 60 and 120 minutes but the 30 and 45 minutes values were intermediate. Representative times during the development of plant response were chosen for the table (Table 11) but at other times with respect to inoculation, the patterns of leakage were the same as the patterns shown.

Table 11a deals with results from the Ia zone. With R1 it can be seen that during the first 15 minutes of incubation, 50 - 60% of the leakage seen over 120 minutes had occurred. By 60 minutes, about 80% of the leakage had occurred and during the last 60 minutes of the experiment the rate of leakage slowed down even more.

With R2, the leakage in the first 15 minutes of incubation ranged from 50 - 70% of the final value at 120 minutes. At 60 minutes the leakage rate had slowed down but 80 - 90% of the total leakage had occurred with the rate of leakage slowing down even more in the final 60 minutes of the experiment.

With Pmp there was again 50 - 70% of the leakage occurring during the first 15 minutes and 80 - 90% by 60 minutes. The rate of leakage during the final 60 minutes was very slow compared with that in the first 15 minutes.

With Xm, Ea and Pf, 50 - 60% of the conductivity increase

Table 11 Rate of electrolyte leakage from disks cut from RM leaves inoculated with R1, R2, Pmp, Xm, Ea or Pf at 10^8 cells/ml and 25°C.

(a) Disks cut from the inoculated zone (Ia)

Bacterium	Time (h) after inoculation	Time during incubation of disks (25°C) 15 min.	60min.	120 min.
R1	2	[b] 51.03	82.24	*100[a] (54.47)
	16	50.65	81.09	100 (68.77)
	264	60.11	88.29	100 (54.40)
R2	7	51.15	82.40	100 (58.70)
	24	50.53	84.27	100 (58.30)
	48	69.15	92.83	100(107.00)
	264	70.24	95.46	100 (66.10)
Pmp	2	53.80	86.99	100 (61.50)
	17	72.20	94.00	100 (73.17)
	192	60.06	90.50	100 (35.17)
Xm	3	50.80	85.96	100 (49.87)
	48	53.40	85.80	100 (42.27)
	192	61.57	79.84	100 (54.57)
Ea	3	60.64	91.35	100 (38.30)
	48	58.90	89.97	100 (56.87)
	192	60.41	95.91	100 (57.27)
Pf	3	53.88	90.29	100(40.83)
	48	55.92	88.08	100 (55.97)
	192	68.39	90.36	100 (37.97)
Water Control	3	50.66	91.76	100 (30.00)
	48	55.99	88.15	100 (25.16)
	192	60.23	80.34	100 (40.67)

* 120 minute value has been made to equal 100 in each case for ease of understanding and corresponding changes have been made in the 15 and 60 minute values.

[a] Actual value of the 120 minute reading. (in μmhos)

[b] Each value is the mean of three results (SE R1 and R2 = 8.0; Xm and Ea = 9.0; Pf and control = 6.5).

Table 11 (contd)

b) Disks cut from the area adjacent to the Ia (Ih)

Bacterium	Time (h) after inoculation	Time during incubation of disks (25°C) 15 min.	60 min.	120 min.
R1	2	[b]50.87	83.06	*100[a] (49.2)
	16	52.58	86.09	100 (52.73)
	264	60.14	90.52	100 (52.77)
R2	24	60.03	89.14	100 (33.80)
	72	55.27	86.63	100 (57.30)
	264	60.26	91.16	100 (109.47)
Pmp	2	53.79	88.72	100 (59.17)
	17	58.44	86.79	100 (54.07)
	192	57.92	90.00	100 (40.00)
Xm	3	54.61	88.65	100 (47.00)
	48	59.78	87.13	100 (38.43)
	192	60.57	80.88	100 (41.43)
Ea	3	60.64	91.35	100 (38.30)
	48	58.82	92.30	100 (39.57)
	192	69.24	100.00	100 (53.43)
Pf	3	50.88	81.65	100 (34.16)
	48	56.78	82.50	100 (43.78)
	192	60.00	81.65	100 (41.89)
Water Control	3	58.88	90.00	100 (35.90)
	48	56.75	89.15	100 (29.50)
	192	61.91	80.78	100 (30.68)

*, [a], [b]. For explantion of footnotes see previous page.

noted at 120 minutes, occurred in the first 15 minutes and 80 - 90% in the first 60 minutes of incubation.

Table 11b deals with the Ih zones adjacent to the Ia zones dealt with above. It can be seen from these results that with R1, R2, Pmp, Pf and Xm 50 - 60% of leakage occurred in the first 15 minutes of incubation and 80 - 90% in the first 60 minutes. Rates of leakage in the final 60 minutes of the experiment were much slower than in the first 15 minutes.

With Ea leakage from disks cut from the Ih zone ranged from 50 - 70% in the first 15 minutes and was 90 - 100% by 60 minutes. Leakage had almost stopped by 60 minutes and very little electrolyte loss was noted in the final 60 minutes of the experiment.

It can be seen that in every case the rate of electrolyte leakage follows the form of Rate B in Materials and Methods IIIa2 with roughly 50 - 60% of the conductiwity increase noted over the 120 minute incubation period occurring in the first 15 minutes.

This experiment showed that the practice of taking only two conductivity readings (at Time 0 and Time 3h) is reasonable in these cases because the same rate pattern occurred in all of them. It should be established, however, in experiments of this kind, that rate patterns for electrolyte leakage are similar before much work is carried out.

C The effect of temperature of incubation on electrolyte leakage from disks cut from RM tissue.

In order to gain more information on the nature of the leakage phenomenon, the effect of temperature of incubation of the disks was studied. This technique is often used in physiological studies of membranes (Siegel, 1969; Toprover and Glinka, 1976). Plant cells have distinctive patterns of leakage in response to

to temperature. It was hoped to deomonstrate that the action of R1 or R2 on RM plants was to alter this pattern.

The method is described in Material and Methods IIIa3 but was basically the same as in the previous experiment except that several different incubation temperatures were used.

Disks (0.2 cm diameter) were cut from the Ia zone of plants inoculated with R1 or R2 at 10^8 cells/ml and placed in flasks of water (10 ml in each) which had been incubated at 4, 15, 20, 25, 37, 50°C for one hour prior to addition of disks. Incubation of the flasks plus disks continued at these temperatures for one hour. Preliminary experiments indicated that the rate of leakage in all cases took the form of Rate B as above so the result are given only for the final conductivity reading in Table 12. Again, for ease of understanding, the value for leakage at 50°C has been made to equal 100 and the values for other temperatures have been adjusted similarly with respect to the 50°C value.

The results in Table 12 show that temperature of incubation does influence the leakage of electrolytes from disks of RM tissue. As disk tissue becomes more necrotic and desiccated during development of HR or SR , the influence of temperature becomes slightly less. The massive differences seen between 37°C and 50°C in all cases (including controls) due possibly to heat damage to plant cell membranes, is less obvious with increasing desiccation of the Ia as a result of the action of R1 or R2. If membrane permeability changes of the disk cells are fully responsible for the electrolyte leakage, then even when desiccated and necrotic the cells are still not totally leaky.

Table 12 The effect of temperature of incubation on electrolyte leakage (in 1h) from disks cut from inoculated RM leaves.

a) Leaves inoculated with R1

Temperature of incubation (°C)	Time (h) from inoculation. 4	24	48	72	120
4	[a]25.1	23.65	31.0	39.3	40.41
15	32.7	31.6	32.9	48.0	49.1
20	35.8	43.8	35.7	51.1	54.88
25	41.65	60.0	48.9	62.6	62.8
37	56.63	69.0	57.0	89.59	88.9
50	*100.0	100.0	100.0	100.0	100.0
Actual value of 50°C result (μ mhos)	106.83	155.0	92.0	58.9	63.77

b) Leaves inoculated with R2

Temperature of incubation (°C)	Time (h) from inoculation. 4	24	48	72	120
4	[a]35.62	25.0	25.5	30.0	34.8
15	37.7	34.0	30.0	37.27	42.2
20	40.0	59.0	43.61	39.92	55.6
25	43.23	68.0	57.1	43.21	68.38
37	59.32	71.0	64.0	59.1	87.52
50	*100.0	100.0	100.0	100.0	100.0
Actual value of 50°C result (μ mhos)	87.5	116.13	138.33	95.5	68.58

c) Leaves inoculated with sterile water (control). One typical result only is given.

Time (h) after inoculation	Temperature of incubation (°C) 4	15	20	25	37	50	Actual value of 50°C result (μmhos)
24	[a]18.6	28.1	35.5	39.69	58.4	*100.0	80.5

* 50°C value has been made to equal 100 in each case for ease of understanding and corresponding changes have been made in the values at other temperatures.

[a] Each value is the mean of three replicates (SE R1 = 5.5; R2 = 6.3; controls = 5.8)

D Patterns of electrolyte leakage following damage to plants by means other than injection of bacteria.

1 a) Triton X-100

Triton X-100 is a non-ionic detergent which disrupts membranes and has been shown to result in massive leakage of electrolytes from potato tissue disks when the disks are placed in a 2% solution (Stephens, 1974).

Plants were injected with 2% Triton X-100. The Ia remained water-soaked and translucent and became desiccated over the next four days. The Ia was necrotic and very brittle by 6 - 7 days. The Ih also reacted. The tissue began to dry up and became pale green in colour by day 2. This response spread over the next 5 - 6 days to cover the whole leaf. The leaf abscissed after 7 days. It was not noted that the effect spread to the rest of the plant.

Measurements of rate of electrolyte leakage from disks cut from the Ia or Ih zones were made and the results are presented in Table 13. For ease of understanding, the values of conductivity at 120 minutes of incubation at 25°C have been made equal to 100 and corresponding alterations have been made to the other values.

It can be seen from Table 13 that leakage decreases during the development of the response in the Ia zone. However, whatever the final conductivity value at 120 minutes, 50 - 60% of the conductivity increase occurred in the first 15 minutes of incubation. 80 - 90% of the leakage occurred by 60 minutes and leakage in the last 60 minutes of incubation was much slower that in the previous 60 minutes.

In the Ih zone, leakage became very high at the time of the spread of the response from the Ia zone into the Ih zone (48h). However in each case the rate pattern of leakage was as before ie 50 - 60% in the first 15 minutes and 80 - 90% in the first 60 minutes.

Table 13 Rate of electrolyte leakage from disks cut from RM plants injected with 2% Triton X-100 at 25°C.

a) Ia zone.

Time (h) after inoculation	Time during incubation of disks (25°C)			
	15 min.	60 min.	120 min.	
0	[b]50.7	86.0	*100	[a](95.0)
6	69.5	93.9	100	(33.23)
24	65.1	87.1	100	(8.7)
144	56.6	92.7	100	(13.83)
Water control 24h **	53.6	87.9	100	(31.67)

b) Ih zone

Time (h) after inoculation	Time during incubation of disks (25°C)			
	15 min.	60 min.	120 min.	
0	[b]50.0	81.6	*100	[a](31.7)
6	52.97	85.06	100	(60.27)
24	55.7	91.94	100	(49.67)
48	60.92	89.97	100	(199.5)
168	65.31	89.06	100	(152.33)
Water control 24h**	54.05	90.67	100	(37.0)

* 120 minute value has been made to equal 100 in each case for ease of understanding and corresponding changes have been made in the 15 and 60 minute values.

a Actual value of the 120 minute reading (in μmhos)

b Each value is the mean of three results (SE Ia = 6.5; Ih = 10.

** This result is typical of other time intervals after injection.

It can be seen therefore that visually and by electrolyte leakage, responses of RM leaves to Triton X-100 (2% solution) are different from responses to R1 and R2. It seems likely that the response to Triton X-100 is so rapid that the leakage occurs almost immediately ie at Time 0 in Table 13(a). In that case, the massive leakage at around 18h with R1 and 48h with R2 can be compared with the action of Triton X-100.

The results from zone Ih of the RM plants inoculated with Triton X-100, have in common with the results from plants inoculated with R2 the sudden and massive rise around 48h. Visually, Ih zone (Triton X-100) becomes pale green and dry from 48h and Ih (R2) becomes chlorotic from 48h onwards.

b) The effect of temperature of incubation on leakage from disks cut from RM plants inoculated with Triton X-100 (2% solution)

In the same way as in C above the effect of temperature of incubation of disks from plants injected with 2% Triton X-100 was studied.

Disks (0.2 cm diameter) were cut from the Ia zone and placed in flasks of water (10 ml in each) which had been incubated at 4, 15, 25, 37 and 50°C for one hour prior to addition of the disks. Preliminary experiments indicated that the rate of leakage at all temperatures took the form of Rate B as before so the results are given only for the final conductivity increase (ie from Time 0 to Time 1h). Results are presented in Table 14. The results for 50°C incubation have all been made equal to 100 for ease of understanding and corresponding changes have been made for the results at other temperatures.

With increasing temperature of incubation, there was a corresponding increase in electrolyte leakage from the disks. At Time 0 when most leakage occurred the effect of temperature was

Table 14 The effect of temperature of incubation on electrolyte leakage from disks cut from RM plants injected with 2 % Triton X-100.

Temperature of incubation (°C)	Time after injection with Triton X-100			
	1h	24h	72h	120h
4	[a]44.3	66.8	44.66	41.4
15	47.26	62.24	56.29	42.57
25	54.96	62.78	62.78	46.56
37	81.5	81.78	72.94	67.42
50	*100.0	100.0	100.0	100.0
Actual value of 50°C result (μ mhos)	34.13	24.1	25.1	25.6

Water control results were as in Table 12 (c)

* 50°C value has been made equal to 100 in each case for ease of understanding and corresponding changes have been made in the values at other temperatures.

a Each value is the mean of three replicates (SE = 3.7)

most marked. The effect noted with disks from plants inoculated with R1, R2 or H_2O of a very large increase of losses at temperatures greater than 37°C was not quite so marked in this case.

2 $ZnSO_4.7H_2O$.

RM plants were injected in the usual way with 1% w/v $ZnSO4.7H_2$ (referred to from now on as Zn). The reponses of the plants were at first visually similar to HR and this was the reason for carrying out this experiment. At 18h from inoculation tissue collapse in the Ia zone was noted. The Ia was pale green in colour and at 48h became bronzed and later necrotic and desiccated. At 72h however (unlike HR) an effect in the Ih zone was seen and the tissue around leaf veins became dark brown and necrotic. No other effects were noted.

Disks from zones Ia and Ih were cut for measurements of leakage of electrolytes as before. Preliminary experiments indicated that the leakage pattern took the form of Rate B as before so the results in Table 15 give only the differences between the Time 0 reading and the Time 1h reading.

Table 15 shows that the pattern of electrolyte losses is not the same as with disks cut from plants inoculated with R1 or R2. This is not unexpected because of the effect Zn must have on the conductivity measurements itself. There is an early rise in leakage from Time 0 onwards with a peak about 2h in Ia disks. At 96h however there is practically no leakage at all. With the Ih zone, electrplyte losses increase until 24h and then fall but do not reach the low levels seen in the Ia.

3 Electrolyte losses from disks following autoclaving.

RM leaves were inoculated with bacteria (R1 or R2) at 10^8 cells/ml and at intervals disks were cut from either the Ia zone or the Ih zone and floated on 10 ml sterile water. Conductivity

Table 15 Electrolyte leakage (μmhos) from disks (in 1h) cut from RM plants inoculated with 1% w/v $ZnSO_4.7H_2O$ (Zn)

Time (h) from injection[a]	Water Control	Ia (Zn)	Ih (Zn)
0	*43.5	64.27	45.0
2	39.93	78.56	55.83
5	41.16	66.0	62.0
24	38.33	63.83	118.0
48	34.67	61.0	-
96	35.33	5.2	52.83
144	26.5	5.6	41.83
168	37.83	8.16	66.83

* Each value is the mean of three results (SE CONTROL = 5.0; Zn = 5.5)

[a] Temperature 25°C.

Table 16 Electrolyte losses (μmhos) from disks cut from plants inoculated with 10^8 cells/ml R1 or R2 during autoclaving.

Time (h) from inoculation	Ia			Ih		
	CON[a]	R1	R2	CON[a]	R1	R2
0	*76.6	71.32	75.32	79.12	80.72	82.12
2	77.0	76.0	81.3	78.1	80.5	76.5
6	78.62	79.62	80.6	79.6	80.0	79.5
10	81.67	79.5	79.5	79.62	83.62	84.62
18	79.6	74.37	81.33	82.33	91.0	79.33
24	91.67	70.27	78.13	85.7	102.0	80.07
48	74.67	70.0	73.77	69.0	87.83	81.0
72	101.8	87.83	107.23	87.83	113.4	110.43
96	79.0	62.33	76.0	76.33	94.83	93.17
144	87.0	63.5	76.33	81.67	96.0	119.0

* Each value is the mean of three results (SE = 6.0)

[a] Water injected control results

readings were made immediately and after flasks were autoclaved at 15 lb/in^2 for 15 minutes and then cooled to 25°C. This process took c. 1h to carry out. In this way it was hoped to find an indication of the total amount of electrolytes in the disks at each stage in the development of HR or SR. Autoclaving was found to disrupt the disk cells completely.

Results are given in Table 16. Controls are injected with sterile water. Table 16 shows that with increasing necrosis of the Ia of R1 inoculated plants, the total amount of electrolytes falls below control values but not very greatly. With the Ia of R2 inoculated plants, electrolyte levels do not differ greatly from control values.

For the Ih zone results, R1 and R2 levels remain higher than control levels throughout with the levels of electrolytes in plants inoculated with R2 being slightly higher than in the case of R1.

Overall however, the differences are not very great considering the results from non-autoclaved tissue. These results indicate that infection with R2 or R1 does not increase the pool of electrolytes in the cells.

E The effect of changing the ambient solution during incubation of disks cut from RM plants.

In these experiments, disks were cut from RM plants 3 days after inoculation with R2 or injection with water and electrolyte leakage into distilled water was observed. After 35 minutes when the leakage rate reached a plateau, disks were removed from the first ambient solution and placed in a second (also distilled water). It was hoped to establish whether or not the typical hyperbolic Rate B leakage patterns could be induced to occur a second time with the same disks by replacement of the ambient solution.

In Table 17, the results for R2 Ia can be taken as being

representative of results taken at other times and also those using R1. It was not found possible to repeat the initial rapid leakage from any of the disks. In the first solution, conductivity of control disks increased by 32 μmhos in 35 minutes. When the disks were taken out of the first solution and placed in the second, instead of again increasing the conductivity by 32 μmhos in 35 minutes, the conductivity increased by only 10.13 μmhos in 60 minutes. The same pattern was observed with R2 disks. The usual rate of leakage continued throughout the experiment despite the fact that the ambient solution was changed in the middle of the experiment.

Table 17 The effect of changing the ambient solution during the period of incubation of leaking leaf disks cut from inoculated RM plants. (25°)C.

Time (minutes) during incubation	first solution conductivity (μmhos)		a second solution conductivity (μmhos)	
	R2**/	Control*	R2**/	Control*
0	[b]6.33	5.33	3.3	3.77
3	91.0	19.0	4.5	4.97
10	137.0	28.0	6.43	6.33
30	171.0	30.0	-	-
35	175.0	32.0	-	-
60	-	-	10.13	10.3

* Water controls at three days from injection.

** R2 disks cut from plants three days from inoculation.

[a] At 35 minutes disks were taken from the first solution and the same disks were put into the second solution for measurement to continue.

[b] Each value is the mean of three results (SE = 6.8)

These results raised questions about the nature of the leakage from the disks - especially the control disks where cell membranes were not damaged by the action of bacteria.

Passage of electrolytes through a cell membrane by diffusion should continue until the concentrations of the ions are equal on

each side of the membrane. Hence, once the diffusion stops because of this equilibrium, the replacement of the ambient solution by fresh solution (distilled water) should cause the diffusion to begin again and to continue until equilibrium is reached again.

As can be seen from Table 17, this did not occur and it was decided that it would be worth while investigating the situation further. It was thought that the initial rapid leakage might only be coming from the damaged cells at the cut edges of the disks (ie damaged by the process of disk cutting) and might not be a direct result of damage to cell membranes within the disk itself.

F Electrolyte leakage from excised tissue as affected by volume and length of cut edge of the tissue.

In the last experiment, doubts were raised about the origin of the electrolyte leakage. The main questions were:-

a) Is leakage primarily coming from the damaged cells at the cut edges of the disks?

b) Is leakage a direct result of membrane permeability with leakage coming from all parts of the disk tissue, leaving the disk at the damaged cut edge through intercellular spaces?

It was reasoned that if the answer to question (a) was yes, then leakage should be directly proportional to the length of cut edge. If the answer to question (b) was yes, then leakage should be affected both by the length of cut edge and by disk volume.

An experiment was set up using the following pieces of excised RM leaf tissue.

A 20 disks, 0.4 cm in diameter.

B One disk, 1.6 cm in diameter.

C One rectangular piece of tissue, 6.3 x 0.4 cm^2 in surface area.

D 16 disks, 0.4 cm in diameter.

E Three disks. 1.2 cm in diameter.

F Two rectangular pieces of leaf tissue each 5.8 x 0.4 cm^2 in surface area.

The different lengths of cut edge and volumes of the tissue pieces are given in Table 18.

Table 18 Length of cut edge and volumes of tissue pieces used in experiment VIF.

Tissue Piece.	Cut edge (e) in mm	Volume (v) in mm^3	Ratio e/v
A	*251.2	251.2	1
B	50.24	2oo.96	0.25
C	134.0	252.0	0.53
D	200.96	200.96	1
E	113.04	339.12	3
F	248.0	464.0	1.87

* Each value is the mean of three measurements, SE = 2 (e) and SE = 8 (v).

The tissue pieces were cut from different nine day old monofoliate leaves but from the same areas of these leaves. The tissue was not inoculated or injected with anything. Measurements of rates of electrolyte leakage from these tissue pieces in 10 ml water were made over 120 minutes at 25°C and are presented in Table 19. For ease of understanding the results for 120 minutes have been made equal to 100 and the other results have been adjusted accordingly.

Table 19 Rates of electrolyte leakage from RM tissuePieces of sizes detailed in Table 18.

Tissue piece	Time and leakage (mhos) during incubation. 15 min.	60 min.	120 min.	(Actual valu
A	[a]73.02	90.19	*100.0	33.37
B	60.45	89.0	100.0	6.17
C	33.28	71.85	100.0	7.0
D	63.07	90.3	100.0	27.54
E	48.66	79.72	100.0	12.33
F	26.32	69.69	100.0	16.07

* 120 minute value made equal to 100 and other results altered correspondingly.

a Each value is the mean of three results, SE = 5.

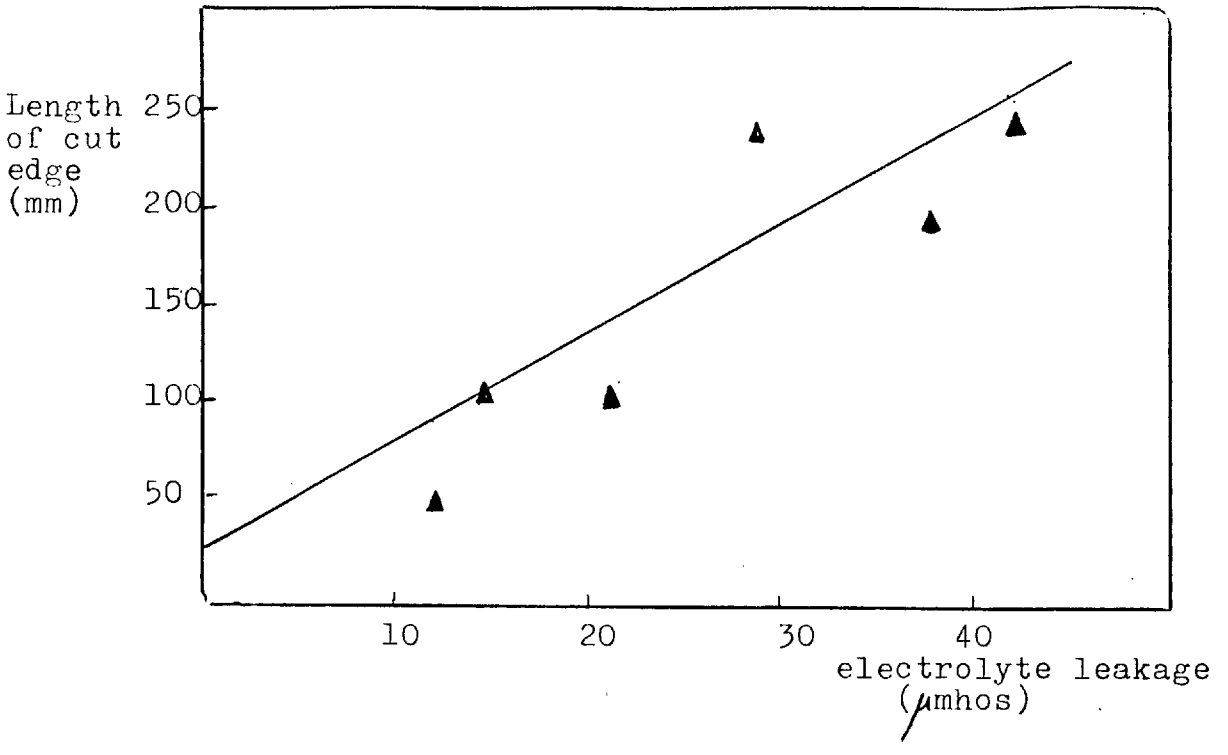
The situation here is not at all clear cut. A, B, D, and E follow Rate B pattern leakage with more than half of the leakage over 120 minutes incubation occurring in the first 15 minutes. With samples C and F, the leakage pattern is slightly different in the less than half of the total leakage occurring in 120 minutes takes place in the first 15 minutes. The difference between the two groups is that A,B,D and E are all samples made up of disks and C and F are made up of rectangles of tissue. It is not easy to see why shape of tissue should have influence on leakage but there is another difference. Rectangles were cut using a razor blade and the edges were less damaged that the disks cut with blunt cork borers.

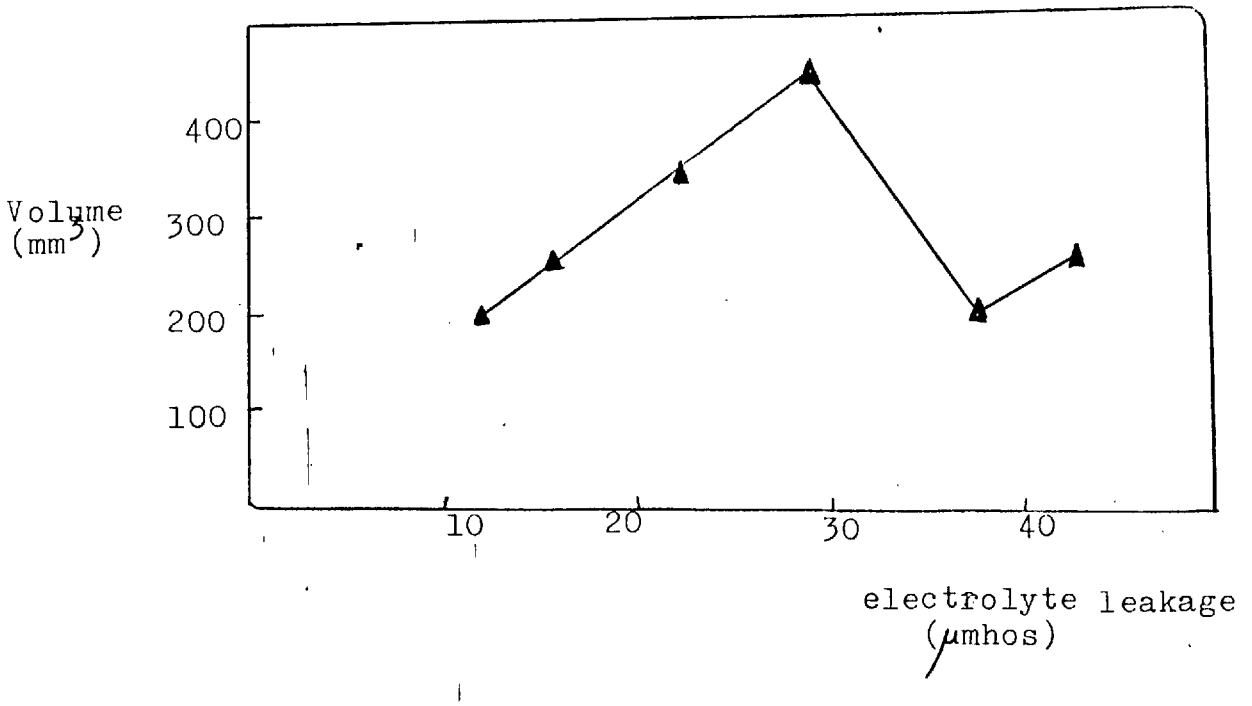
When graphs of leakage vs. edge length and leakage vs. volume were drawn (Figure 3), there seemed to be more of a relation ship between cut edge and leakage than between volume and leakage although volume seemed to be having some influence. The best straight line drawn through the points on the graph of leakage vs. edge length was found using linear regression and had a co-efficient of determination (r^2) of 0.7. When $r^2 = 1.0$ there is 'perfect fit' of data to straight line and when $r^2 = 0$ there is 'no fit'. A value of 0.7 for r^2 is clearly not perfect fit but it would suggest that there is a fairly good relationship between cut edge and amount of leakage.

This experiment does not completely answer the questions (a) and (b) at the beginning of this section but it seems that length of cut edge and amount of damage of cut edge are important in determining the level of electrolyte leakageas well as volume of tissue and care should be taken in interpretation of results.

The experiment was repeated with tissue from plants inoculated with R1, R2, Pf and Ea and the results were very similar.

Figure 3 The relationship between cut edge of leaf tissue and electrolyte leakage and between tissue volume and electolyte leakage.





b Leaf disks

The technique of floating disks of RM tissue on water was used here as in Section III. RM leaves were inoculated with 10^9 cells/ml R1 or R2 and after 1h (to allow water-soaking to disappear) disks were cut from the leaves. These were of two kinds. The first were of 1cm diameter and cut such that the whole of the disk was inoculated. The second were 1.2 cm in diameter and cut so that a small rectangle of tissue 0.4 x 0.5 cm^2 in the disk centre was inoculated. Controls were Blank, Injured, Water or Dead Cell as before. Disks were floated on 20ml sterile water in petri dishes, five disks per dish. Measurements of conductivity of the ambient solutions were made at intervals and as disks were not removed from dishes the results were cummulative. In Table 20, Water control results are given only because all the different control-type results were similar.

In fully inoculated disks electrolyte losses from R2 disks rose to a peak at 72h and another at 168h. Losses from disks inoculated with R1 increased to some extent over the period of the experiment but the losses in attached leaves at the time of tissue collapse were much greater.

In disks inoculated only in the central area, losses from disks inoculated with R1 increased over the first 24h of incubation and reached a stationary phase which lasted until 72h when a rapid increase in electrolyte loss began. This stage was marked by the onset of browning of the Ia and may be compared with the sudden increases in electrolyte loss seen in attached leaves at this stage.

Losses from disks partly inoculated with R2 rose steadily until 96h after which a more rapid rate of increase in losses began. At this point the spread of the chlorotic halo around the Ia could be seen. In this respect the results from R2 disks (partly inoculated) may be compared with those from the Ih zone of

Table 20 Conductivity readings of water on which leaf disks (RM) floated (at 25°C).

a) Disks 1cm in diameter and fully inoculated with R1 or R2 at 10^9 cells/ml

Time (h) after addition of disks to water (20ml)	Control (water) μmhos	R1 μmhos	R2 μmhos
0	*18.0	24.5	29.0
2	21.0	25.0	30.0
8	25.0	25.0	33.0
10	30.0	27.0	44.0
24	39.0	29.0	60.0
72	39.0	96.5	230.0
120	40.0	128.5	230.0
168	41.0	133.5	260.0

b) Disks 1.2 cm in diameter and inoculated in central 0.4 x 0.5 cm^2 area with R1 or R2 at 10^9 cells/ml

Time (h) after addition of disks to water (20ml)	Control (water) μmhos	R1 μmhos	R2 μmhos
0	* 24.0	29.0	32.0
2	24.0	32.0	32.0
8	24.0	36.0	33.0
10	24.0	40.0	37.0
24	29.0	60.0	56.0
48	50.0	60.0	67.0
72	50.0	60.0	86.0
96	50.0	95.0	90.0
168	80.0	241.5	273.0

* Each value is the mean of three results, each result from six disks. (SE = 6.0 for controls and SE = 8.0 for R1 and R2)

attached leaves. Common to both is the sudden rise in electrolyte losses associated with the spread of chlorosis from around the Ia.

These experiments were repeated with similar results.

c Calibration of the conductivity meter.

It was thought that it would be useful to have some idea of how the conductivity meter used extensively in this work, responded to a dilution series of ionic solutions of known concentration.

Solutions of KCl and of $CaCl_2.2H_2O$ at concentrations of 20 mg/ml were made up and diluted in stages down to 39.9μg/ml and 20μg/ml respectively, with conductivity measurements made at each stage. Dilution was carried out using distilled water because that was what was used in the main experiments involving the meter.

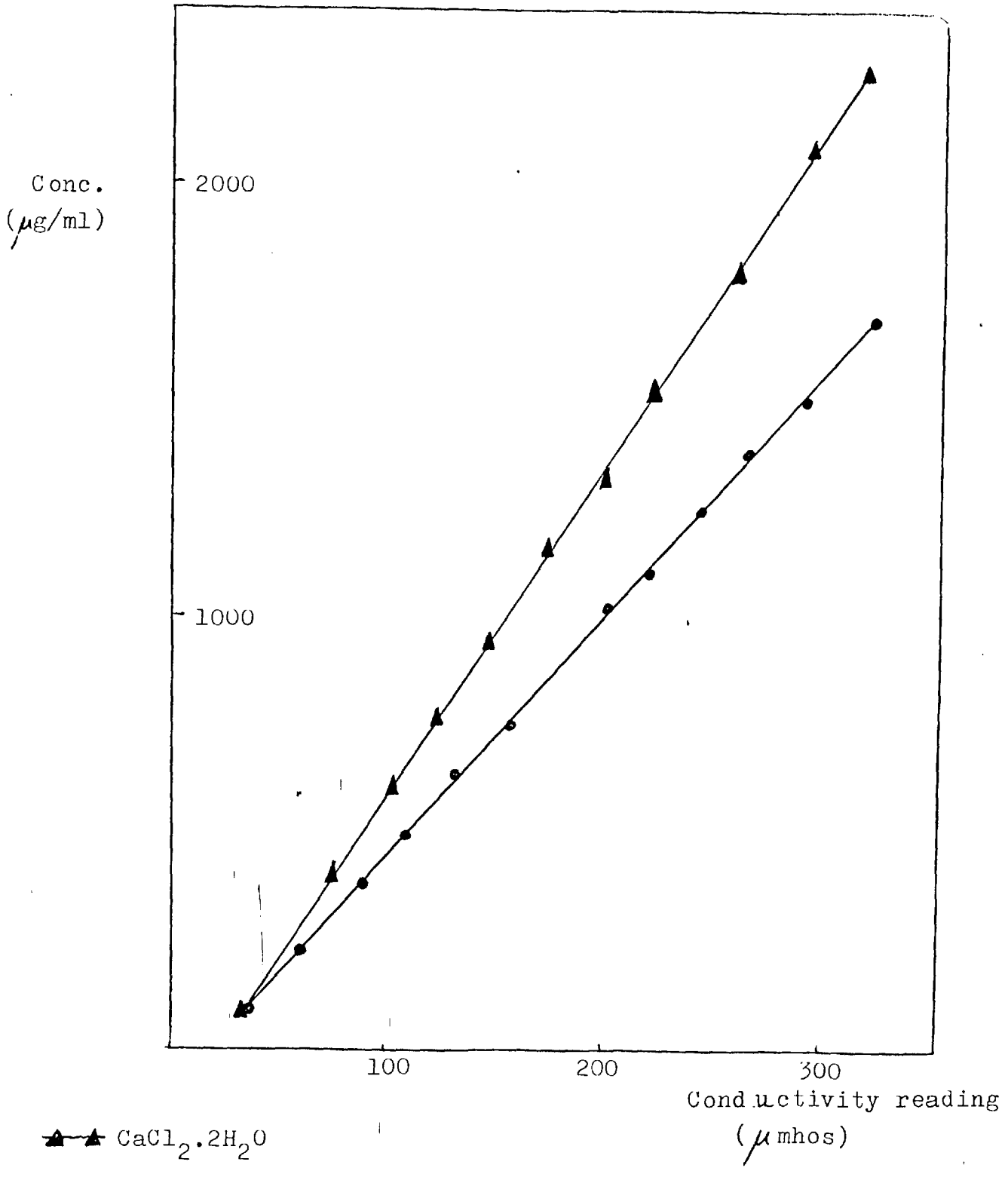
The results are graphed in Figure 4 and it can be seen that there is a linear relationship between concentration of ions and conductivity measurements.

VII Physiological changes in the host as measured by leakage of ions using flame photometry.

A Leaves

(a) RM leaves were injected with Rl, R2, Pmp, Pf, Xm or Ea at 10^8 cells/ml. At intervals 20 disks (0.2 cm diameter) were cut from the Ia and washed in water. Using the method described in Materials and Methods IVa levels of potassium (K^+), sodium (Na^+) and calcium (Ca^{++}) ions in the electrolyte losses were measured by use of flame photometry during 3h incubation in water (10 ml per 20 disks) at 25°C.

Figure 4 Calibration of conductivity meter using (a) $CaCl_2.2H_2O$ and (b) KCl solutions at 25°C.



Controls were Blank, Injured, Water or Dead Cell as usual with the results from the water controls being subtracted from the inoculated plant results to give the final results in Figures 5 to 10 inclusive. Control results varied very little over the course of the experiment and were 11 - 15 parts per million (ppm) for K^+, 0.3 - 1.5 ppm for Na^+ and 10 - 15 ppm for Ca^{++}. Each treatment was replicated five times and although each Figure has graphs of electrolyte loss for comparison, the conductivity and flame photometry results are from two different experiments.

1 R1 (Figure 5)

The initial electrolyte loss peak (at 2h) seems to involve K^+ and Ca^{++} ions. Levels of sodium ions leaking from the disks were very much lower at this time. The electrolyte peak at 13h is reflected only in K^+ levels and the 72h electrolyte peak is not reflected in any of these ions. After 72h the levels of K^+, Na^+ and Ca^{++} were not high enough to account for the continuing high electrolyte loss and other ions must be involved.

2 R2 (Figure 6)

The first increase in electrolyte loss at 7h involved an increase in Ca^{++} ion leakage. Leakage of K^+ was not higher than in controls at this point and did not show an increase until 13h. This contrasts with the results for R1. K^+ and Ca^{++} leakage rose to a peak at 48h and leakage of Na^+, although always very low, peaked at 72h. This was a reflection of the increasing rise in electrolyte losses over this time.

3 Pmp (Figure 7)

The rise in electrolyte loss over the first 24h of development of HR is reflected in a rise in leakage of K^+. Na^+ involvement was again very little and Ca^{++} seemed only to be involved in leakage at the 72h peak of electrolyte loss. The pattern with Pmp does not seem to be similar to that of R1. Other ions

must be involved.

4 Xm (Figure 8)

The results seem to indicate that leakage from disks cut from plants inoculated with Xm is due to both K^+ and Ca^{++} leakage. Na^+ leakage again is very low.

5 Ea (Figure 9)

Levels of K^+, Na^+ and Ca^{++} released from disks peaked at 48h. This reflected the general rise in electrolyte loss noted at 48h. The higher levels of electrolyte loss noted at 120 - 144h may be partly due to increasing release of K^+ and Ca^{++} but other ions may be involved too.

6 Pf (Figure 10)

Electrolyte losses were low as were leakages of K^+, Na^+ and Ca^{++}.

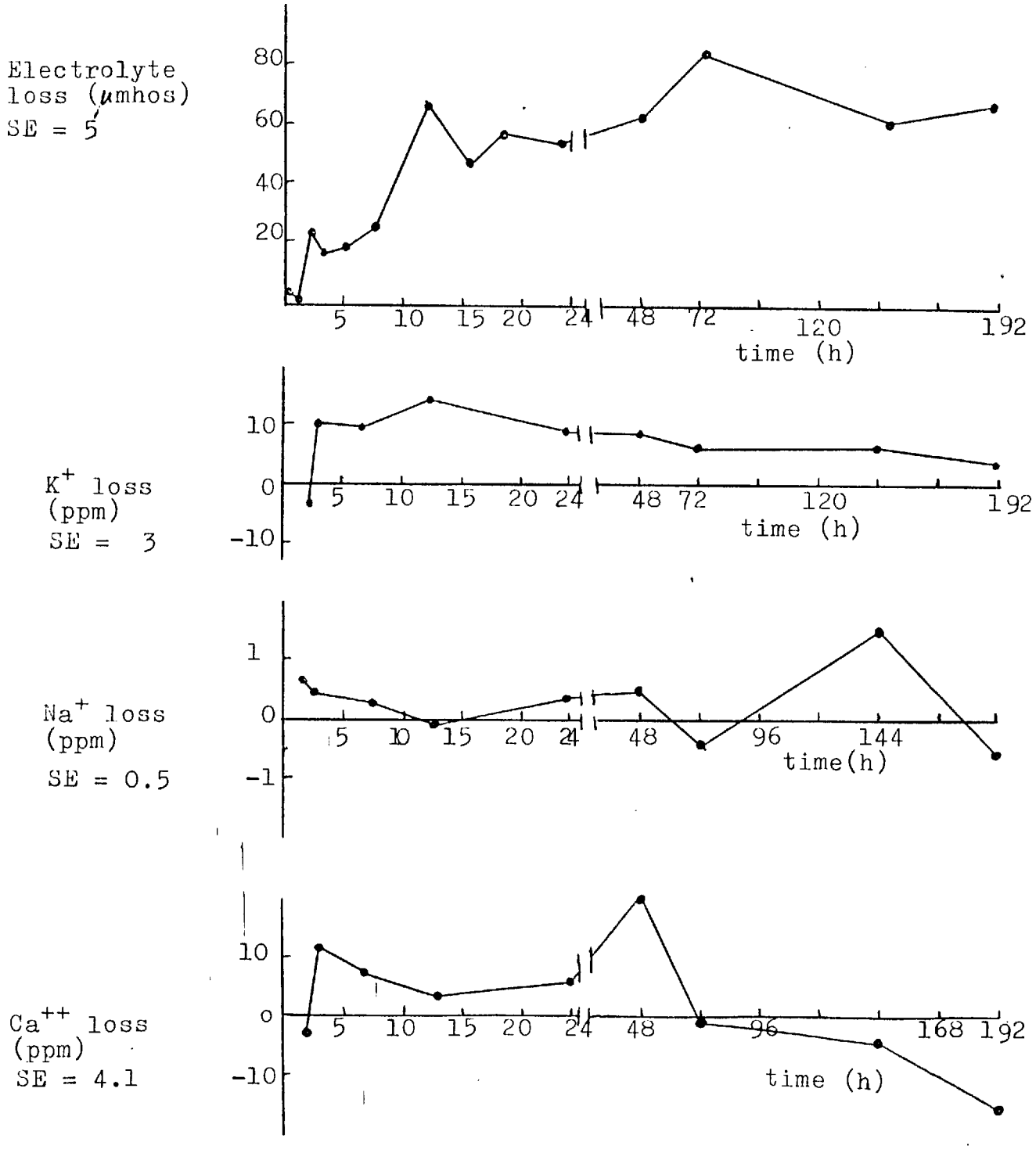
In summary, an involvement of K^+ and Ca^{++} was found in the electrolyte losses from disks cut from inoculated RM leaves. Na^+ was found not to be very important in this respect and results implied that other ions were involved.

The experiment was repeated with similar results.

(b) RM leaves were inoculated with R2 at 10^8 cells/ml. At intervals measurements were made on electrolyte losses from disks cut from the Ih zone adjacent to the Ia zone. Previous work indicated that massive electolyte losses occurred in zone Ih when the chlorotic halo typical of SR spread into this region. It was hoped to show involvement of K^+, Na^+ or Ca^{++} in this leakage.

Controls were Injured, Blank, Water or Dead Cell as before and all the measurements from controls were similar over the period of the experiment. Leakage of K^+ from control disks was 11 - 15 ppm; leakage of Na^+ was 0.3 - 1.5 ppm; leakage of Ca^{++} was 10 - 15

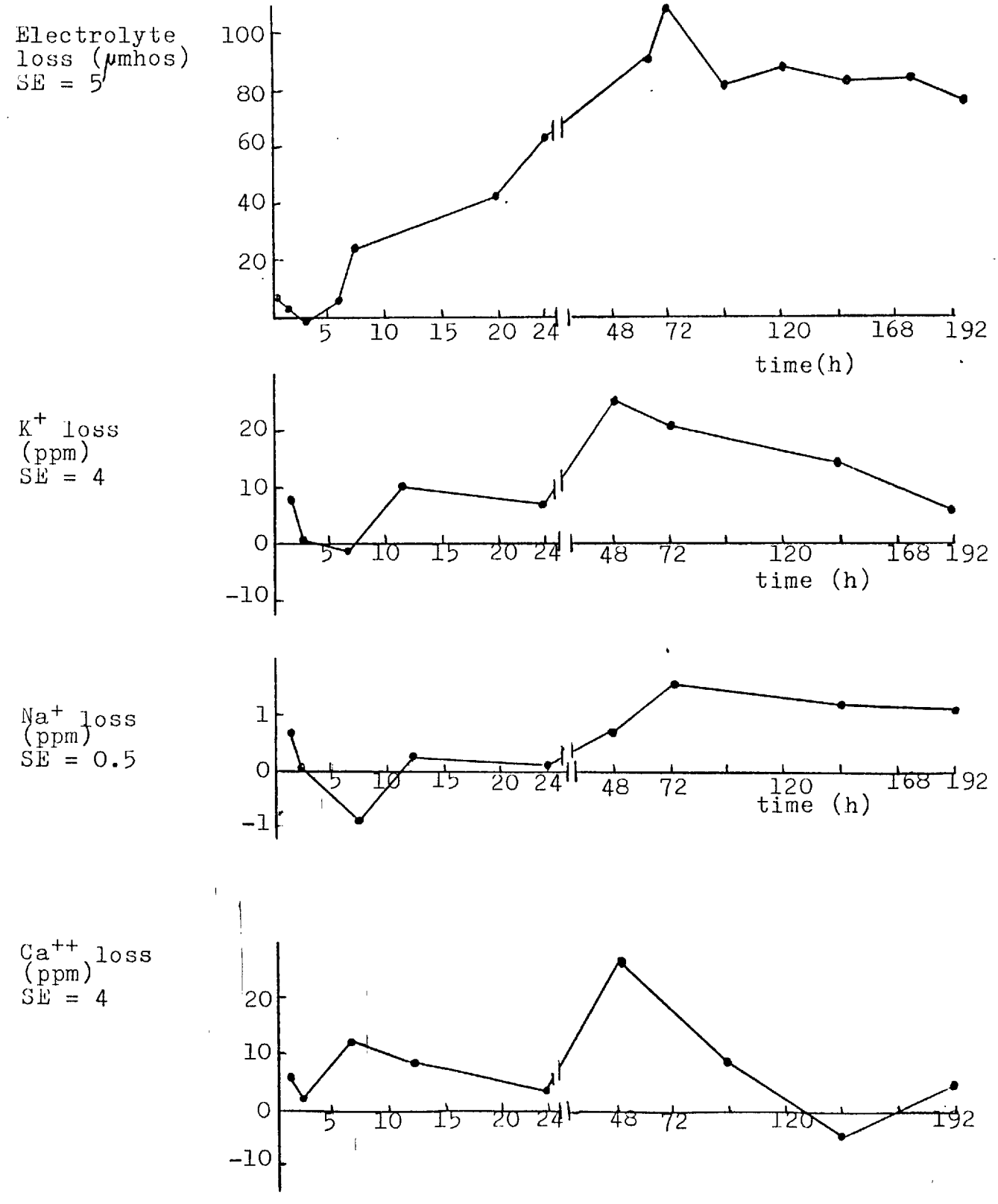
Figure 5 Levels of K^+, Na^+ and Ca^{++} leaked into water from leaf disks cut at various times after inoculation with R1 at 10^8 cells/ml.
(Electrolyte loss included for comparison.)



All points are the means of six results with controls subtracted.

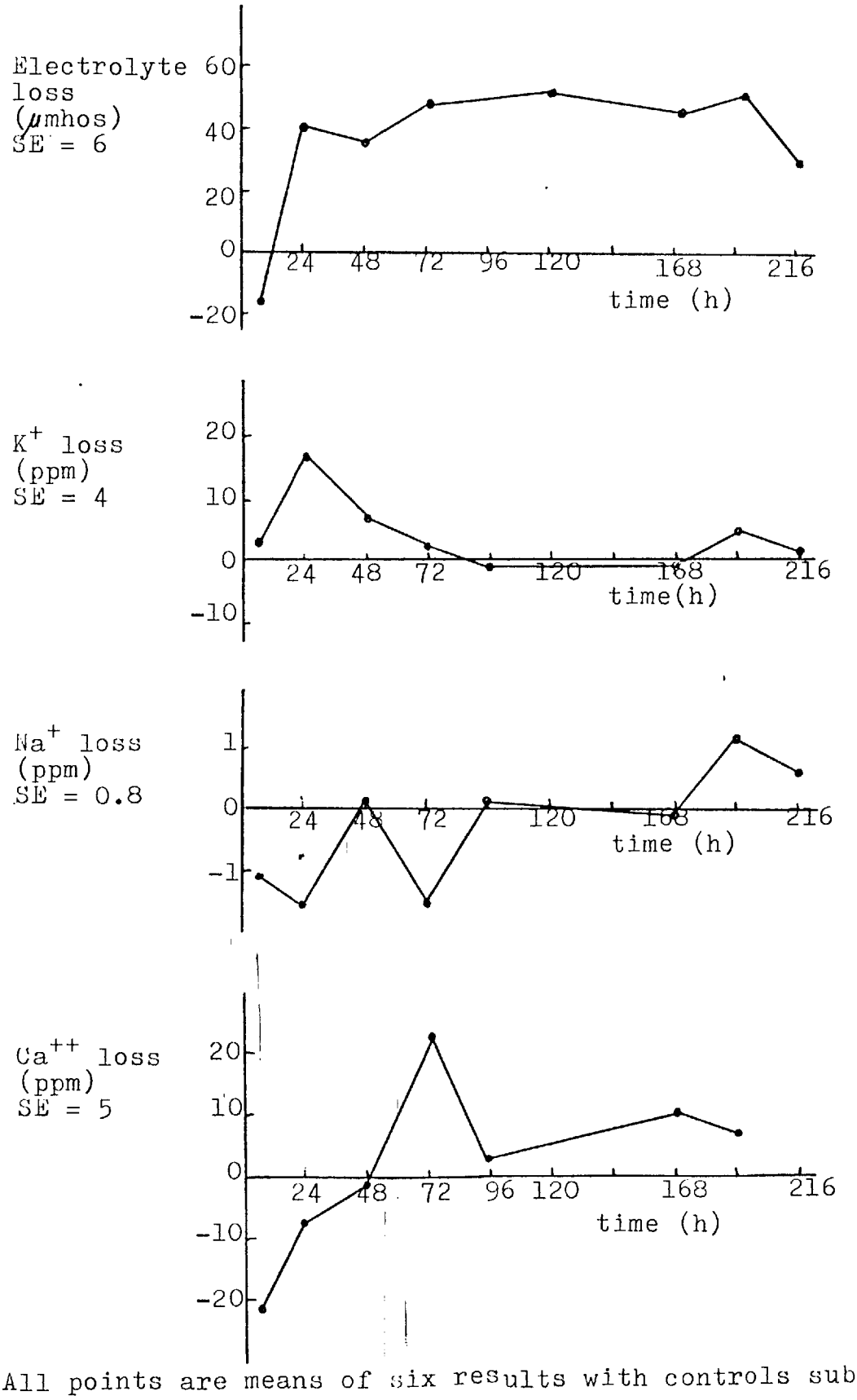
Figure 6 Levels of K^+, Na^+ and Ca^{++} leaked into water from leaf disks cut at various times after inoculation with R2 at 10^8 cells/ml.

(Electrolyte loss included for comparison.)



All points are the means of six results with controls subtracted.

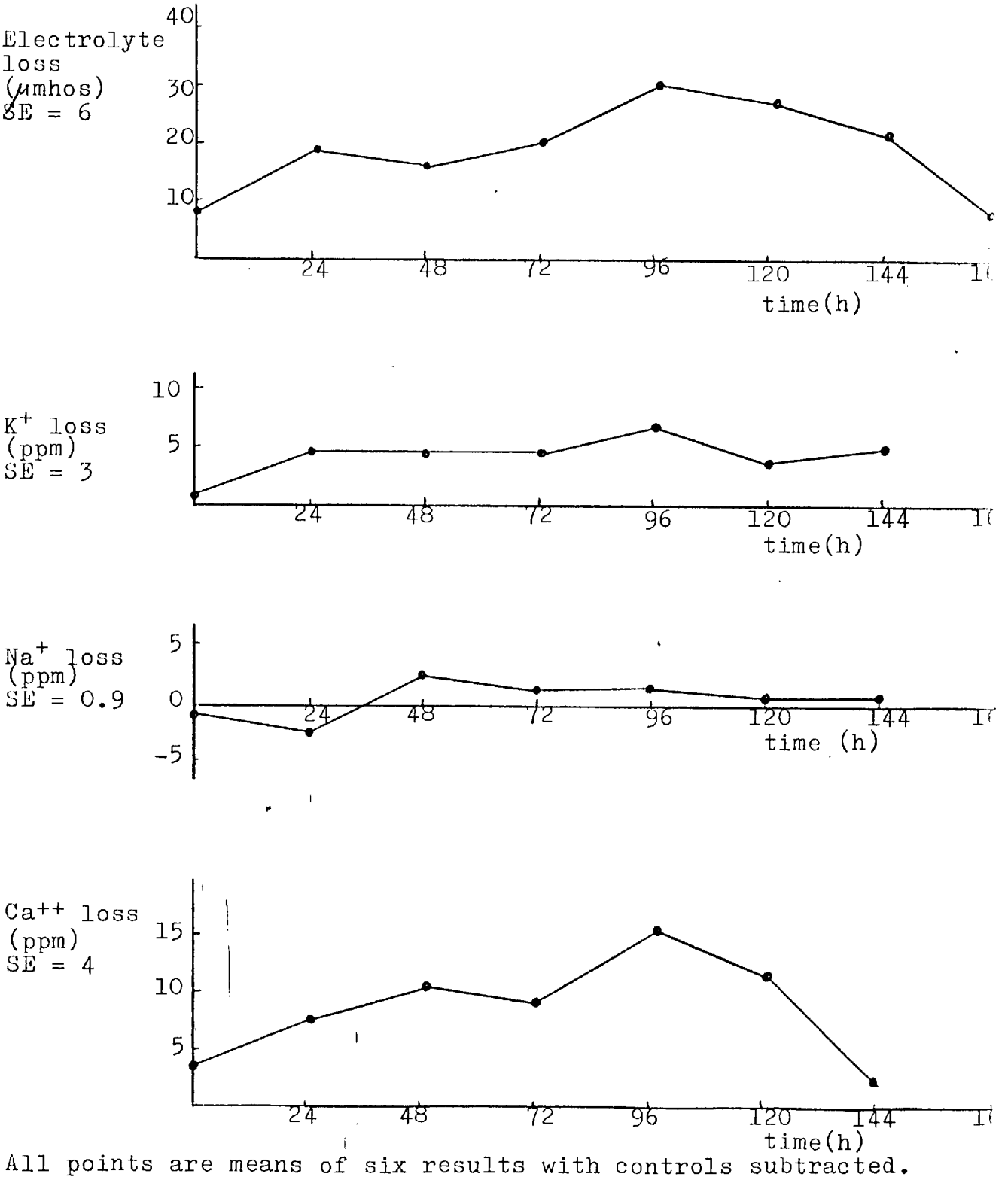
Figure 7 Levels of K^+, Na^+ and Ca^{++} leaked into water from leaf disks cut at various times after inoculation with Pmp at 10^8 cells/ ml.
(Electrolyte loss included for comparison.)



All points are means of six results with controls subtracted.

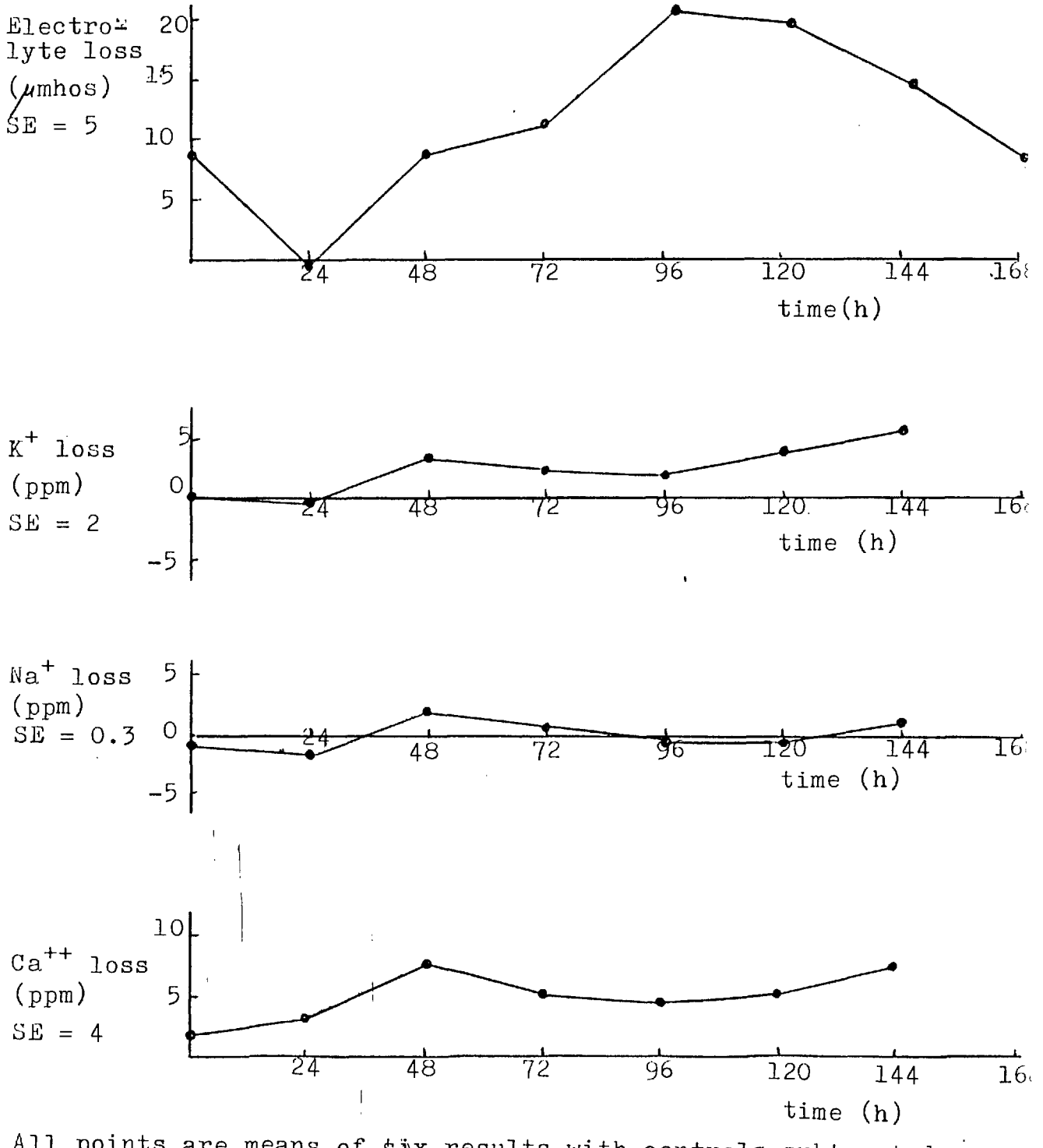
Figure 8 Levels of K^+, Na^+ and Ca^{++} leaked into water from leaf disks cut at various times after inoculation with Xm at 10^8 cells/ml.

(Electrolyte loss included for comparison.)



All points are means of six results with controls subtracted.

Figure 9 Levels of K^+, Na^+ and Ca^{++} leaked into water from disks cut at various times after inoculation with Ea at 10^8 cells/ml.
(Electrolyte loss included for comparison.)



All points are means of six results with controls subtracted.

Figure 10 Levels of K^+, Na^+ and Ca^{++} leaked into water from disks cut at various times after inoculation with Pf at 10^8 cells/ml.

(Electrolyte loss included for comparison.)

All points are means of six results with controls subtracted.

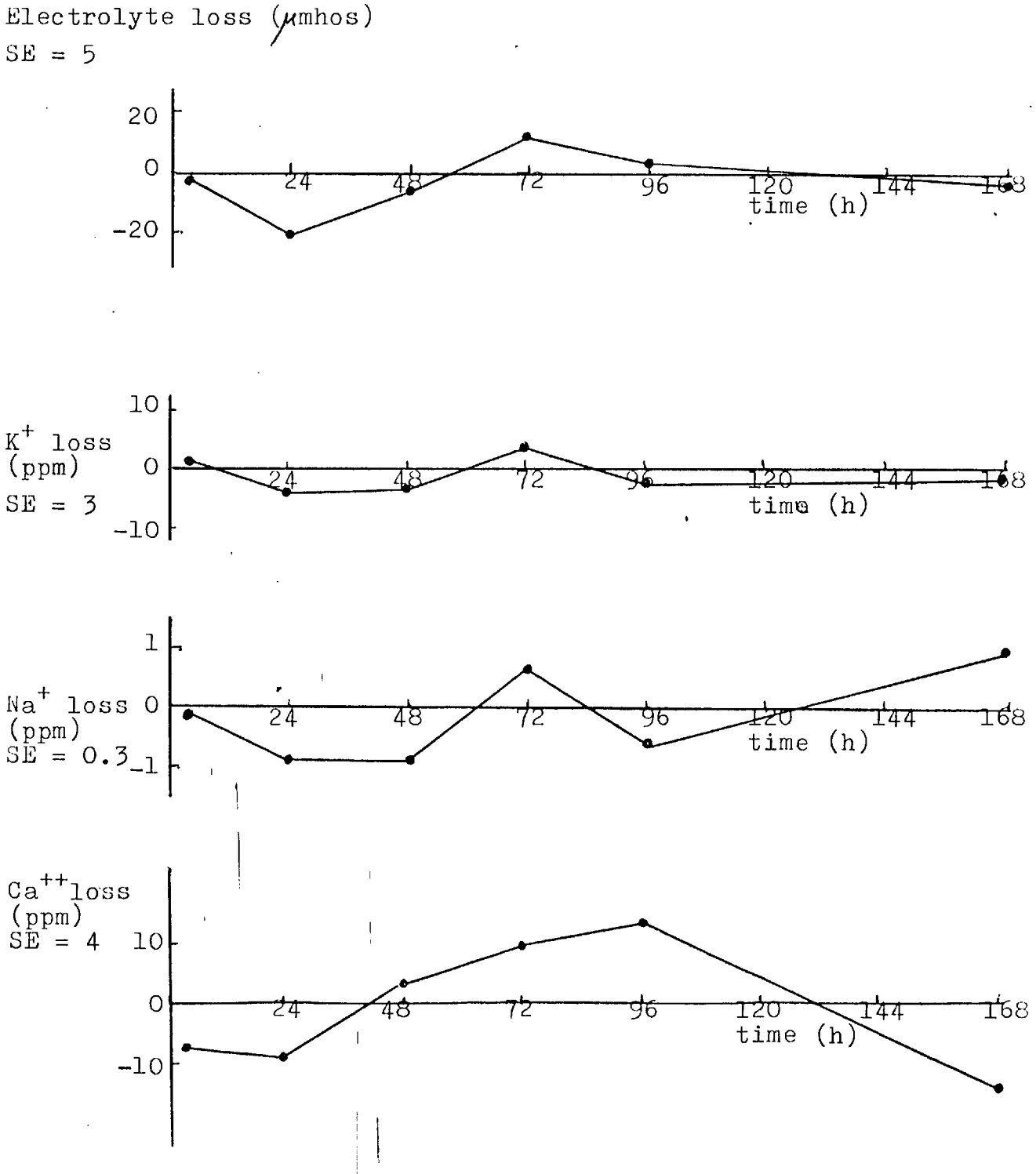
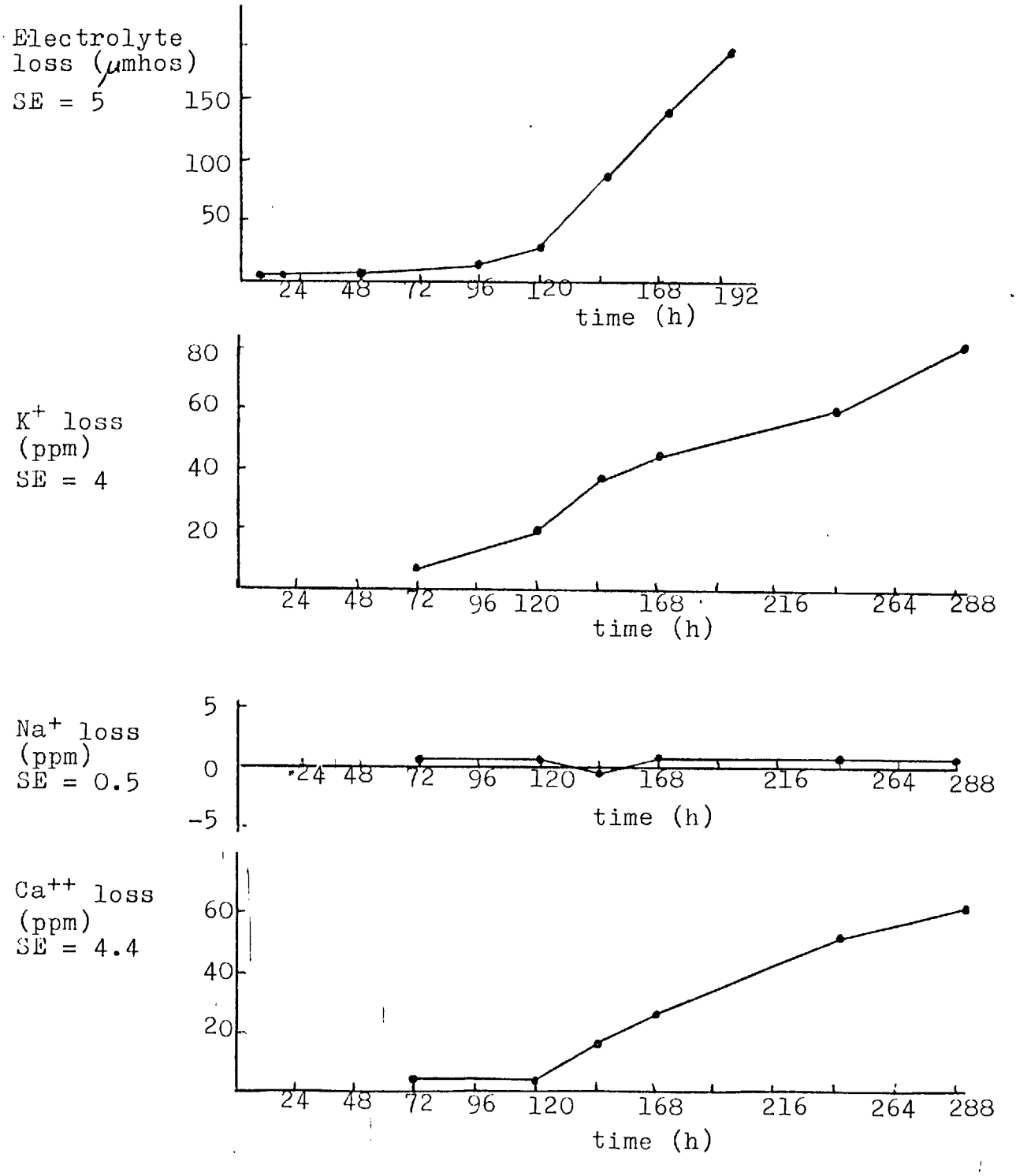


Figure 11 Levels of K^+, Na^+ and Ca^{++} leaked into water from disks cut at various times after inoculation with R2 at 10^8 cells/ml in the Ia zone (disks cut from the Ih zone). (Electrolyte loss included for comparison.)

All points are means of six results with controls subtracted.



ppm. Control results were subtracted from the results from inoculated plants to give the final results in Figure 11. Electrolyte losses are also presented for comparison but these results come from a different experiment.

It can be seen from Figure 11 that both K^+ and Ca^{++} are involved in the increase in electrolyte losses noted. K^+ ion loss increased first at 72 - 120h and Ca^{++} loss followed at 120 - 144h. Na^+ involvement did not seem significant.

This experiment was repeated with similar results.

(c) Measurement of the rate of ion leakage from disks cut from inoculated RM leaves.

As described in Materials and Methods IVb, the experiments above have limitations on interpretations because the rate of ion release from the disks is not measured. As Figure 2 shows, Rate A and Rate B very different from each other would appear to be the same with flame photometer measurements only taken at Time 0 and Time 3h during incubation as in (a) and (b) above.

The following experiments were designed and carried out in order to investigate in some detail the rates of ion leakage from the disks cut from inoculated RM leaves. 1 ml samples were removed from the ambient solution at intervals over the incubation period and analysed with the flame photometer for the presence of K^+, Na^+ and Ca^{++}. Adjustments to the calculations of leakage of ions were made to account for the decreasing amount of ambient solution over the period of incubation.

Plants were inoculated with R1 or R2 at 10^8 cells/ml and disks were cut from both Ia and Ih zones and results are presented in Table 21. For ease of understanding, the values at 120 minutes have been made equal to 100 and corresponding changes have been made in the other values. Only certain representative results have been given but other results followed the same pattern.

Table 21 Rate of ion leakage from disks cut from inoculated RM leaves at 25°C.

a) Ia zone

1) Plants inoculated with R1

Time (min.) during incubation	Time after inoculation. 2h K^+	2h Ca^{++}	12h K^+	12h Ca^{++}	72h K^+	72h Ca^{++}
15	*55.2	53.5	52.6	62.0	47.9	52.5
60	83.8	91.8	93.3	90.0	81.2	97.5
120	[a]100.0	100.0	100.0	100.0	100.0	100.0
Actual value of 120 min. result (ppm)	10.5[b]	11.0	15.0	5.0	9.6	4.0

2) Plants inoculated with R2

Time (min) during incubation	Time after inoculation. 7h K^+	7h Ca^{++}	48h K^+	48h Ca^{++}
15	*48.1	62.5	53.9	50.0
60	85.84	99.16	96.77	90.8
120	[a]100.0	100.0	100.0	100.0
Actual value of 120 min result (ppm)	10.6[b]	12.0	31.0	25.0

b) Ih zone

1) Plants inoculated with R1

Time (min) during incubation	Time after inoculation. 24h K^+	24h Ca^{++}	192h K^+	192h Ca^{++}
15	* 53.3	53.6	53.4	51.6
60	96.6	92.6	93.0	93.5
120	[a] 100.0	100.0	100.0	100.0
Actual value of 120 min result (ppm)	3.0[b]	4.1	4.3	3.1

2) Plants inoculated with R2

Time (min) during incubation	Time after inoculation. 120h K^+	120h Ca^{++}	288h K^+	288h Ca^{++}
15	*54.7	67.0	53.5	52.5
60	95.2	94.3	99.5	86.4
120	[a]100.0	100.0	100.0	100.0
Actual value of 120 min result (ppm)	21.0[b]	20.0	55.1	59.0

*,a,b see next page.

Table 21 (contd) Footnotes

* Each value is the mean of three results (SE K^+=4.0; SE Ca^{++}=5.0)

a 120 minute value has been made equal to 100 for ease of understanding and corresponding changes have been made in other values.

b Actual value of 120 minute value with water control subtrated. (Mean control values : K^+ = 15ppm; Ca^{++} = 10ppm)

(contiued from p 114)

Results for Na^+ leakage were not included in the table because they were very low (1 - 2ppm) and were not considered of great importance. However Na^+ leakage was of the same pattern as K^+ and Ca^{++} leakage.

Table 21 shows that in the Ia zone of plants inoculated with R1 (a1), 50 - 60% of total leakage of K^+ and Ca^{++} over 120 minutes occurs in the first 15 minutes. 80 - 90% of leakage of both ions occurs in the first 60 minutes with leakage in the last 60 minutes of the experiment being much slower.

Plants inoculated with R2 show the same pattern of leakage in the Ia and also in the Ih zone. The Ih zone of plants inoculated with R1 behaves similarly. This pattern of 50 - 60% leakage in the first 15 minutes and 80 - 90% by 60 minutes is exactly the same pattern as that found with electrolyte leakage measured by conductivity.

The experiment was repeated using plants inoculated with Xm, Pmp, Ea and Pf at 10^8 cells/ml and the rate patterns were always similar.

d) The effect of temperature of incubation on ion leakage.

Using the same methods as used in VIC above, the effect of temperature of incubation of the leaking disks was studied.

Several different temperatures of incubation were used and

disks (0.2 cm diameter) were cut from the Ia zone of plants inoculated with R1 or R2 at 10^8 cells/ml and placed in flasks of water (10 ml in each) which had been incubated for 1h prior to addition of disks at the required temperatures (3°, 25°, 37° and 50°C). Incubation of the flasks plus disks continued for one hour more with samples removed for ion analysis at Time 0 and Time 1h. Preliminary experiments indicated that leakage of each ion at the four different temperatures was of Rate B form as before so the results are given only for the final readings in Table 22. Table 22 shows only the results for plants at 24h after incubation but the patterns at this time were similar to those at other times with respect to inoculation and can be taken as representative.

It can be seen from Table 21 that with water controls, R1 and R2, leakage of K^+ increased with tamperature and that there was a very large increase in leakage between 37° and 50°C (of c. 6ppm) compared with the rises between for instance 3° and 25°C (of c. 2ppm). Leakage of Na^+ was as usual very low (c. 2ppm) in all cases but there was a slight tendency to increase in level of leakage with increasing temperature. In the case of Ca^{++} Controls showed that there was increased leakage with temperature but the massive leakage associated with electrolyte loss between 37° and 50°C was not obvious in Ca^{++} leakage. With R1 leakage increased from 20.8 to 23.0ppm between 3° and 37°C but decreased again to 19.7ppm at 50°C. With R2, leakage increased from 19.5 to 25.7ppm between 3° and 50°C but again there was no very large increase in leakage at 50°C as in electrolyte loss.

As these patterns were repeated at 48h, 72h, 120h and 168h it can be seen that inoculation with R1 and R2 has some effect on the normal pattern of temperature sensitivity of disks of RM tissue and that the large increases in electrolyte loss associated

Table 22 The effect of temperature of incubation on leakage of ions from disks 24h after inoculation with R1 or R2.

a) Potassium leakage (K^+) in ppm in 1h of incubation.

Temperature °C	Control	R1	R2
3	*10.7	23.2	16.0
25	16.3	24.7	18.3
37	18.3	27.42	28.7
50	24.0	33.4	38.3

b) Sodium leakage (Na^+) in ppm in 1h of incubation.

Temperature °C	Control	R1	R2
3	* 2.1	2.8	2.7
25	2.3	2.4	2.4
37	2.5	2.7	2.6
50	2.3	2.7	2.5

c) Calcium leakage (Ca^{++}) in ppm in 1h of incubation

Temperature °C	Control	R1	R2
3	*8.3	20.8	19.5
25	10.0	20.7	21.25
37	12.1	23.0	23.7
50	12.91	19.7	25.7

* Each value is the mean of three results. SE (K^+) = 5; SE (Na^+) is 0.5; SE (Ca^{++}) = 4.

with high temperature (50°C) are reflected only in K^+ leakage patterns.

e Leakage of ions from disks cut from RM tissue injected with Triton X-100.

In order to make comparisons between the patterns of ion leakage found in tissue inoculated with bacteria, with leakage from tissue damaged chemically, RM plants were injected with 2% Triton X-100 solution. At intervals disks (o.2 cm diameter) were cut from Ia zone and measurements of the leakage of K^+, Na^+ and Ca^{++} were made as before.

Controls were inoculated with sterile water and the results are presented in Table 23 for incubation of disks for one hour.

After initial high levels of leakage each of the ions fell in leakage and by 2h was much the same as controls. As the development of the response continued, the leakage of ions fell to very low levels indeed (Table 23).

This pattern is not the same as the pattern of leakage found in tissue from inoculated plants where ion leakage is low at first and K^+ and Ca^{++} leakage is seen to come to a peak at times of tissue collapse. It is possible that the action of Triton X-100 is to decrease the pool size of the ions very rapidly.

f The effect of temperature on ion leakage from disks cut from plants injected with Triton X-100.

RM plants injected with Triton X-100 (2%) had, at intervals, disks (0.2 cm diameter) cut from their Ia's. Measurement of ion leakage was made as before and incubation temperatures were 3°, 25°, 37°and 50°C with flasks (10 ml water in each) kept at the required temperatures for one hour prior to addition of disks.

Table 23 Leakage of ions from disks cut from RM tissue in one hour after injection with Triton X-100 at 25°C.

Time after injection (h)	Amount (ppm) of ion leaked in one hour. K^+	Na^+	Ca^{++}
a) Water control plants.			
0	*11.0	1.2	10.6
1	11.6	0.9	10.1
2	12.1	0.3	9.7
24	13.6	0.6	10.5
48	14.5	0.5	13.7
120	15.2	0.5	18.9
b) Plants injected with 2% Triton X-100.			
0	*35.3	3.9	31.0
1	10.5	1.2	15.1
2	11.6	0.8	6.5
24	5.7	0.3	5.2
48	3.9	1.7	4.2
120	4.6	0.8	6.1
SE	4.0	o.3	4.0

* Each value is the mean of three results.

Preliminary experiments indicated that with all ions at each temperature, leakage followed the standard Rate B pattern as before so the results in Table 24 are only for the one hour reading and are therefore amount(ppm)/h. Results are given only for time 0 and time 24h as these illustrate well the effects noted. Other results for other times with respect to injection of leaves were of intermediate value. Controls are not included in Table 24 as these were not significantly different from the control results given before in Table 22.

The effect of temperature on Triton X-100 tissue is to alter amounts of leakage of each ion. However the alteration is not the same as in RM tissue inoculated with R1 or R2. In the case of inoculated tissue leakage of K^+ increased with temperature but in Triton X-100 treated tissue leakage of K^+ increases only between 3^oC and 37^oC and decreases at 50^oC at time 0. At time 24h leakage of K^+ decreases between 3^oC and 37^oC and increases at 50^oC. In tissue inoculated with R1 or R2, Na^+ leakage although very low (c. 2ppm) tended to increase with temperature. However in Triton X-100 treated tissue, leakage of Na^+ is 2.8 ppm at 3^oC and increases to 3.55ppm at 25^oC. After this there is a decrease in leakage with increasing temperature.

In the case of Ca^{++} in tissue inoculated with R1 leakage increased between 3^o and 37^oC and decreased at 50^oC. In tissue inoculated with R2, leakage of Ca^{++} increased with temperature. Table 24 shows that in tissue injected with Triton X-100 leakage of Ca^{++} increased between 3^o and 25^oC but decreased at 37^o and 50^oC at time 0. At time 24h leakage of Ca^{++} hardly changed at all with increasing temperature.

This experiment has been repeated with similar results.

Table 24 The effect of temperature of incubation on leakage of ions (in 1h) from disks cut from plants injected with Triton X-100.

a) Time 0 ie immediately after injection.

Temperature of incubation °C	Amount (ppm) of ion leaked in one hour. K^+	Na^+	Ca^{++}
3	* 28.67	2.87	21.2
25	32.25	3.55	26.0
37	35.33	2.8	19.0
50	30.67	1.5	12.33

b) Time 24h after injection.

Temperature of incubation °C	Amount (ppm) of ion leaked in one hour. K^+	Na^+	Ca^{++}
3	*10.5	1.6	6.0
25	8.7	0.7	6.1
37	6.3	1.2	5.0
50	9.6	0.8	7.1
SE	5.0	o.5	5.0

* Each value is the mean of three results.

Control values are as in Table 22.

B Leaf disks

Leaf disks were cut from RM plants at one hour from inoculatio with R1 or R2 (10^8 cells/ml) or injection with sterile water. Disks were of two sizes. The first were 1cm in diameter and cut such that the whole disk was inoculated; the second were 1.2 cm in diameter and cut such that only the central area (0.4 x 0.5 cm^2) was inoculated. Five disks per treatment were floated on sterile water and measurements of K^+, Na^+ and Ca^{++} levels were made at intervals on the ambient solution. The results were therefore cummulative. The results are given in Table 25 and from this table and with reference to section VI(b) it can be seen that electrolyte losses from fully inoculated disks was best reflected in the K^+ leakage pattern. Losses from disks partly inoculated with R1 were reflected in K^+ leakage and those from disks inoculated with R2 in both K^+ and Ca^{++} leakage.

Na^+ ion leakage was higher than with disks cut from attached leaves but seemed to be involved more in the leakage from partly inoculated disks.

This experiment was repeated with similar results.

VIII Leakage of protein from disks cut from inoculated plants

RM plants were injected with 10^8 cells/ml R1, R2, Pf or Ea. At intervals 80 disks (0.3 cm in diameter) were cut from the Ia, washed and placed in distilled water in closed flasks. With R1 and R2 there was 10 ml per flask and with Pf and Ea there was 5 ml per flask. Measurements of total protein content of the ambient solution were made at time 0 and time 2h during incubation at 20^oC using the method described in Materials and Methods Va. The difference in protein levels was used as an estimate of the protein leaking from the disks in 2h. Controls were inoculated with sterile water or Dead R1, R2, Pf or Ea Cells and the water control

Table 25 Measurements of changes in ion concentration of water on which floated inoculated RM leaf disks at 25°C.

A) Disks fully inoculated (1cm in diameter)

Time (h) from addition of disks.	Absolute level(ppm) of each ion at each time. Control K^+	Control Na^+	Control Ca^{++}	R1 K^+	R1 Na^+	R1 Ca^{++}	R2 K^+	R2 Na^+	R2 Ca^+
0	*2.5	9.0	3.5	3.0	9.0	5.0	6.0	9.0	6.0
24	6.5	9.0	3.5	8.0	9.0	5.0	11.0	9.0	12.0
48	6.5	9.0	3.5	12.0	10.7	8.0	13.0	13.0	12.0
72	7.0	9.0	4.0	12.0	10.7	17.0	15.0	13.0	12.0
96	9.5	9.0	6.0	15.0	10.7	17.0	17.5	13.0	12.0
120	9.5	9.0	7.5	15.0	10.7	18.0	21.5	13.0	12.0
144	9.5	9.0	7.5	15.0	10.7	23.0	21.0	16.0	14.0
168	9.5	9.0	12.5	15.0	10.7	28.0	21.0	16.0	24.0

B) Disks inoculated in central 0.4 x 0.5 cm^2 only (diameter)

Time (h) from addition of disks	Absolute level (ppm) of each ion at each time. Control K^+	Control Na^+	Control Ca^{++}	R1 K^+	R1 Na^+	R1 Ca^{++}	R2 K^+	R2 Na^+	R2 Ca^{++}
0	* 2.0	6.0	6.0	8.0	6.0	6.0	4.0	6.0	6.0
24	2.0	8.0	6.0	10.0	8.0	10.0	8.0	8.0	9.0
48	6.0	8.0	10.0	10.0	8.0	10.0	14.0	8.0	9.0
72	6.0	8.0	10.0	10.0	8.0	18.0	17.0	8.0	16.0
96	12.0	8.0	12.0	20.0	8.0	18.0	17.0	8.0	16.0
120	13.0	8.0	18.0	34.0	8.0	19.0	17.0	13.0	27.0
144	13.0	10.0	18.0	40.0	9.0	21.0	47.0	17.0	47.0
168	14.0	12.0	26.0	48.0	10.0	21.0	47.0	23.0	48.0

* Each value is the mean of three results SE (K^+ and Ca^{++} = 4; Na^+ = 3)

results are presented only because there were no significant differences amongst the controls.

Preliminary experiments indicated that Rate B leakage of protein occurred from the disks with more than 50% of the protein leakage in 2h occurring in the first 15 minutes. The results in Table 26 are the total amounts (in g/ml) in 2h of incubation.

With R1 and R2 some equation of leakage of protein can be made with the visible symptoms on the leaves. There are rises in R1 plants at 3h and 17h which were also noted as times of high electrolyte loss. 17h was noted as the time of first development of HR in this experiment. When the onset of tissue browning of the Ia became apparent (48h) protein leakage dropped.

In R2 plants there was a massive increase in protein leakage at 48h - a time of first symptom development and of electrolyte leakage. At 7 - 10h when electrolyte leakage rises had been noted, there was no leakage of protein above that of the control values. After 48h protein leakage remained very high.

In Pf plants the values of protein leakage were very low compared with controls and are probably not significant.

Leakage of protein from disks from plants inoculated with Ea was fairly high. There were no sudden rises and no macroscopic symptoms to relate to the protein leakage.

This experiment was repeated with similar results.

IX Leakage of phenols from disks cut from inoculated plants

RM plants were inoculated with R1, R2, Ea or Pf at 10^8 cells per ml. At intervals 80 disks (0.3 cm diameter) were cut, washed and placed in 5ml distilled water in flasks. Measurements of total phenol content of the ambient solution were made using the method of Addy (1976) as modified and described in Materials and Methods VIa. Measurements were made at time 0 and after 24h incubation in darkness at 20°C. The difference between the two measurements

Table 26 Total protein leakage from RM disks cut from inoculated plants in 2h at 20°C.

Time (h) after inoculation	Leakage (µg/ml ambient solution) with control results subtracted				Water Control (µg/ml)
	R1	R2	[a]Pf	[a]Ea	
0	*22.8	9.8	5.2	-	58.2
2.5	1.2	-7.7[b]	-	-	73.29
3	32.0	-4.2	0	19.4	62.8
10	40.0	-2.0	-	-	65.3
17	86.5	-0.6	-	-	85.5
24	24.4	-3.1	9.93	33.4	81.0
48	34.0	73.9	0	31.1	57.9
72	15.1	66.6	12.0	25.1	90.6
96	30.2	65.2	21.4	22.4	63.9
168	71.4	71.4	12.4	30.3	62.4

* Each values are means of three results SE = 10µg/ml.

[a] Results corrected because of different volumes of ambient solutions

[b] Negative results were lower than controls in value

Table 27 Total phenol leakage from RM disks cut from inoculated plants in 24h at 20°C.

Time (h) after inoculation	Leakage (µg/ml ambient solution) with control results subtracted				Water control (µg/ml)
	R1	R2	Pf	Ea	
1	*52.61	25.2	-	-	77.28
2	6.0	47.1	89.3	84.9	92.1
4.5	-12.1[b]	3.3	-	-	77.3
6.5	5.48	35.1	-	-	73.9
18	55.1	29.0	26.1	69.0	80.57
24	35.3	73.9	27.9	41.6	73.4
48	-20.2	48.8	36.9	49.6	87.1
120	-42.5	53.1	64.1	81.1	81.1
168	14.2	123.3	-	-	57.6

* Each value is the mean of three results SE = 15µg/ml.

[b] Negative results were lower than controls in value.

was used as an estimate of total phenol leakage in 24h.

Experiments designed to find the rate of phenol leakage determined that 50 - 60% of the phenols leaked in 24h had leaked from the disks in the first 2h of incubation. This was Rate B leakage but slower than with electrolyes, ions or proteins.

Controls were water injected, injured, blank or injected with dead bacterial cells. All were approximately the same and the water control results only are presented in Table 27 with the other results.

Variation in this experiment was quite high but some trends were fairly clear.

With disks from plants inoculated with R1, there was a high level of leakage of phenols at 1h. This fell to very low values and then rose to a peak at 18h only to fall then so low as to be significantly lower than controls. The peak at 18h corresponds with peaks of electrolyte leakage and with onset of tissue collapse.

With disks from plants inoculated with R2, levels of phenol release increased dramatically at 24h which is 24h sooner than peaks of electrolyte and protein leakage and than symptom development.

Disks from plants inoculated with Pf or Ea had very high phenol leakage over the whole course of the experiment. There was no macroscopically visible tissue browning.

This experiment was repeated with similar results.

X Phenoloxidase activity of ambient solution of disks cut from inoculated RM plants.

Using the method as described in Materials and Methods X, ambient solutions of disks cut from inoculated plants were assayed for phenoloxidase activity.

Plants were inoculated with 10^8 cells/ml R1, R2, Pf or Ea

Table 28 Phenoloxidase activity of the ambient solution of disks cut from inoculated RM plants at 20°C.

Time (h) after inoculation	Change in absorbance at 495nm in 1 min; ability to oxidise catechol. Control results subtracted.				Water control
	*R1	*R2	*Pf	*Ea	
1	[a] 0	0.01	0.02	0	0.01
4	-0.01[b]	0.02	0.02	0	0.02
10	0.09	0.01	0	0	0.03
18	0.13	0.01	-0.01	-0.01	0.02
24	0.22	0.06	0	-0.01	0.03
48	0.20	0.21	-0.03	-0.03	0.04
96	0.21	0.25	0.01	0.01	0.01
120	0.14	0.13	0.02	0.02	0.02

[a] Each value is the mean of three results.

SE 0.05 0.05 0.03 0.03 0.03

* Bacteria injected into RM leaves at 10^8 cells/ml.

[b] Negative results were lower than control values.

and disks were cut from the la for analysis. The ambient solution was 5ml per flask with 80 disks (0.3 cm diameter) per flask.

At time 0 and time 2h, 0.2ml of ambient solution was removed from each flask and added to 2ml 0.05M catechol and 0.5ml 0.2M sodium phosphate buffer at pH 7.0 and 0.3ml of water. One minute later absorbance at 495nm was measured against a reagent blank as an estimate of phenol oxidase activity. Results in Table 27 are expressed as change in absorbance at 495nm in one minute. Controls were inoculated with sterile water.

Leakage of phenoloxidase relate well to tissue browning. With plants inoculated with R1 or R2, phenoloxidase activity increased in the ambient solution just before the onset of tissue browning. At 10 - 24h with R1, phenoloxidase activity increased from 0.09 to 0.22 and at 24 - 48h with R2, phenoloxidase activity increased from 0.06 to 0.21.

With control disks and those from plants inoculated with Pf or Ea there was very little phenoloxidase activity and no tissue browning visible macroscopically.

XI Attempts to modify HR using bacteria

A Leaves

RM leaves injected with either R1 or Pmp at 10^8 cells/ml. At various times with respect to inoculation ie before, simultaneously or afterwards, the same leaves were injected in the same place with 10^6 cells/ml of R1, R2, Pmp, Pf, Xm or Ea.

There were three plants per treatment and incubation was in the growth cabinet at 25°C. Controls were Blank, Injured, Water or Dead Cell as before. In no case was protection from HR noted in controls.

1 Prior injection

a 3h

1 Prior injection

a 3h

Plants were first injected with Ea, Xm, Pmp, R1, R2, or Pf at 10^6 cells/ml. Three hours later the same plants were injected with 10^8 cells/ml R1 or Pmp.

In all cases development of HR was normal.

b 24h

Plants were first injected with Ea, Xm, Pmp, R1, R2 or Pf at 10^6 cells/ml and 24h later were re-injected with 10^8 cells/ml R1 or Pmp.

With plants previously injected with Ea, Xm, R2 or Pf development of HR was normal.

With plants previously injected with R1 or Pmp then development of HR was suppressed to some extent. Necrosis was not confluent but was patchy.

2 Simultaneous injection

P lants were inoculated with bacterial suspensions made by mixing 5ml of 10^6 cells/ml Ea, Xm, Pmp, R1, R2 or Pf with 5ml of 10^8 cells/ml R1 or Pmp.

In allcases development of HR was normal.

3 Prior injection with R1 or Pmp at 10^8 cells/ml.

a) 3h

Plants were first injected with R1 or Pmp at 10^8 cells/ml and 3h later were re-injected with Ea, Xm, Pmp, R1, R2 or Pf at 10^6 cells/ml.

In all cases development of HR was normal.

b 12h

Plants were first injected with R1 or Pmp at 10^8 cells/ml and 12h later were re-injected with Ea, Xm, Pmp,R1, R2 or Pf at 10^6 cells/ml. In all cases development of HR was normal.

The experiment was repeated with similar results. It was

only found possible to reduce the effect of HR with 24h previous injection of 10^6 cells/ml Rl or Pmp.

B Disks.

Plants were injected with Rl or Pmp (10^8 cells/ml) in an area 0.4 x 0.5 cm^2 as described previously. The plants were inoculated in the same position with 10^6 cells/ml Xm, Ea, Pmp, Rl, R2 or Pf before, simultaneously or after the Rl or Pmp injection. (at 10^8cells/ml)

Disks of 1.2 cm diameter with the Ia in the disk centre were cut one hour after the second injection to allow water soaking to disappear and were floated on sterile water in petri dishes (four disks and 20 ml per dish). Incubation was in the growth cabinet at 25°C.

Controls were as before and in no case gave protection to the HR to Rl or Pmp which began to develop visibly at around 22h after injection.

1 Prior injection

a 3h

Plants were first injected with Xm, Ea, Pmp, Rl, R2 or Pf at 10^6 cells/ml and then re-injected 3h later with 10^8 cells/ml Rl or Pmp. In all cases development of HR was normal.

b 24h

Plants were first injected with Xm, Ea, Pmp, Rl, R2 or Pf at 10^6 cells/ml and 24h later were re-injected with Rl or Pmp at 10^8 cells/ml.

In the disks from plants previously injected with Xm, Ea, R2 or Pf development of HR was normal.

In the disks from plants previously injected with Rl or Pmp development of HR was delayed until 28h and did not develop normally. The Ia was necrotic only in patches.

2 Simultaneous injection

Plants were inoculated with bacterial suspensions made by mixing 5ml of 10^8 cells/ml R1 or Pmp with 5ml of 10^6 cells/ml Xm, Ea, Pmp, R1, R2 or Pf. In all cases development of HR was normal.

3 Prior injection of R1 or Pmp at 10^8 cells/ml

a 3h

Plants were first injected with R1 or Pmp at 10^8 cells/ml and 3h later were re-injected with 10^6 cells/ml Ea, Xm, Pmp, R1, R2 or Pf. In all cases development of HR was normal.

b 24h

Plants were first injected with R1 or Pmp at 10^8 cells/ml and 12 hours later were re-injected with Ea, Xm, Pmp, R1, R2 or Pf at 10^6 cells/ml. In all cases development of HR was normal.

The development of HR in disks was delayed and lessened by prior injection (24h previously) of 10^6 cells/ml R1 or Pmp only. This experiment was repeated with similar results.

XII Attempts to modify the response of leaf disks floating on suspensions of R2.

In section V1 it was shown that leaf disks floating on suspensions of R2 (10^8 cells/ml) showed sysmptoms of chlorosis at the disk edges at day three. This chlorosis spread towards the disk centre over the next few days. Disks floating on suspensions of R1, Pmp, Xm, Ea and Pf (at 10^8 cells/ml) and on sterile water (control) remained green and healthy for eleven days.

In this experiment, disks were first floated on suspensions of R1, Pmp, Xm, Ea or Pf (10^8 cells/ml) and after 1, 2, 3, 4 or 5 days were removed , washed and floated on suspensions of R2 (10^8 cells/ml.

Disks which had been floated on suspensions of other bacteria for 1 and 2 days responded normally to suspensions of R2. Disks floated on suspensions of other bacteria for more than 2 days showed no response to R2. However the same was true of disks which had floated on sterile water for more than 2 days so this result was not found to be significant.

The resistance of disks floating on suspensions of R1, Pmp, Ea, Xm or Pf or on water was thought to be due to the fact that the cut edges of the disk had by 2 days formed a brown reaction - the wounds had 'healed' and perhaps there was no means of entry into the disk for R2 bacteria after 2 days.

XIII Attempts to modify HR using chemicals.

1 Leaves

RM plants were inoculated with R1 at 10^8 cells/ml . 24h previously, plants had been injected in the same Ia with solutions of various chemicals.

There were three plants per treatment and incubation was at 25°C in the growth cabinet. Controls were plants injected with R1 at 10^8 cells/ml alone or with sterile water. Test compounds were used at levels not toxic to the plants.

The results are presented in Table 29. It can be seen that with the divalent ions calcium, magnesium, strontium, barium, berilium, zinc and molibdenum, HR was suppressed.

Protamine sulphate which has been shown to alter the charge on protoplast membranes (Grout and Coutts, 1974) caused HR development to be only patchy but it was visible.

2 Disks

Disks were cut from RM plants such that their central area only had been injected with R1 at 10^8 cells/ml. This was an area of 0.4 x 0.5 cm2 in a disk of diameter 1.2 cm.

Disks were then floated on solutions (those detailed in Table 29) and responses were noted. It was found that only when disks were floated on 0.1M $CaCl_2.2H_2O$; 0.1 M $Mg(NO_3)_2$; 0.05 M $SrCl_2$; $BaCl_2$; $BeSO_4$; $ZnCl_2$ (last three at 0.05M); MoO_3 at 0.005M. was HR suppressed. When disks were floated on 100µg/ml protamine sulphate HR was visible but patchy. All the other solutions detailed in Table 29 were used but HR development was normal in the disks.

Table 29 The effect of various substances on the development of HR to R1 (10^8 cells/ml)

Substance injected 24h before R1	Concentration	Response of RM leaves
H_2O (Control)		HR
Mannitol	0.1 M	HR
"	0.05 M	HR
Nutrient broth	-	HR
Casein hydrolysate	1g/ml	HR
Sucrose	1%	HR
Glucose	1%	HR
Ringer's solution	25%	HR
Macerase	0.2%	HR
Casamino acids	4.6g/l	HR
K_2HPO_4	1g/l	HR
Glycine	1%	HR
Leucine	1%	HR
Glutamic acid	1%	HR
Arginine	1%	HR
Aspartic acid	1%	HR
Protamine sulphate	100µg/ml	Patchy HR
"	250µg/ml	Patchy HR
$CaCl_2.2H_2O$	0.1M	NR*
$Mg(NO_3)_2$	0.1M	NR
$SrCl_2$	0.05M	NR
$BaCl_2$	0.05M	NR
$BeSO_4$	0.05M	NR
$ZnCl_2$	0.005M	NR
$MnCl_2$	0.005M	HR
$FeCl_3$	0.005M	HR

Table 29 (contd)

Substance injected 24h before R1	Concentration	Response of RM leaves
MoO_3	0.005M	NR*
$CuCl_2$	0.001M	HR

* Suppression of HR.

XIV Production of isolated RM protoplasts.

The methods used to produce isolated RM protoplasts were based on the method devised by Pelcher et al.for Phaseolus vulgaris L. var. Pinto. The following enzyme medium was advocated

0.3M Mannitol

0.25% Cellulase

0.25% Pectinase

pH adjusted to pH 7.0 using 0.2N KOH.

When this medium was used on RM leaves using Macerase and Driselase as pectinase and cellulase, the yield of protoplasts was very low and the presence of large numbers of free chloroplasts in the medium indicated extensive damage to the protoplasts.

When the mannitol concentration was increased to 0.4M and the medium otherwise unchanged, yield of protoplasts was about 1.0×10^4/ml of medium and there were few free chloroplasts to be seen.

The presence or absence of potassium dextran sulphate had little or no effect on yield.

A range of pH was tested but pH 7.0 was found to be best in terms of survival of protoplasts.

A selection of different enzyme media were tested as detailed in Materials and Methods IX. It was found that using the cellulase Onozuka SS instead of Driselase, increased yield of protoplasts

to around 4.4 x 10^4/ml. Driselase for reasons unknown, did not dissolve completely on addition to distilled water and therefore did not pass through millipore filtering properly. Onozuka SS on the other hand did dissolve well and passed through the filters. The higher yield of protoplasts using Onozuka SS was thought to be due to the higher concentration of cellulase present as a result of the ability of Onozuka SS to dissolve more readily than Driselase.

The protoplasts were found to be extremely fragile and damaged very easily by the centrifugation necessary to transfer them to Cell Incubation Medium (CIM). It was found that making up the enzyme medium in CIM with 0.4M Mannitol stabilised the protoplasts to a certain extent. They were still very fragile and even mild shaking of the flasks in which they incubated resulted in damage to the protoplasts. However the use of CIM lessened this greatly.

The use of CIM however, made necessary the increased concentration of the enzymes to 0.45%. They seemed to be slightly less active .

In later experiments it was found necessary to make use of sucrose instead of mannitol. 0.4M sucrose was used and the yield of protoplasts was around 4.0 x 10^4/ml.

The enzyme medium finally decided upon was as follows.

0.45% Macerase

0.45% Onozuka SS

Made up in CIM with 0.4M mannitol or sucrose

pH adjusted to 7.0 using 0.1N NaOH

Yield of protoplasts was 4.4 x 10^4/ml when mannitol was used and 4.0 x 10^4/ml with sucrose. After isolation protoplasts were incubated in CIM with mannitol or sucrose at 0.4M and pH 7.0. Survival of protoplasts was around three days.

XV Production of isolated RM leaf cells.

In this work isolated cells were defined as protoplasts with cell walls. The shape of the cell is retained after tissue digestion whereas protoplasts are spherical.

The method used to produce isolated RM cells was modified from that of Takebe et al.(1968) and is given in detail in Materials and Methods VIII. The method of Takebe et al.was devised for tobacco mesophyll cells and advocates the following enzyme medium:-

0.5% Macerozyme
0.8M mannitol
0.3% potassium dextran sulphate
pH adjusted to 5.4 with 2N HCl

When this medium was used on RM leaves using Macerase instead of Macerozyme, maceration of the leaf tissue took place but isolated cells did not survive at all well.

With further work it was found that the Mannitol concentration was too high. 0.4M Mannitol was found to be the optimum concentration. Below this, cells were damaged and, above this were plasmolysed.

The pH of the enzyme medium was found to be too low. Experiments involving the use of a range of pH indicated that pH 6 - 7.5 was suitable and pH 7.0 was always used.

It was not found necessary to use potassium dextran sulphate as its presence or absence made no difference to yield or survival of cells.

The enzyme medium used was then as follows:-

0.5% Macerase
0.4M Mannitol
pH adjusted to 7.0 with 0.1N NaOH

The yield of cells was found to be around 10^4 cells/ml enzyme

medium. Later experiments required the use of sucrose instead of mannitol and yield fell slightly to 9.0 x 10^3 cells/ml.

After isolation the cells were incubated in Cell Incubation Medium(CIM) detailed in Materials and Methods VIII. This is a modification of the medium used by Takebe et al.(1968) and was supplied by Dr. RHA Coutts of Imperial College. The mannitol concentration (0.4M) and pH (7.0) were as used in the enzyme medium.

Survival of cells in CIM was 2 - 3 days.

XVI Growth of bacteria in CIM

The intention behind the production of isolated RM cells and protoplasts was to study the responses of suspensions of cells or protoplasts to bacteria. Comparisons made between responses of RM cells in leaves and in suspension were thought to be potentially of interest.

It was therefore necessary to devise a medium which would allow the survival of both suspensions of cells or protoplasts and bacteria. Cell Incubation Medium (CIM) itself would not allow the survial of R1.

When a suspension of 10^6 cells/ml R1 in CIM was set up, no movement of the bacteria was seen within a few hours and after 18 hours incubation when 0.1 ml of medium was plated on nutrient growth agar, no colonies developed at all.

The most likely explanation of this was that R1 required some additional nutrient to allow growth. Other reasons could have been the concentration of mannitol or the pH of the medium being inhibitory.

a Bacterial tolerance of pH

Flasks containing 100ml of nutrient broth or 100ml of Medium A (Materials and Methods p25) at a range of pH were inoculated with 1 ml of 10^8 cells/ml R1 giving a final concentration of c. 10^6 cells/ml bacteria. The pH was raised using 0.1N NaOH and lowered

using 0.1 N HCl.

Flasks were incubated at 25°C in an orbital incubator. After 48h, 10ml of culture medium was removed from each flask and centrifuged for 20 minutes at 10,000g. Supernatants were discarded and bacterial pellets were resuspended in distilled water. Bacterial concentration was estimated by use of nephelometry and the suspensions were then injected into RM leaves to ensure that the bacteria were still able to induce HR. The results are presented in Table 30. The growth of R1 in the pH range 5.4 - 8.0 was not affected by pH and the bacteria were still able to induce HR in RM leaves

Table 30 Growth of R1 in nutrient broth or Medium A at a range of pH at 25°C.

Medium and initial pH	Log concentration bacteria per ml. after 48h incubation [b]	RM leaf response	Final pH
Medium A			
pH 7.3	9.21*	HR*	7.75
4.4	8.64	[a] HR⅔	5.63
5.4	9.26	HR	7.40
6.4	9.10	HR	7.45
6.9	9.28	HR	7.90
7.5	9.10	HR	7.40
8.0	8.90	HR	7.20
Nutrient Broth			
pH 5.4	9.52	HR	7.60
6.2	9.54	HR	7.70
6.9	9.41	HR	7.30
7.9	9.50	HR	7.90

* Mean of three replicates

[a] HR in two out of three replicates only

[b] Initial inoculum concentration was 10^6 cells/ml.

b) Bacterial tolerance of mannitol

In the same way as described in (a) above nutrient broth and Medium A were made up with 0.3 - 0.8M mannitol. Flasks were inoculated such that the initial concentration of bacteria (R1 was 10^6 cells/ml.

The concentration of bacteria was estimated after 48h grpwth at 25°C by nephelometer measurements. Suspensions were injected into RM leaves to test the ability to induce HR. The results are given in Table 31 which shows that the tolerance of high levels of mannitol by the bacteria is very good and the ability to produce HR in RM leaves is not affected by mannitol.

Table 31 Growth of R1 in nutrient broth or medium A supplemented with Mannitol at 25°C.

Culture medium and mannitol concentration	Log concentration bacteria/ml after 48h growth[b]	RM leaf response.
Nutrient broth (NB)	*9.31	*HR
NB + 0.3M mannitol	9.28	HR
NB + 0.5M mannitol	9.19	HR
NB + 0.8M mannitol	8.98	HR
Medium A (MA)	9.21	HR
MA + 0.3M mannitol	9.27	HR
MA + 0.5M mannitol	9.17	HR
MA + 0.8M mannitol	9.05	HR

* Means of three replicates
[b] Initial inoculum concentration 10^6/ml.

c) To find if the Cell Incubation Medium contains a substance toxic to R1.

In this experiment, R1 was grown in Medium A plus CIM (ratio 1:1) and in Medium A supplemented with KI, $CaCl_2.2H_2O$ and KNO_3 - all constituents of CIM. All media were at pH 7.0 and incubation was at 25°C for 48h.

The results obtained as in (a) and (b) above are presented in Table 32 which shows that addition of CIM to Medium A resulted in reduced growth of R1 but bacteria were subsequently able to induce normal HR in RM plants. These results did not indicate a toxic effect in CIM to R1.

Table 32 Tests of growth of R1 in Medium A (MA) supplemented with Cell Incubation Medium(CIM) or its constituents (25°C.

Medium pH = 7.0	Log concentration bacteria/ml in 48h incubation[b]	RM leaf response
MA	*9.22	*HR
CIM	0	[a]NR
MA + CIM(1:1)	8.85	HR
MA + 1 M KI	8.99	HR
MA + 0.1mM $CaCl_2$	9.19	HR
MA + 1mM KNO_3	9.2	HR

* Each value is the mean of three replicates.
[a] No macroscopically visible response
[b] Initial inoculum concentration 10^6 cells/ml

Table 33 Growth of R1 in 48h at 25°C in CIM supplemented with various substances (allmedia pH 7.0 and with 0.4M mannitol).

Culture Medium	Log concentration bacteria/ml [b]	RM leaf response	Final pH of medium
Medium A	*9.22	*HR	*7.37
CIM	0	[a]NR	5.75
Medium A + CIM	8.85	HR	6.33
CIM + 4.6 g/l casamino acids	8.43	HR	6.4
CIM + 1g/l K_2HPO_4	0	NR	7.0
CIM + 0.5g/l $MgSO_4.7H_2O$	0	NR	7.1
CIM + 1% trace elements	0	NR	7.3

(contd)

Table 33 (contd)

Culture Medium	Log concentration Bacteria/ml[b]	RM leaf response	Final pH of medium
CIM + 2g/l $NaCO_3$	*0	*NR[a]	*7.1
CIM + 0.6g/L sodium dodecyl sulphate	0	NR	6.23
CIM + 2.3g/l Na_2HPO_4	0	NR	7.1
CIM + 5g/l NH_4Cl	0	NR	7.1
CIM + 10g/l sucrose	0	NR	7.0
CIM + 1g/l casein hydrolysate	8.64	HR	7.0
CIM + 1% glycerol	0	NR	7.1
CIM + 0.4g/l ribose	0	NR	7.0
CIM + 1g/l dioctyl sulpho-succinate	0	NR	7.1
CIM + 10g/l sodium tartrate	0	NR	7.1
CIM + 7.5g/l cellobiose	0	NR	7.1
CIM + 0.01g/l Thiamine HCl	0	NR	6.9
CIM + 0.13g/l $CoCl_2$	0	NR	6.9

(contd)

Table 33 (contd)

Culture Medium	Log concentration bacteria/ml[b]	RM leaf response	Final pH of medium
CIM + 0.01g/l Hydroxy L proline	*0	*NR[a]	*7.02
CIM + 1mg/l Pyridoxine HCl	0	NR	7.1

* Each value is the mean of three replicates.

a No macroscopically visible response.

b Initial inoculum concentration 10^6 cells /ml Rl.

d) Growth of R1 in CIM supplemented with various substances

From previous results it seemed likely that R1 could not grow in CIM because of nutrient defficiency.

In this experiment, R1 were grown in 50ml flasks in CIM with various supplements. The substances used as supplements are detailed in Table 33 and were constituents of different bacterial growth media used in the same concentrations recommended for the original medium. The methods used for growth and for obtaining the results in Table 33 were the same as described in (a) and (b) above.

The results in Table 33 show clearly that R1 will grow in CIM when it is supplemented with casein hydrolysate or casamino acids.

Having found a medium which would support the growth of of R1 under these conditions, it was then necessary to find out if RM cells or protoplasts would isolate or survive in medium supplemented with casein hydrolysate.

It was found that the presence of casein hydrolysate in CIM did not have an adverse affect on the survival of isolated RM cells or protoplasts but it presented another problem. The medium was now suitable not only for the growth of R1 but also for other bacteria and fungi. Contamination was a major problem.

The use of antibiotics was not possible in this case because the growth of R1 and R2 was desired. The answer was scrupulous care in sterile techniques. The levels of saprophytic organisms in leaf tissue are fairly high however and some contamination was always found. However over three days the contamination was not great and it was decided that further experimentation was possible. It must be borne in mind however that the results may have been affected by these contaminating organisms.

XVII Survival of RM cells in suspension with R1 or R2

A suspensions of RM cells was produced as described previously at a concentration of c. 10^4 cells/ml estimated by use of a haemocytometer. The cells were suspended in CIM with 0.4 M mannitol.

The suspension was divided into nine flasks each of which had a supplement of 0.001 g/ml casein hydrolysate. There was 10 ml suspension per flask and to three of these was added suspension of R2 such that the final concentration of R2 was 10^7 cells/ml; the remaining three flasks were controls.

The numbers of RM cells and R1 or R2 cells were followed over a period of 72h by use of haemocytometer and nephelometry respectively. Bacteria, isolated, washed and concentrated for nephelometry measurements were subsequently injected into RM plants to ascertain their ability to produce HR or SR normally. The results are in Table 34 which shows that in control flasks a drop in levels of RM cells was not seen to be marked until 72h of incubation and bacteria were not detectable until 48h. These bacteria did not when injected at 10^8 cells/ml into RM plants elicit any macroscopically visible response.

In flasks inoculated with R1, RM cell numbers were seen to drop appreciably by 48h and numbers of bacteria increased. Cells starting at 10^4/ml fell to 10^2/ml at 48h and bacteria increased from 10^7/ml to c. 10^8/ml at 48h. The bacteria when injected into RM leaves at 10^8 cells/ml produced normal HR.

In flasks inoculated with R2 the situation was much the same. RM cell numbers dropped from 10^4/ml to c. 10^2/ml by 48h. Numbers of bacteria increased steadily from 10^7/ml to c. 5.0×10^8/ml by 72 hours. The bacteria when injected into RM leaves at 10^8 cells/ml produced normal SR.

In the RM cell suspensions there were no signs at all of HR or

Table 34 Survival of RM cells in suspension with R1 or R2 at 25°C.

Time (h)	Bacterium	Log conc. Rm cells/ml	Log conc. bacterial cells/ml	Response of RM leaves
0	R1	*4.0	*7.0	**HR
24		4.0	7.9	HR
48		2.0	8.1	HR
72		0.4	8.5	HR
0	R2	*4.0	*7.0	**SR
24		3,9	7.9	SR
48		2.3	8.1	SR
72		0.9	8.5	SR
0	Water	*4.0	None detectable	**NR
24	control[a]	4.0	"	NR
48		3.9	*2.3	NR
72		2.9	3.6	NR

* Each value is the mean of three results.

** Three RM plants tested.

[a] No inoculum added.

SR as known in the whole plant. RM cells died at the same rate in suspension with R1 and with R2 and showed no signs of browning. There were no obvious differences between R1 and R2 in their action in this case.

The experiment was repeated with similar results.

It was noted during microscopic examination of the suspensions that bacteria seemed much more active in the near vicinity of air bubbles than in the main suspension and it was thought that the absence of obvious differences between R1 and R2 action might be due to aeration problems. Attempts to shake cultures while incubation took place were not satisfactory as the only equipment available for the purpose was much too fast in its action. The rough treatment given by even mild shaking caused death of RM cells.

XVIII Survival of RM protoplasts in suspension with R1 or R2.

The same experiment as described previously for RM cells was set up using RM protoplasts. Relative numbers of RM protoplasts and R1 or R2 bacteria were followed for 72h using a haemocytometer and nephelometry respectively. The bacteria were washed and concentrated for nephelometry measurements and then injected into RM leaves to assess their ability to produce HR or SR. Three plants (six leaves) were used per treatment.

The results in Table 35 show that in control flasks levels of RM protoplasts were not seen to drop until 72h of incubation and bacteria were not detectable by nephelometry until 48h. These bacteria when injected into RM leaves at 10^8 cells/ml did not elicit any macroscopically visible response.

In flasks inoculated with bacteria, protoplast levels fell appreciably by 48h to c. 10^2/ml with R1 and 10^3/ml with R2. Numbers of bacteria increased by around 10^2 cells/ml in 72h and the bacteria from flasks inoculated with R1 or R2 produced HR or SR respectively when injected into RM leaves at 10^8 cells/ml. In

Table 35 Survival of RM protoplasts in suspension with R1 or R2 at 25°C.

Time (h)	Bacterium	Log conc. RM protoplasts/ml	Log conc. bacterial cells/ml	Response of RM leaves
0	R1	*4.00	*7.0	**HR
24		3.66	8.7	HR
48		2.32	8.81	HR
72		1.00	9.1	HR
0	R2	* 4.00	*7.0	**SR
24		3,55	8.5	SR
48		3.0	8.9	SR
72		0,96	8.9	SR
0	Water control[a]	*4.00	None detectable	**NR
24		4.00	"	NR
48		3.92	1.92	NR
72		2.36	2.68	NR

* Each value is the mean of three results

** Three RM plants tested.

[a] No inoculum added.

no case was there any sign of HR or SR as seen in whole plants. There were no obvious differences in the action of R1 and R2 in this case as with the previous case.

This experiment was repeated with similar results.

As in the previous study, it was noted that bacteria in inoculated flasks were more active in the near vicinity of air bubbles on microscope slides. However because the protoplasts were so fragile it was not possible to aerate the cultures.

XIX Survival of RM cells and protoplasts in modified culture media with R1 and R2.

The work of Kopp et al.(1977) showed that, in suspension cultures of tobacco leaf protoplasts, mannitol inhibited the enzyme phenyl alanine ammonia lyase (PAL). The concentration of mannitol needed for 100% inhibition of PAL was 0.2M. The inhibition could be reversed by increasing concentrations of the substrate of PAL - L-phenylalanine. Kopp et al. also found that sucrose did not inhibit PAL activity until 0.7M was used. This concentration produced 7% inhibition of PAL..

As the enzyme PAL is involved in the production of phenolic compounds associated with necrotic responses in plants, it was thought that the 0.4M mannitol used in the CIM might be partly the cause of the failure of the cells in particular to show necrosis in their responses to R1 or R2.

Growth and survival of plant and bacterial protoplasts and cells were observed using media with (a) sucrose instead of mannitol and (b) mannitol(0.4M) but supplemented with 80 mM L-phenyl alanine. The experiment was set up as described before and bacterial numbers were estimated by nephelometry. Plant cell or protoplast numbers were estimated by use of a haemocytometer. The results are in Table 36.

Table 36 shows that the addition of 80 mM L-phenylalanine to

Table 36 Survival of RM protoplasts or cells in suspension with R1 or R2 in various culture media at 25°C.

1) RM cell suspensions

Treatment	Time (h)	Log conc.RM cells/ml	Log conc. bacterial cells per ml	Response of RM leaves.
Medium 1 control	0	*4.00	None detectable	**NR
	24	4.00	"	NR
	48	3.86	1.52	NR
	72	3.00	2.16	NR
Medium 1 + R1 (10^7/ml)	0	4.01	7.00	HR
	24	3.66	8.61	HR
	48	3.05	8.90	HR
	72	1.15	9.00	HR
Medium 1 + R2 (10^7/ml)	0	4.00	7.00	SR
	24	3.51	8.50	SR
	48	2.90	8.75	SR
	72	0.90	8.99	SR
Medium 2 control	0	4.00	None detectable	NR
	24	4.00	"	NR
	48	3.55	1.90	NR
	72	2.92	2.66	NR
Medium 2 + R1 (10^7/ml)	0	4.00	7.00	HR
	24	3.41	8.60	HR
	48	2.90	8.91	HR
	72	1.00	9.00	HR
Medium 2 + R2 (10^7/ml)	0	4.00	7.00	SR
	24	3.50	8.59	SR
	48	3.00	8.80	SR
	72	2.05	8.90	SR

(contd)

Table 36 (contd)

1) RM cells suspensions (contd)

Treatment	Time (h)	Log conc. RM cells/ml	Log conc. bacterial cells per ml	Response of RM leaves.
Medium 3 control	0	*4.00	None detectable	**NR
	24	4.00	"	NR
	48	3.56	*1.00	NR
	72	3.00	2.01	NR
Medium 3 + R1 (10^7/ml)	0	4.00	7.00	HR
	24	3.21	8.20	HR
	48	2.90	8.55	HR
	72	1.00	8.80	HR
Medium 3 + R2 (10^7/ml)	0	4.00	7.00	SR
	24	3.56	8.10	SR
	48	2.80	8.41	SR
	72	1.o5	8.65	SR
2) RM protoplasts suspensions				
Medium 1 control	0	4.00	None detectable	NR
	24	4.00	"	NR
	48	3.60	1.50	NR
	72	3.10	2.96	NR
Medium 1 + R1 (10^7/ml)	0	4.00	7.00	HR
	24	3.50	8.50	HR
	48	3.10	8.66	HR
	72	2.21	8.80	HR
Medium 1 + R2 (10^7/ml)	0	4.00	7.00	SR
	24	3.40	8.60	SR
	48	3.0	8.70	SR
	72	2.10	8.90	SR

(contd)

Table 36 (contd)

2) RM protoplasts suspensions (contd)

Treatment	Time (h)	Log conc RM protoplasts per ml	Log conc bacterial cells/ml	Response of RM leaves
Medium 2 control	0	*4.00	None detectable	**NR
	24	4.00	1.01	NR
	48	3.50	1.90	NR
	72	3.00	2.32	NR
Medium 2 + R1 (10^7/ml)	0	4.00	7.00	HR
	24	3.21	8.62	HR
	48	2.91	8.80	HR
	72	1.01	8.90	HR
Medium 2 + R2 (10^7/ml)	0	4.00	7.00	SR
	24	3.30	8.50	SR
	48	2.80	8.75	SR
	72	0.91	8.89	SR
Medium 3 control	0	4.01	None detectable	NR
	24	4.00	"	NR
	48	3.61	1.91	NR
	72	2.50	3.00	NR
Medium 3 + R1 (10^7/ml)	0	4.00	7.00	HR
	24	3.02	8.20	HR
	48	2.40	8.60	HR
	72	1.20	8.80	HR
Medium 3 + R2 (10^7/ml)	0	4.00	7.00	SR
	24	3.01	8.10	SR
	48	2.50	8.75	SR
	72	1.01	8.90	SR

Medium 1 CIM with mannitol at 0.4M
Medium 2 CIM with sucrose at 0.4M
Medium 3 CIM with mannitol at 0.4M and L-phenylalanine at 80mM

* Means of three results.
** Three plants used per test.

the medium or replacement of mannitol by sucrose made no difference to the numbers of cells or protoplasts of RM leaves surviving, nor did this affect the mutiplication of the bacteria or influence their subsequent ability to produce HR or SR in RM leaves.

Again there were no affects noted that were comparable with HR or SR in the suspended RM cells or protoplasts.

XX Levels of total phenols in suspension cultures of RM cells or protoplasts with R1 or R2.

Suspensions of RM cells or protoplasts were set up in the following media:-

1 CIM with 0.4M Mannitol

2 CIM with 0.4M sucrose

3 CIM with 0.4M mannitol and 80mM L-phenylalanine

At intervals 1 ml of medium was removed from each flask and was centrifuged at 12,000g to pellet cell fragments and bacterial cells. The sample was finally filtered through millipore filters before being analysed for total phenols as described in Materials and Methods(IVb). Samples were taken at Time 0 and Time 24h during incubation and the difference was noted as the change in total phenol levels in 24h. This time was chosen because in controls RM cells or protoplasts had not begun to die and contaminating levels of bacteria were still too low to detect. With inoculated cell or protoplast suspensions, the cells or protoplasts showed marked signs of decrease in number at 24h.

Out of interest total phenol levels were analysed in the medium during enzymic dgradation ie during the time of isolation of cells and protoplasts. In the case of protoplasts it was the difference between Time 0 and Time 24h and with cells it was the difference between Time 0 and Time 4h as in each case the times of isolation were different.

Table 37 Levels of total phenols (as increase during incubation times) in RM cell or protoplasts isolation or incubation suspensions with or without R1 or R2.

1) RM cell suspensions.

a Enzyme media.

Medium	Total phenol Time 0 (µg/ml)	Total phenol Time 4h (µg/ml)	Difference in 4h incubation (µg/ml)
1 + E*	[a]138.95	141.14	2.19
2 + E	134.87	127.48	-7.40
3 + E	123.37	123.37	0

* Enzyme Macerase at 0.5%

b) Incubation media.

Medium	Total phenol Time 0 (µg/ml)	Total phenol Time 24h (µg/ml)	Difference in 24h incubation (µg/ml)
1	[a]63.32	60.86	-2.46
2	29.64	37.87	8.23
3	30.64	31.36	0.74
1 + R1[b]	107.46	77.32	-30.14
2 + R1[b]	60.88	45.26	-15.62
3 + R1[b]	64.17	46.09	-18.09
1 + R2[b]	100.51	80.50	-20.01
2 + R2[b]	68.98	57.46	-11.52
3 + R2[b]	69.00	55.10	-13.90

2) RM protoplast suspensions

a) Enzyme media.

Medium	Total phenol Time 0 (µg/ml	Total phenol Time 24h (µg/ml)	Difference in 24h incubation (µg/ml)
1 + E2**	[a]179.26	182.55	3.31
2 + E2	185.84	228.53	42.69
3 + E2	222.01	228.59	6.57

b) Incubation media.

Medium	Total phenol Time 0 (µg/ml)	Total phenol Time 24h (µg/ml)	Difference in 24h incubation (µg/ml)
1	[a]58.68	41.14	-17.53
2	98.69	74.03	-24.66
3	64.82	46.13	-18.69

** Enzymes Macerase and Onozuka at 0.45%

(contd)

Table 37 (contd)

Medium	Total phenol Time 0 (µg/ml)	Total phenol Time 24h (µg/ml)	Difference in 24h incubation (µg/ml)
1 + R1[b]	[a] 30.73	24.15	-6.57
2 + R1[b]	66.63	222.01	155.38
3 + R1[b]	62.53	68.27	5.75
1 + R2[b]	39.63	39.63	0
2 + R2[b]	61.00	161.53	100.53
3 + R2[b]	63.83	74.21	10.39

Medium 1 CIM + 0.4M mannitol

Medium 2 CIM + 0.4M sucrose

Medium 3 CIM + 0.4M mannitol + 80mM L-phenylalanine

[a] All values are the means of three results SE = 10.5

[b] Inoculum concentration 10^7 cells/ml.

The media used for isolation were as Media 1, 2 and 3 named above but with the addition of the necessary enzymes (Macerase and Onozuka SS). The results are presented in Table 37.

During the four hour isolation of cells from RM leaves there was very little change in levels of phenols in the suspension This was also true of the incubation of the cells in the different media alone.

When bacteria were added to the suspension at 10^7 cells/ml levels of total phenols in the suspensions fell in all cases, mostly so in suspensions containing only mannitol ie medium 1. The variation between samples was such that the significance of this result was doubtful. However the experiment was repeated with similar results and the addition of L-phenylalanine seemed to result in slightly less of a drop in phenol levels.

There was no obvious difference between suspensions with R1 and those with R2.

During the 24h isolation of protoplasts from RM leaves suspensions in Media 1 and 3 showed little or no increase in phenol levels but there was quite a marked increase in phenol levels in medium 2 (42.69µg/ml).

During incubation of protoplasts in fresh medium without enzymes, levels of phenols in all media fell over 24h. This drop was about 20µg/ml in each case. If bacteria wwere added to the suspensions, in media 1 and 3, levels of phenols did not change much over 24h but in medium 2 there was always a very great increase (more than 100µg/ml) in levels of phenols in 24h.

Thus although the behaviour of suspensions of RM cells and RM protoplasts is quite different, in each case, the response to addition of bacteria is the same whether the bacteria are R1 or R2.

XXI Levels of phenoloxidase in suspensions of RM cells or protoplasts with R1 or R2.

Suspension cultures of RM cells or protoplasts were set up in CIM with 0.4M sucrose or mannitol. Some flasks had R1 at 10^7 cells/ml added; some had R2 at 10^7 cells/ml added; some were left as controls with no bacteria added. At Time 0 and Time 24h during incubation, 0.2ml of medium was removed and added to 2ml of 0.05M catecho 0.5ml 0.2M sodium phosphate butter at pH 7.0 and 0.3 ml water; one minute later absorbance at 495 nm was measured against a reagent blank as an estimate of phenoloxidase activity.

The results in Table 38 are given as absorbance at 495 nm after 1 minute. The table shows that there is very little difference in levels of phenol oxidase activity over 24h in these suspension cultures. This goes some way to explaining why no browning of cells in suspension or of cell debris in suspensions of both cells and protoplasts occurs.

Table 38 Phenoloxidase activity of suspensions of RM cells or protoplasts with R1 or R2.

Medium	Absorbance at 495nm in 1 min.	Time 0	Time 24h
CIM + mannitol	a	0.01**	0.02
	b	0.015	0.015
CIM + sucrose	a	0.03	0.03
	b	0.02	0.03
CIM + mannitol + R1*	a	0.11	0.13
	b	0.14	0.15
CIM + sucrose + R1	a	0.14	0.14
	b	0.12	0.13
CIM + mannitol + R2*	a	0.12	0.14
	b	0.14	0.14
CIM + sucrose + R2	a	0.14	0.16
	b	0.15	0.17

a RM cells

b RM protoplasts

* at 10^7 cells/ml

** Each value is the mean of three results.

DISCUSSION

Interactions between plants and bacteria have been divided into five categories by Kelman and Sequeira (1972). In this work some of these categories were studied using Phaseolus vulgaris var. Red Mexican (RM). The bacteria were as follows.

a) Non pathogenic or saprophytic eg Pseudomonas fluorescens (Pf)

b) Pathogenic bacteria with a non-host plant

   1 Resulting in hypersensitive response (HR) from the plant eg P. phaseolicola Race 1(R1) and P. mors-prunorum (Pmp)

   2 Resulting in no macroscopically visible response from the plant eg Xanthomonas manihotis (Xm) and Erwinia carotovora var. atroseptica (Ea).

c) Pathogenic bacteria with a host plant eg P. phaseolicola Race 2 (R2).

It was found that RM plants were resistant to Pf, Xm and Ea with no visible response and resistant to R1 and Pmp in the form of HR. These results agree with Lallyett (1977) working with the same plants and bacteria. RM plants were susceptible to R2 and the halo blight symptoms were as described by Patel and Walker (1963).

In 1971 Klement postulated that HR was the normal response of a plant to pathogenic bacteria to which it was resistant. The exceptions to this rule were the soft rot bacteria. Ea is a soft rot organism and as no HR resulted from the injection of Ea into RM leaves, Klement's view is so far supported. However Xm is not a soft rotting species and does not cause HR in RM leaves. This absence of HR was also noted in RM plants to Xm inoculated by high pressure spray was noted by Ikotun(1975).

In this study the threshold levels of R1 and Pmp needed to produce HR in RM leaves was 5.0×10^6 cells/ml suspension. This was also the threshold found for confluent necrosis in tobacco

inoculated with P. syringae (Klement, 1964) and 6.8×10^7 cells/ml was the threshold level of Pmp needed to produce HR in beans (Klement,1967). 5.0×10^6 cells/ml of bacteria in RM leaves has been calculated to be a ratio of bacterial to plant cells of 2:1 (O'Brien,1973) and so this work seems to support the hypothesis of Ercolani (1973) that the pattern of infection observed in bacteria in non-host plants is due to the co-operative action of several cells of the bacteria to overcome the plants' defenses against HR. Synergism of this kind is known in vivo with drug molecules (Meynell and Stocker, 1957) and Ercolani (1973) thought that the interaction between inoculated incompatible bacteria in plant tissues, reflects the joint cummulative action of some bacterial component on the plant.

The numbers of R1 and R2 recovered from RM leaves following inoculation were similar to those found by Omer and Wood(1969). The numbers of R2 increased to levels far higher than R1 or Pmp and in all cases bacterial multiplication stopped at the onset of necrosis. This has also been noted by Stall and Cook (1966) in pepper inoculated with X. vesicatoria.

Numbers of Pf did not increase in RM tissue although low levels of the saprophyte persisted for several days.

Numbers of Xm and Ea recovered from RM leaves remained fairly steady for seven days at around $10^5/cm^2$ leaf tissue. These levels were much higher than levels of Pf recovered but the figures for Ea agree with those found by O'Brien (1973). The work of Ikotun (1975) using Xm on RM plants gave a much lower figure (c. $10^2/cm^2$) for survival of Xm over a few days. However the levels did persist and did not increase.

When inoculum concentrations below threshold level were used interesting results were obtained with R1 and Pmp. Although the bacteria in RM leaves increased in 24h and 72h respectively to levels high enough to cause HR no confluent necrosis developed.

This was also found by Novacky *et al.* in 1972.

This result would seem to contradict Ercolani's theory (1973) of co-operative action. The bacteria have multiplied to the required bacterial/plant cell ratio but no necrosis has developed. However this result was thought to be an indication of a protective effect developing during the time taken for bacteri to multiply. Goodman (1971) inoculated tobacco with concentrations of bacteria too low to cause HR and found that subsequent inoculation with 10^8 cells/ml *P. tabaci* failed to produce the expected HR. This implies that a protective effect can be achieved by injection of levels of bacteria below threshold levels.

In this work, low (10^6 cells/ml) inoculations of R1 and Pmp prevented the expected HR to 10^8 cells/ml R1 and Pmp 24h later. This was also found in disks of RM tissue floating on water. In 1972 Sequeira *et al.* proposed that incompatible bacteria in tobacco leaves released both the HR inducing factor and the factor protecting against HR. The response to the first at threshold levels would be so rapid that the effect of the second would be masked. At lower than threshold concentrations, the protection factor would act and prevent HR when a second inoculation of threshold level was given. The work in this thesis supports this idea.

However prior injection of R2, Pf, Ea or Xm did not have a protectant effect. Cook (1971) found that protection was not given in tobacco by xanthomonads, but in 1973 O'Brien found that R2 inoculations protected against HR caused by inoculations of R1 18h later.

During the growth of R2 in RM leaves no lag phase was noted. This does not confirm the work of Mobley *et al.* (1972) but O'Brien (1973) found a similar result.

Ercolani (1970) has explained the different patterns of growth of bacteria *in vivo* by postulating a "unified hypothesis of induced resistance against bacterial infection". Saprophytic

bacteria are invisaged as lacking a pathogenicity factor essential for growth in living tissue and thus occupy "transit" sites within the host. Phytopathogenic bacteria possess the factor and are able to occupy "multiplication" sites within the host. Multiplication sites are composed of "sensitivity" and "susceptibility" determinants. Activation of the former results in HR whereas activation of a susceptibility determinant counteracts HR and enables the pathogen to develop. All pathogenic bacteria are thought to possess virulence factors which activate susceptibility determinants in compatible combinations. However this idea does not explain why Xm and Ea persist at such high levels in RM tissue but do not cause HR or SR. This kind of resistance is probably very common and deserves more attention.

The results from electrolyte leakage from disks agree with Goodman (1968) using tobacco and Cook and Stall (1971) using pepper. The three stages of HR as defined by Klement (1971) were detected. Induction phase ended with a small rise in electolyte leakage (at 2 - 3 hours), latent phase corresponded with an increase in electrolyte loss but no visible symptoms. In this phase respiration rate may rise (Nemeth et al. 1969). The maximum electrolyte loss corresponded with the phase of expression or time of tissue collapse.

Electrolyte losses from susceptible lesions (plants inoculated with R2) were far greater but developed more slowly. It has been suggested that electrolyte leakage contributes to the in vivo nutrition of phytopathogens (Link and Wilcox. 1936; Thatcher, 1939).

Losses from disks cut from plants inoculated with Xm or Ea were greater than controls but not markedly so. With Pf losses of electrolytes were extremely low and compared well with the losses from disks cut from plants inoculated with below threshold levels of R1.

When electrolyte losses were examined using disks cut from a zone adjacent to the inoculated area (Ia) and called the Ih zone there were no significant losses from this area of plants injected with R1, Pmp, Pf, Xm or Ea. This tends to contrast with the findings of Tomiyama (1958) who found that a certain amount of healthy tissue needed to be involved of plant (potato) tissue was to be completely resistant to blight. However the involvement need not have involved electrolyte losses.

In the Ih zone of plants injected with R2, electrolyte losses from disks related very well with the spread of the chlorotic halo, typical of the susceptible response, into the Ih zone.

Measurements of the rate of electrolyte losses from disks cut from inoculated leaves, showed that rates in all cases (plants inoculated with R1, R2, Pmp, Xm, Ea, Pf in Ia and Ih zones) were hyperbolic and reached a plateau in approximately one hour. About 50 - 60% of the total loss in 120 minutes of incubation occurred in the first 15 minutes and this did not depend on the final levels reached but was always true. This effect was also demonstrated byEiseman et al.(1973) using lipid bilayer membranes but contrasts with the work of Pegus (1976) who, using tomato leaf and pith tissue disks with Fusarium sp. found a slow linear rate of electrolyte leakage. Linear rates were also found by Williams and Keen (1967) working with cucumber and P. lachrymans.

On closer inspection of the work mentioned in the last paragraph, it was found that the methods used in each case were not common. In this work, 20 disks of 0.2cm diameter were cut from the required areas, washed briefly and put into 10ml water for the first measurement. In the work of Pegus (1976), leaf disks and pith tissue disks were first washed in several changes of distilled water until the conductivity of the washings was constant. Then disks were placed in distilled water for the first measurement. Williams and Keen (1967) used whole detached leaves of cucumber

which they put in 200ml of water and measured the rate of leakage over a few hours. Another method, that of Toprover and Glinka (1976) used 8mm diameter disks washed in running tap water for one hour prior to use. The rate of electrolyte leakage (from beet root cells) was slow at first and then became very fast. The metho of giving disks a brief washing before using seems to be the most common eg Cook and Stall (1968); Goodman (1968); O'Brien (1973) and Lallyett (1977) but the practise of examining leakage from excised plant tissue is open to question as to the source of the electrolyte leakage.

In this work, the leakage was hyperbolic at first. Does this originate from the damaged cells at the disk edges or is it a result only of membrane permeability changes as has always been assumed? It was found that the hyperbolic leakage could not be induced to happen twice with the same disks in fresh water and so it seemed unlikely that the effect was due to a balance or equilibrium being set up across the leaf cell membranes. If this had been the case, it would perhaps have been possible in replacing the disks in fresh water, to force them to leak more electrolytes at a hyperbolic rate. However, if the leakage originates only from the disk edges then results would seem to indicate that cell contents were increasing in levels of electrolytes during development of HR or SR. Experiments using autoclaved RM tissue disks showed however that the total electrolyte levels do not change much during HR or SR from levels in controls. Other experiments indicated that the length of cut edge of disks had slightly more influence on the amount of disks leakage than had volume of disks. Leakage was not proportional to edge/volume ratio This, too, is not conclusive as either the damaged edge cells are causing the leakage or leakage from cells within the disk is leaving the disk via the cut edge.

Electrical resistance (reciprocal of conductance) was used

by Osterhaut (1912) as a method of studying penetration of various ions into cells. He determined that penetration was slower into living cells than into dead cells and that conductivity could be used to "measure the permeability of protoplasm" (Osterhaut, 1918) because "resistance of the cell wall remains unaltered (as) the protoplasm undergoes great variations" (Osterhaut, 1921). More recently work by Goodman and Plurad (1971) has shown that at times of maximum electrolyte leakage from disks cut from inoculated plants, membranes became deranged and it seems very likely that their permeability increases.

It has been suggested that ions liberated from cell walls could contribute to conductivity changes (Friedman and Jaffe, 1960) but Stephens (1974) demonstrated that in potato tissue this was not so. Disks of potato tissue were killed with ethanol and left to leach completely in water. After this point, when treated with pectic enzymes, no leakage occurred.

On balance the evidence suggests that at least part of the electrolyte losses from these disks is a result directly of membrane permeability. However there is some influence of the cells of the disks at the cut edges and this should always be borne in mind.

Using similar techniques (leakage from disks) but with healthy plants, the effect of temperature of incubation on leakage has been studied by membrane physiologists. Generally temperature of 40°C and over cause irreversible damage to plant cell membranes (Toprover and Glinka, 1976). Siegel (1969) suggest that initially, elevating temperatures bring about reversible conformational changes in the membranes. With continuing increase in temperature in the presence of oxygen, membrane chemical groups susceptible to oxidation are exposed and upon oxidation render conformational changes irreversible. This means that any plant tissue has a distinct temperature-leakage relationship and in this

work, the influence of R1 and R2 on the relationship was studied.

In the experiments reported here, control RM disk leakage at 50°C corresponded with the figures obtained for leakage from autoclaved disks (80 - 100μmhos). The effect on the disks is quite clear with increase in temperature corresponding with a small increase in leakage until at between 37° and 50°C leakage increased at a far greater rate.

When disks were cut from plants inoculated with R1 or R2 , the increase in leakage at 50°C became much less marked at 3d and 5d respectively - the time of browning and desiccation of the Ia. The losses in general at these times are lower than losses from controls at 50°C but the temperature effect is still noticeable at a time when membranes are totally deranged (Goodman and Plurad, 1971).

Triton X-100 is a non-ionic detergent which disrupts membranes and has been shown to influence leakage from disks of potato tissue(Stephens, 1974). The effect on RM leaf tissue was rather similar in that massive leakage of electrolytes occurred at the time of injection and very little leakage occurred after this. In the Ih zone adjacent to the inoculated zone, disks showed great electrolyte loss at the time of spreading of the TritonX-100 symptoms into the zone. In this respect the response was similar to that in plants inoculated with R2.

The effect of Triton X-100 on the temperature leakage relationship was to markedly reduce the effect of the large rise in conductivity between 37° and 50°C. In necrotic tissue formed as a results of injection of R1 or R2 this effect was never so noticeably removed. There are some similarities between bacterial and Triton X-100 action on RM tissue in that leakage in both cases occurs at the point of tissue collapse.

When RM leaves were injected with 1% w/v $ZnSO_4.7H_2O$ the

response was similar to HR. Tissue collapse occurred at 18h in the Ia. Zinc is known as an inhibitor of enzymes acting as an ion competitor and of sulphydryl groups (Tsuba and Hudson, 1951). There was also an effect in the Ih zone which was not found with R1. Tissue became necrotic round veins in the leaves. Leakage from disks cut from the Ia was probably affected by the presence of zinc ions as the levels of conductivity were very high for 2d. Then leakage fell to about one fifth of the controls as the tissue became necrotic.

Electrolyte leakage from plant tissue inoculated with pathogenic organisms has been fairly well investigated but the attempts to determine tne nature or quantities of materials released from the plant tissue have not been so many. In this work levels of potassium (K^+), sodium (Na^+) and Calcium (Ca^{++}), total protein and total phenol leakage were studied in relation to the electrolyte losses.

With disks cut from plants inoculated with R1, leakage of K^+ and Ca^{++} increased at 2h as did electrolyte losses. (This marks the end of induction phase.) The second peak in electrolyte losses at 13 - 18h was reflected only in levels of K^+.

With disks cut from plants inoculated with R2, the 7h electrolyte loss peak involved only Ca^{++}; K^+ did not peak until 13h. Both K^+ and Ca^{++} were involved in the peak of electrolyte loss at the point of tissue collapse at 48h. In the Ih zone, the electrolyte losses associated with the spread of the chlorotic halo involved K^+ and Ca^{++}.

With plants inoculated with Pmp, Pf, Xm or Ea the same involvement of K^+ and Ca^{++} was seen and in all cases Na^+ ions seemed hardly to be involved at all. Very little leakage of this ion occurred. The same lack of involvement of Na^+ ions was found by Stephens(1974) working with disks of potato tissue. Leakage of K^+ and to a lesser extent Ca^{++} followed the conductivity

changes which were results of pectin tras-eliminase (PTE) activity on potato tissue. Levels of Na^+ were consistently low and did not follow the same pattern of leakage.

Black and Wheeler (1966) working with oat tissues treated with victorin found that twice as much K^+ was released from the tissue as in controls but Ca^{++} and Na^+ losses were not significant. K^+ was the chief inorganic ion to be released from tomato cuttings treated with fusaric acid (Gäumann, 1958).

The general finding that Na^+ is not involved in leakage phenomena is given some explanation by Palmer and Civan (1977) who suggest that Na^+ in cells is either bound to macromolecules or contained in subcellular organelles at an activity higher than that in the cytoplasm and that in this way would not be available to diffuse through cell membranes.

The release of K^+ from RM tissue injected with Rl is interesting. There is a peak of release just before the latent phase during which Nemeth et al.(1969) showed that there is often a rise in respiration rate. In 1959, Steward and Sutcliffe found that certain inorganic ions (particularly K^+) caused respiratory rises in plant tissues. There may be a connection between the leakage of K^+ in Rl treated RM leaves and the respiration increases found in the latent phase by Nemeth.

Other work has shown that in many systems intercellular Ca^{++} induces a specific increase in the membrane potassium permeability (Meech, 1976). Again the two ions K^+ and Ca^{++} are connected in activity.

Experiments on the rate of leakage of K^+, Na^+ and Ca^{++} showed that as with electrolyte leakage, 50 - 60% of leakage of each ion in 120 minutes actually took place in the first 15 minutes of incubation. This hyperbolic leakage of ions was also found by Williams and Keen (1967) working with leaves of cucumber and P. lachrymans. Stephens (1974) found that K^+ leakage from potato

tissue treated with PTE was very rapid. Most of the K^+ leaking out in 120 minutes actually did so in the first 45 minutes of incubation. Black and Wheeler (1966) however found that leakage of K^+ from victorin treated oat tissue was at a steady rate for 24h.

Experiments on the effect of temperature on ion leakage showed that increasing temperature did not affect the hyperbolic rate of release of ions but did affect the amount of K^+ released. K^+ leakage showed the same temperature effect (ie very large amounts of leakage at temperatures above 37°C compared with temperatures less than 37°C) as did electrolyte leakage. Ca^{++} leakage was not so greatly affected by temperature and as usual Na^+ levels were very low.

Triton X-100 was injected into RM tissue and ion leakage examined. K^+ and Ca^{++} were released at the same rates and Na^+ leakage was very low. The dual leakage of K^+ and Ca^{++} was similar to the leakage from collapsing tissue inoculated with R2 which involved both K^+ and Ca^{++}.

When the temperature effect was investigated with respect to tissue injected with Triton X-100 (2%) completely different results from before were obtained. Ion leakage was in general less with increasing temperature and the membrane damage effect above 37°C was not apparent. This seems to indicate that the effect of Triton X-100 on membranes is not the same as the effect of R1 or R2.

Studies with thin or bilayer lipid membranes separating two aqueous phases showed that they were highly permeable to "lipophilic" molecules, moderately permeable to water, poorly permeable to hydrophilic solutes and virtually impermeable to ions (Webb, 1966). However cellular membranes have certain specialised molecul or regions of molecules inserted into the bilayer structure to mediate ionic flow across the membrane. These are thought to involve proteins.

In further investigation into the nature of substances leaking from RM disks, release of protein was examined. In general with R1 and R2 leakage of protein related to electrolyte leakage in that peaks of protein leakage occurred at 2 - 3h and 17h with R1 and at 48h with R2. There was no protein leakage at 7 - 10h with R2 when there was some electolyte loss however. Disks cut from plants injected with Pf showed very little protein leakage. The leakage from disks cut from plants injected with Ea was slightly higher than controls but related to the small electrolyte leakages noted.

Stephens (1974) found that leakage of protein from potato disks treated with PTE tended to follow electrolyte leakages ie to occur slightly later.

Leakage of phenols from tissue inoculated with R1, R2, Ea and Pf was examined. When the tissue was from plants inoculated with R1, phenol leakage first occurred at one hour from inoculation ie before electrolyte and protein leakage at 2 - 3h. Another peak of phenol leakage was at 17 - 18h and this co-incided with electolyte and protein leakage. With R2, tissues leaked phenols in a peak at 24h which is 24h sooner than electrolyte and protein leakage and tissue collapse. With disks cut from plants injected with Pf or Ea leakage of phenols was consistently high over the period of the experiment and was rather surprising. In general however it was tissue browning rather than phenol leakage which corresponded with cessation of multiplication of bacteria.

There are a number of reports which suggest that phenolic compounds accumulating during pathogenesis or HR may play a role in HR development (Condon et al.,1963; Farkas and Kiraly, 1962; Matta et al., 1969). Addy (1976) working with apple leaves and Erwinia amylovora found that leakage of phenols from apple leaf disks with the avirulent strain of E. amylovora occurred one hour before leakage of electrolytes. With the virulent strain leakage o

phenols and elecrolytes occurred at the same time. Addy's conclusions are that phenols cause the membrane permeability change indicated by the following electrolyte leakage. However Kosuge (1969) found that involvement of phenolics was secondary to the event that triggers necrosis.

The work in this thesis tends to support that of Addy (1976) in that with R1 and R2 in RM tissue, phenolic leakage was seen to occur just prior to the first small electrolyte peaks. It may be that the first membrane damage is caused by the phenols. With R1 RM plants released phenols in a large peak which corresponds with the time of tissue collapse and so the two are probably not causally related. With R2, phenol leakage occurred 24h before tissue collapse and it may be that the phenols could be involved in the tissue collapse.

The action of phenolics might be affected by changes in membrane structure leading to loss of cell compartmentalisation. Once this occurs regulation of cellular metabolism and respiration would be lost, phenoases would be activated and this and other oxidative and hydrolytic enzymes and their substrates previously separated by compartmentalisation would come together. The resulting degenerative activities would then lead to the formation of lesions (Kosuge, 1969).

The onset of HR is often accompanied by increased activity of oxidative enzymes, increased synthesis of phenolic compounds (Maxwell and Bateman, 1967; Rohringer et al., 1967) and darkening of the necrosing tissue (Rubin and Artsikhouskaya, 1964). The darkening and browning has been shown to be due to the presence of oxidised phenols (Rubin and Artsikhouskaya, 1964) and with accumulation of phytoalexins (Bailey and Deverall, 1971). The main phytoalexins in bean are phenolic and synthesis is dependent on the enzyme phenylalanine ammonia lyase (PAL) (Lyon, 1971), which convert phenylalanine to cinnamic acid in the shikimic acid pathway. Work

by O'Brien (1973) cast doubt on the advisability of examining PAL activity as a means of studying the oxidation of phenolics in plant tissue. This was because O'Brien found that HR was not light dependent and Hadwiger *et al.* (1970) had found that PAL was light dependent.

Investigation into the action of peroxidases have found that HR development is linked with host peroxidases suppressed by bacterial catalases in compatible combinations (Rudolph, 1964). However treatment of RM plants with horse-radish peroxidase did not produce any changes in HR or SR (O'Brien, 1973). In this work, leakage of phenoloxidase was measured from disks cut from inoculated RM tissue. Plants injected with R1 or R2 showed release of phenol oxidase at times just prior to the appearance of tissue browning. This seems to suggest that the browning is caused by the phenoloxidases acting on the phenols in the tissue. With Pf and Ea very little phenoloxidase activity was found in the ambient solutions of the disks thus suggesting a reason why there is no browning in the tissues inoculated with these bacteria.

Stephens (1974) also found that phenoloxidase activity in potato tissue was proportional to the amount of macroscopically visible browning.

Another plant in which phenolics accumulate during HR (eg hydroxyphaseolin, Frank and Poxton, 1970) is soybean and HR to avirulent strains of *P. glycinea* also develops independently of light in this plant (Smith and Kennedy,1970).

In general however there is little to link phenolic compounds directly with resistance (Kosuge, 1969) however in 1977 Sequeira and Webster found that a phenolic compound produced in bean pods inoculated with *P. syringae* reduced growth of the bacteria *in vitro*

The resistance of pathogenic pseudomonads to phenolics has been atributed to their ability to reduce oxidised phenols and inability to produce hydrogen peroxide which affects the oxidation of the reverse reaction. Non-pathogens were inhibited however

(Moustaffa and Whittenby, 1970). The production of phenols in bean plant tissue in response to R1 and R2 is probably a consequence of the more important trigger mechanism of HR.

In experiments designed to find chemicals which delayed or suppressed HR, the most effective substances were those containing divalent ions eg Ca^{++}, Mg^{++}, Sr^{++}, Ba^{++}, Be^{++}, Zn^{++} and Mo^{++}. This result was confirmed by Lallyett (1977) and was found for Ca^{++} by Cook and Stall, (1971). The compound protamine sulphate also delayed HR and when necrosis did develop it was not confluent over the whole of the inoculated area, but was rather patchy. Protamine sulphate has been shown to act by altering the charge from negative to positive on protoplast membranes (Grout and Coutts, 1974). Divalent ions particularly Ca^{++} have also been shown to have important effects on membranes. Ca^{++} has been shown to protect against thermal damage to membranes (Toprover and Glinka, 1976) although the mechanism of heat damage to membranes is unknown. The effect of Ca^{++} is thought to be on the protein element of the membranes as Ca^{++} protects enzymes and other essential cell proteins against heat denaturation by forming links within the protein molecule (Ljunger,1970).

Interestingly enough, Klement and Nemeth (1966) found that high temperature (37°C) suppressed HR in tobacco and in 1972 Klement suggested that there was a heat sensitive period following the induction period in host tissue. The protective effect of Ca^{++} might act to prevent the temperature sensitive period.

It may be that membrane integrity is important in the development of HR ie that membrane collapse resulting in phenolic release, oxidation and cell death causes the visible necroses. However, plants in which HR has been suppressed are still resistant to R1 and indeed Lallyett (1977) has shown that Ca^{++} protects RM plants from R2 induced necrosis too.

A major part of the work in this thesis was concerned with

trying to find a model system simpler than that of the whole plant to investigate HR and SR. The use of excised pieces of plant tissue is common in plant pathology. For instance, Mercer et al., (1974) used excised hypocotyls, leaf disks and pods of Phaseolus vulgaris when studying resistance to Colletotrichum lindemuthianum; Debnam and Smith (1976) used detached red clover leaves when looking at changes in isoflavone and pterocarpans in response to infection; Webster and Sequeira (1977) used bean pods to study resistance to P. syringae and Ready (1977) used segments of barley leaves to study resistance to powdery mildew. The usefulnes: of these systems is that a greater number of experiments can be carried out in more easily defined conditions than when whole plant: are used. However the assumption that the responses of excised plant tissue are the same as those of whole plants is not always true and care should be taken in interpretation of results.

In the work for this thesis leaf disks were used. These were either cut from inoculated tissue and incubated in humid boxes (similar to the technique of Debnam and Smith, 1976) or floating on water (similar to the technique of Ready, 1977) or they were cut from uninoculated tissue and were floated on suspensions of bacteri An important and useful feature was the length of time control disks survived without signs of senescence (18d in humid boxes and 6d on water).

One result from these experiments was that disks cut from plants inoculated with R1 or Pmp needed to have surrounding healthy tissue around the Ia before a response developed. Complete ly inoculated disks showed no response at all. This finding is supported by the work of Tomiyama et al.(1958) who found that healthy tissue was needed around the point of infection of potatoe by Phytophthora infestans before resistance was complete. Yoder and Whalen, 1975, also found that resistance in stored cabbage tissue to infection by Botrytis cinerea was proportional to the

amount of healthy tissue surrounding the point of infection.

Disks floating on water were found to be better and easier to work with as responses were more similar to those of the whole plant than disks in boxes, although in both cases the time of initial response was a little slower. Disks in boxes developed responses at first only on the lower sides of the disks and with disks on water, both sides of the disks responded at the same time - as in whole plants.

The best system was therefore that a small area (c. 0.4 x 0.5 cm^2) of RM leaf tissue should be inoculated and a disk 1.2 cm in diameter should be cut such that the Ia was in the centre, one hour later to allow water soaking to disappear. The disks should have some supply of light because although HR and SR were not dependent on light (also found by Lozano and Sequeira, 1970a; and O'Brien, 1973 using whole plants) development of secondary infections of the disks was more rapid in darkness than in light. The disks were similar to whole plants in that growth of bacteria stopped at the onset of necrosis (found by this author; Stall and Cook, 1966 and Lozano and Sequeira, 1970a using whole plants) but bacteria did not die. Elecrtolyte losses from these disks tended to occur at the onset of browning of the Ia in HR and at the time of spread of the chlorotic halo of the SR into the edge zone of the disk. When specific ions were measured, K^+ was found to be most important followed by Ca^{++} but leakage of Na^+ was still low although higher than with whole plants.

Protection from HR in disks could be achieved if the Ia was inoculated with low levels of R1 or Pmp 24h prior to threshold level injections - again the same result as with whole plants. Protection from HR in disks was also found if disks injected with R1 were floated on solutions of $CaCl_2.2H_2O$ but not if KCl was used - again the same result as with whole plants.

The floating of plant material on suspensions of bacteria

produced some interesting results but was not thought to be such a useful system. Disks floating on 10^9 cells/ml R1 or Pmp suspension showed no sign of infection and remained as green and healthy as controls. Disks on 10^9 Cells/ml R2 suspensions developed chlorosis at the disk edges in a manner similar to the development of chlorosis around the Ia of whole leaves.

The removal of pieces of epidermis did not affect the result above nor did age of leaf or whether it was mono- or tri-foliate. Light had no influence although age of R2 cultures was important. Cultures more than 7d old did not cause the chlorosis response. This supports work by Süle and Klement (1971) who found that culture age was important in the development of plant response.

At first it was thought that exposure of the disks to R1 suspension prevented infection by R2 later. However exactly the same results were obtained by floating disks on sterile water for a few days. There was some browning at the cut edges of the disks and this may have prevented entry of R2.

This method was not considered as useful as floating of inoculated disks on water because of the absence of HR even though R1 could be isolated from the disk tissue.

Protoplasts and cell suspensions have been increasingly used over the last few years as a means to study resistance and susceptibility. Plant protoplasts are a useful tool in viral research since normally the cell wall acts as an efficient barrier to virus particles (Takebe, 1975).

In this work it was hoped to develop a RM protoplast or cell suspension system which would react in a similar manner to bacteria as whole plants. This was not achieved but some progress was made in that a medium was found in which both plant and bacterial cells could grow. The addition of casein hydrolysate to the incubation medium (CIM) allowed growth of bacteria and did not affect survival of RM cells or protoplasts. Casein hydrolysate is

a constituent of some protoplast growth media (Pelcher et al., 1974) and tissue culture media (Linsmaier and Skoog, 1965).

The relative immunity of the bacteria (R1 and R2) to high concentrations of mannitol was surprising at first but Sterne et al.(1976) found that Phytophthora cinnamomi was tolerant of high osmotic potentials as was Endothia sp in the work of Hunter et al. (1976). Erwinia atroseptica was as tolerant of mannitol as were R1 and R2 (Quantick, Imperial College - personnal communication).

Protoplasts differ greatly in the ease with which they can be cultured, for instance tobacco is very tolerant of culturing but petunia is not (Cocking, 1975). RM protoplasts were found to be very fragile indeed and difficult to work with. Any shaking of culture flasks and centrifugation at 100g for more than 2 - 3 minutes would cause them to fracture. Cells were much more robust but even they were not tolerant of rough treatment.

In no case were typical HR or SR symptoms seen to develop although protoplasts and cells survived for a few days. (Survival would have been longer if it had been possible to include antibiotics in the media but this was not possible as the purpose of the experiments was to allow growth of bacteria.)

As in earlier work, development of HR and SR had indicated that phenols were released by RM cells, investigation of the levels of phenols in the suspensions cultures inoculated with R1 or R2 was carried out.

It was found that phenol levels either remained constant or fell over 24h of incubation. The work of Kopp et al.(1977) found that mannitol in protoplast cultures inhibited the activity of any PAL they produced. Sucrose lessened the inhibitory effect if used instead of mannitol. When this was tried, the same result was obtained with cells(constant or decreasing levels of phenols) but with protoplasts a large increase in phenol levels occurred when there were bacteria present. This did not affect growth of R1

or R2 probably because phytopathogenic pseudomonads are resistant to phenols (Moustaffa and Whittenby, 1970). It seems that mannitol may inhibit production of phenols in both protoplast and cell cultures and that sucrose will allow protoplasts to produce phenols but not cells. In suspension cultures of soybean cells however, Ebel et al.(1976) found that elicitors of phytoalexins in Phytophthora megasperma var. sojae stimulated PAL activity even though the medium contained 20g/l sucrose.

Analysis for phenoloxidase activity showed that levels of this enzyme were negligible in all the cultures possibly explaining the lack of browning reactions.

The main problem with this work was that no differences between R1 and R2 were detected at all. One reason for this may be in the lack of aeration of cultures. Under light microscopy bacteria were seen to be much more active near air bubbles than in the main bulk of the suspension. It would not be possible to aerate protoplast cultures by shaking techniques as they fracture so easily but this would probably be possible with cells. For protoplasts it might increase the aeration if drop culture techniques were used ie drops of protoplast/bacterial suspension cultured on microscope slides.

Another possible explanation of the absence of HR in suspension cultures lies in the disk work which demonstrated that a certain amount of un-inoculated tissue was required around the inoculated area before HR would develop normally. In suspension cultures there is no un-inoculated tissue.

However the technique offers great potential for biochemical analysis of the intercellular fluids (not easy with whole plants) and for experiments involving the addition of substances to attempt to induce HR. In this respect cell suspension cultures may be more useful because recent work has shown that the host

cell walls play a part in the development of HR (Goodman, 1974; Sequeira and Donald, 1976; Sequeira and Graham, 1977).

An important point to remember is that even the most finely dispersed cultures of higher plant cells contain a proportion of the cells associated together in aggregates (not truly free-cell cultures). The finer the suspension the smaller the average size of the cell aggregates and in general the higher the proportion of the cell population present as individual cells (Street, 1975).

Any future work on membrane permeability changes during HR or SR development could profitably make use of the liquid ion-exchange microelectrodes and micropipettes described by Palmer and Civan (1977). Use of micropipettes would remove the problem of the source of the electrolyte leakage when disks are used.

The disk system developed in this work has a potential use as a system for quick screening of compounds for their ability to influence the development of HR.

REFERENCES

ADDY, SK , 1976 Leakage of electrolytes and phenols fromapple leaves caused by virulent and avirulent strains of Erwinia amylovora. Phytopathology, 66, 1403-1405.

AVERRE, CW 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, PA and DeVAY, JE, 1971. Studies on the mode of action and biogenesis of the phytotoxin syringomycin. P hysiological Plant Pathology, 1, 215 - 235.

BAILEY, JA and DEVERALL, 1971. Formation and activity of phaseolli in the interaction between bean hypocotyls (P. vulgaris) and physoilogical races of Colletotrichum lidemuthianum. Physiological Plant Pathology, 1, 435 - 44

BAJAJ, YPS and SAETTLER, AW, 1970. Effect of Halo Toxin containing filtrates of Pseudomonas phaseolicola on the growth of bean callus tissue. Phytopathology, 60, 1065 - 1067.

BALDWIN, CH and GOODMAN, RN, 1963. Prevalence of E. amylovora in apple buds as detected by phage typing. Phytopathology, 53, 1299 - 1303

BLACK, HS and WHEELER, H, 1966. Biochemical effects of victorin on oat tissues and mitochondria. Amer. J. Bot., 53, 1108 - 1112.

BOND, G, 1968. Some biological aspects of nitrogen fixation. In'Recent Aspects of Nitrogen Metabolism in Plants' eds. EJ Hewitt and CV Cutting. Academic Press, New York.

BONDAR, G, 1912. Una nova molestia bacteriana das hastes da mandioca. Chacaras e Quintores Sao Paulo,5, 15 - 18.

BRAITHWAITE, CWD and DICKEY, RS, 1970. Synergism between Pseudomonas caryophyll and a species of Corynebacterium Phytopathology, 60, 1046 - 1051.

BURKHOLDER, WH, 1926. A new bacterial disease of the bean. Phytopathology, 16, 915.

CARLSON, PS and CHALEFF, RS, 1975. Heterogeneous associations of cells formed in vitro. In 'Genetic Manipulations with Plant Material' ed. Lucien Ledoux. Plenum.

COCKING, EC, 1975. Plant protoplasts as genetic systems. In 'Genetic Manipulations with Plant Meterial.' ed. Lucien Ledoux.Plenum.

COOK,AA,1971. Alteration of hypersesitivity in plants to

bacterial infection. Proc. 3rd Int. Conf. on Plant Path. Bacteria, Wageningen, 171 - 178.

CROSSE, JE, 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

CRUICKSHANK, IAM and PERRIN, DR, 1964. Pathological function of phenolic compounds in plants. In 'Biochemistry of phenolic compounds. ed JB Harborne. Academic Press, New York.

DAVEY, MR and COCKING, EC, 1972. Uptake of Bacteria by isolated higher plant protoplasts. Nature, 239, 455 - 456.

DEBNAM, JR and SMITH, IM, 1976. Changes in the isoflavones and Pterocarpans of red clover on infection with Sclerotinia trifoliorum and Botrytis cinerea. Phys. Plant Path. 9, 9 - 2

DeVAY, JE; CHARUDATTAN, R and WIMALAJEE, DLS, 1972. Common antigenic determinants as a possible regulator of host pathogen compatibility. American Nature, 106, 185 - 194.

DEVERALL, BJ, 1972. Phytoalexins and disease resistance. Proc. R. Soc. Lond. B.,181, 233 - 246.

DIXON, ROD, 1969.Rhizobia with particular reference to relationship with host plants. Ann. Rev. Microbiol., 23, 137 - 158.

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

EBEL, J; AYERS, AR and ALBERSHEIM, P, 1976. Host-pathogen interactions XII Response of suspension-cultured soybean cells to the elicitor isolated from Phytophthora megasperma var. sojae a fungal pathogen of soybeans. Plant Physiol., 57, 775 - 779.

ERCOLANI, GL, 1967. Bacterial canker of tomato I Analysis of some factors affecting the response of tomato to Corynebacterium michiganese. Phytopathologia Mediterranea, 6, 19 - 29.

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

ERCOLANI, GL, 1970b. 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, GL, 1973. Two hypotheses on the aetiology of response of plants to phytopathogenic bacteria. J. Gen. Microbiol., 75, 83 - 95.

FARABEE, GJ and LOCKWOOD, JL, 1958. Inhibition of E. amylovora

by bacterium species isolated from fire blight cankers. Phytopath. 43, 209 - 211.

FARKAS, GL and KIRALY, Z, 1962. Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopathol. Z., 44, 105 - 150.

FARKAS, GL and SOLYMOSY, F, 1965. Host metabolism and symptom production in virus-infected plants. Phytopathol. Z., 53, 85 - 93.

GARDNER, JM and KADO, CI 1972a. Induction of the hypersensitive reaction in tobacco with specific high molecular weight substances derived from the osmotic shock fluid of Erwinia rubrifaciens. Phytopath. 62, 759 (Abstr.)

GARDNER, JM and KADO, CI 1972b. Deoxyribonucleic acid and homologies between Erwinia species and between other members of the Enterobacteriaceae as determined by molecular hybridisation and renaturation techniques. Phytopath., 62, 759 (Abstr.)

GAUMANN, E , 1958. The mechanisms of fusaric acid injury. Phytopath., 48, 760 - 786.

GAMBORG, OL and MILLER, RA 1973. Isolation, culture and uses of plant protoplasts. Can. J. Bot., 51, 1795 - 1799.

GNANAMANICKAM, SS and PATIL, SS, 1977. Phaseotoxin suppresses bacterially induced hypersensitive reaction and phytoalexin synthesis in bean cultivars. Phys. Plant Path., 10, 169 - 179.

GOODMAN, RN, 1968. The hypersensitive reaction in tobacco: a reflection of changes in host permeability. Phytopath., 58, 872 - 873.

GOODMAN, RN, 1971. Re-evaluation of the role of NH_3 as the cause of the hypersensitive reaction. Phytopath., 61, 893 (Abstr.)

GOODMAN, RN, 1972. Electrolyte leakage and membrane damage in relation to bacterial population, pH and ammonia production in tobacco leaf tissue inoculated with Pseudomonas pisi. Phytopath., 62, 1327 - 1331

GOODMAN, RN and PLURAD, SR, 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Phys. Plant Path., 1, 11 - 16.

GOODMAN, RN; HUANG, S and HUANG, PY, 1974. A host specific phytotoxic polysaccharide from apple tissue infected by Erwinia amylovora. Science, 183, 1081 - 1082.

GROGAN, RG; LUCAS, LT and KIMBLE, KA, 1965. The correlation of pathogenicity in Pseudomonas lachrymans and P. phaseolicola with capsular antigens. Phytopath., 55, 1060

GROUT, BWW and COUTTS, RHA, 1974. Additives for the enhancement of fusion and endocytosis in higher plant protoplasts: an electrophoretic study. Plant Science Letters, 2, 397 - 403.

HADWIGER, LA and SCHWOCHAU, ME, 1969. Host resistance responses - an induction hypothesis. Phytopath.,59,223 - 227.

HALE, CN; 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.

HILDEBRAND, EM, 1942. A micrurgical study of crown gall infection in tomato. J. Agr. Res. 65, 45 - 59.

HILDEBRAND, DC and SCHROTH, MN, 1971. Isolation of P. phaseolicola from bean leaves exhibiting systemic sysmptoms. Phytopath., 61, 580 - 581.

HOITINK, HAJ; PELLETIER, RL and COULSON, JG, 1966. Toxemia of halo blight of beans. Phytopath.,56, 1062 - 1065.

HOWES, NK; KIM, WK ; ROHRINGER, R and SAMBORSKI, DJ, 1973. Evidence for a gene specific RNA determining resistance in wheat to stem rust. Nature, 249, 585 - 588.

HSU, ST and DICKEY RS, 1972. Interaction between Xanthomonas phaseoli, Xanthomonas vesicatoria, Xanthomonas campestris and Pseudomonas fluorescens. Phytopath., 62, 1120 - 1125.

HUNTER, PP; GRIFFIN, GJ and STIPES, RJ, 1976. The influence of osmotic water potential on the linear growth of Endothia species. Phytopath., 66, 1418 - 1421.

IKOTUN, B, 1975. Cassava bacterial blight disease caused by Xanthomonas manihotis (CBB). PhD thesis. Univ. London.

KADO, CI and HESKETT,MG, 1970. Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas. Phytopath., 60, 969 - 976.

KASSANIS, B,1963. Interactions of viruses in plants. Advan. Virus Res., 10, 219 - 255.

KELMAN, A and SEQUEIRA,L, 1972. Resistance in plants to bacteria. Proc. R. Soc. Lond. B., 181, 247 - 266.

KLEMENT, Z, 1963. Method for the rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature, 199, 299 - 300.

KLEMENT, Z, 1965. Method of obtaining fluid from the intercellular spaces of foliage and the fluid's merit as substrate for phytobacterial pathogens. Phytopath., 55, 1033 - 1034.

KLEMENT, Z, 1971. The hypersensitive reaction of plants to bacterial infections. Acta Phytopath. Acad. Sci. Hung.,6, 115-118.

KLEMENT, Z; FARKAS, GL and LOVREKOVICH, L, 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopath., 54, 474 - 477.

KLEMENT, Z and GOODMAN, RN, 1967. The role of the living cell and induction time in the hypersensitive response of tobacco plants. Phytopath., 57, 322 - 323.

KLEMENT, Z and GOODMAN, RN, 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Ann. Rev. Phytopath 5, 17 - 44.

KOPP, M; FRITIG, B and HIRTH, L, 1977. Pheylalanine ammonia-lyase activity of protoplasts: in vitro inhibition by mannitol and sorbitol. Phytochemistry, 16, 895 - 898.

KOSUGE, T, 1969. The role of phenolics in host response to infection. Ann. Rev. Phytopathol., 7, 195 - 222.

LALLYETT, CIK, 1977. Resistance and susceptibility in bean to bacterial pathogens. PhD thesis. Univ. London.

LEBEN, C, 1963. Multiplication of Xanthomonas vesicatoria on tomato seedlings. Phytopath., 53, 778 - 781.

LINK, SK and WILCOX, HW, 1936 . Relation of nitrogen - carbohydrat nutrition of Stayman apple trees to susceptibility to fire blight. Phytopath., 26, 643 - 655.

LINSMAIER, EM and SKOOG, F, 1965. Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum, 18, 100 - 127.

LIPPINCOTT, JK and HABERLEIN, GT, 1965. The quantitative determination of the infectivity of Agrobacterium tumefaciens. Amer. J. Bot., 52, 856 - 863.

LIPPINCOTT, JA and LIPPINCOTT, BB, 1975. The genus Agrobacterium and plant tumorigenesis. Ann. Rev. Microbiol., 29, 377 - 405.

LJUNGER, C, 1970. On the nature of the heat resistance of thermophilic bacteria. Physiol. Plant., 23, 351 - 364.

LOBENSTEIN, 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 - 597.

LOVREKOVICH, L and FARKAS, GL, 1965. Induced protection against wild fire disease in tobacco leaves treated with heat-killed bacteria. Nature, 205, 823 - 824.

LOVREKOVICH, L and LOVREKOVICH, H and GOODMAN, RN, 1969. Role of ammonia in wild fire disease of tobacco caused by P. tabaci. Phytopath., 59, 1713 - 1716.

LOVREKOVICH, L; LOVREKOVICH, H and GOODMAN, RN, 1970. Ammonia as a necrotoxin in HR caused by bacteria in tobacco. Can. J. Bot., 48, 167 - 171.

LOZANO, JC and SEQUEIRA, L, 1970a. Differentation of races of P. solanacearum by leaf infitration. Phytopath., 60, 833 - 838

LOZANO, JC and SEQUEIRA,L,1970b. Prevention of the hypersensitive reaction in tobacco leaves by heat killed bacterial cells. Phytopath., 60, 875 - 879.

LOZANO, JC and BOOTH, RH, 1974. Diseases of cassava (Manihot esculenta Crantz.) Pans., 20, 30 - 54.

LYON, GD, 1972. Studies on metabolism of phaseollin and other phytoalexins in beans infected by Colletotrichum lindemuthianum and other pathogens. Phd thesis. Univ. Lond.

MAINO, AL; SCHROTH, MR and VITANZA, VB, 1974. Synergy between Achromobacter sp and Pseudomonas phaseolicola resulting in increased disease. Phytopath., 64, 277 - 283.

MERCER, PC; WOOD, RKS and GREENWOOD, AD, 1974. Resistance to anthracnose of French bean. Phys. Plant Path., 4, 291 - 306

MEYNELL, GG and STOCKER, BAD,1957. Some hypotheses on the actiology of fatal infections of partially resistant hosts and their application to mice challenged with Salmonella paratyphi - B or Salmonella typhimurium by intraperitoneal injection. J. Gen. Microbiol., 16, 38 - 58.

MITCHELL, RE, 1976. Bean halo blight toxin. Nature, 260, 75 - 76.

MITCHELL, RE, 1976. Isolation and structure of a chlorosis inducing toxin of Pseudomonas phaseolicola. Phytochem., 15, 1941 - 1947.

MITCHELL, RE and PARSONS, EA, 1977. A naturally occurring analogue of phaseolotoxin (bean halo blight toxin). Phytochem., 16, 280 - 281.

MULLER, KO, 1958. Studies on phytoalexins 1 the formation and the immunological significance of phytoalexins produced by Phaseolus vulgaris in response to infections with Sclerotinia fructicola and Phytophthora infestans. Aust. J. Biol. Sci., 11, 275 - 300.

McINTYRE, JL; KUĆ, J and WILLIAMS, EB, 1975. Protection of Bartlett pear against fireblight with deoxyribonucleic acid from virulent and avirulent Erwinia. Phys. Plant Path., 7, 153 - 170.

NEMETH, J; KLEMENT Z and FARKAS, GL, 1969. An enzymological study of the hypersensitive reaction induced by Pseudomonas syringae in tobacco leaf tissue. Phytopath.Z., 65,267-278

O'BRIEN, FM, 1973. Resistance of French bean leaves to bacteria. Phd thesis. Univ. London.

O'BRIEN, FM and WOOD, RKS, 1973. Role of ammonia in infection of Phaseolus vulgaris by Pseudomonas spp. Phys. Plant Path., 3, 315 - 326.

OMER, MEH, 1966. A study of halo blight of beans. Phd thesis. Univ. London.

OMER, MEH and WOOD, RKS, 1969. Growth of P. phaseolicola in susceptible and resistant bean plants. Ann. appl. Biol., 63, 103.

OSTERHAUT, WJU, 1912. The permeability of protoplasm to ions and the theory of antagonism. Science, 35, 112 - 115.

OSTERHAUT, WJU, 1918. Conductivity as a measure of permeability. J. Biol. Chem., 36, 485 - 487.

OSTER HAUT, WJU, 1921. Conductivity and permeability. J. Gen. Physiol., 4, 1 - 9.

PALMER, LG and CIVAN, MM, 1977. Distribution of Na^+, K^+ and Cl^- between nucleus and cytoplasm in Chironomus salivary gland cells. J. Membrane Biol., 33, 41 - 61.

PATEL, PN and WALKER, JC 1963. Relation of air temperature and age and nutrition of the host to the development of halo and common bacterial blights of bean. Phytopath., 53, 407-411.

PATIL, SS, 1972. Purification of the phytotoxin from P. phaseolicola. Phytopath., 62, 782 (Abstr.)

PATIL, SS and TAM, LQ, 1970. Mode of action of P. phaseolicola toxin. Mechanism of ornithine accumulation. Phytopath., 60, 1536. (Abstr.)

PEGUS, JE, 1976. Aspects of disease resistance in Fusarium wilt of tomato. PhD thesis. Univ. London.

PELCHER, LE; KAO, KN; GAMBORG, OL and YODER, OC and GRACEN, VE, 1975. Effects of Helminthosporium maydis race T toxin on protoplasts of resistant and susceptible corn (Zea mays) Can. J. Bot., 53, 427 - 431.

PERAMBELON, MCM, 1971. A quantitative method for assessing virulence of Erwinia carotovora var. carotovora and Erwinia carotovora var. atroseptica to rotting of potato tuber tissue. In Proc. 3rd Int. Conf. Plant Path. Bacteria, Wageningen.

PEREIRA, ALG, and ZAGATTO, AG 1967. Etiologia da 'Mancha angular' na folha da mandioca (Manihot utilissima). Archivos Instituto Biologico, Sao Paulo, 34, 153 - 160.

RATHMELL, WG and SEQUEIRA, L, 1975. Induced resistance in tobacco leaves: The role of inhibitors of bacterial growth in the intercellular fluid. Phys. Plant Path., 5, 65 - 73.

READY, PA. 1977. Studies on race non-specific resistance of barley to powdery mildew. Erysiphe graminis DC. PhD thesis. Univ. London.

ROHRINGER, R and SAMBORSKI, DJ, 1967. Aromatic compounds in the host-parasite interaction. Ann. Rev. Phytopath., 5, 77 - 8

ROLFS, FM, 1915. A bacterial disease of stone fruits. Cornell Univ. Agr. Exp. Sta. Mem., 8, 377 - 436.

RUBIN, BA and ARTSIKHOVSKAYA, EV,1964. Biochemistry of pathological darkening of plant tissues. Ann. Rev. Phytopathol.,2, 157 - 178.

RUDOLPH, K, 1972. The halo blight toxin of Pseudomonas phaseolicol : Influence on host parasite relationship and counter effect on metabolites. In 'Phytotoxins in Plant diseases' Ed. RKS Wood, A. Ballio and A. Graniti. Academic Press, London and New York.

SAMADDER, KR and SCHEFFER, RP, 1968. Effect of the specific toxin of Helminthosporium victoriae on host cell membranes. Plant Physiol., 43, 21 - 28.

SEQUEIRA, L, and AINSLIE, V, 1969. Bacterial cell-free preparation that induce or prevent the hypersensitive reaction in tobacc Eleventh Int. Bot. Congr. (Abstr.) 195.

SEQUEIRA, L; AIST, S and AINSLIE, V, 1972. Prevention of the hyper sensitive reaction in tobacco by proteinaceous constituents of Pseudomonas solanacearum. Phytopath. 62, 536 - 542.

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. RKS Wood and A. Graniti. Plenum Press, New York and London.

SIEGEL, SM, 1969. Further studies on factors affecting the efflux of betacyanin from beet root:A note on thermal factors. Phys. Plant., 22, 327 - 331.

SINCLAIR, MG; SASSER, JM and TJ GLINKA, 1970. Pepper leaf intercellular fluid composition after inoculation with Xanthomonas vesicatoria. Phytopath., 60, 1314 (Abstr.)

SKOOG, HA, 1952. Studies on host-parasite relations of bean varieties resistant and susceptible to P. phaseolicola. and toxin production by the parasite, Phytopath, 42, 475 (Abstr.

SLEESMAN, HC; PERLEY, JE and HOITINK, HAJ, 1970. Susceptibility

and hypersensitivity in tobacco and their prevention by cell free extracts of P. tabaci and P. glycinea. Phytopath.,60, 1314 (Abstr.)

STAKMAN, EC, 1915. Relation between Puccinia graminis and plants highly resistant to its attack. J. Agr. Res.,4, 193.

STALL, RE; BARTZ, JA and COOK, AA, 1972. Induced susceptibility in pepper to Xanthomonas vesicatoria. Phytopath., 62, 791 (Abstr.)

STALL, RE and COOK, SA, 1973. Hypersensitivity as a defense mechanism against natural infection. Proc. 4th Int. Congr. Plant Pathol., Abtsr.

STEAD, DE, 1973. Rotting of potato tubers by the blackleg bacterium Erwinia atroseptica. PhD thesis. Univ. London.

STEPHENS, GJ, 1974. Killing of plant cells by Erwinia carotovora. PhD thesis. Univ. London.

STERNE, RE; ZENTMEYER, GA and BINGHAM, FT.1976. The effect of osmotic potential and specific ions on growth of Phytophthora cinnamomi. Phytopath., 66, 1398 - 1402.

STEWARD, FC and SUTCLIFFE, JF, 1959. Plants in relation to inorganic salts. In 'Plant Physiology Vol. II' ed. FC Steward. Academic Press, New York and London.

Street, HE. 1975. Plant cell cultures: present and projected applications for studies in cell metabolism. In 'Genetic Manipulations with Plant Material. ed Lucien Ledoux.

STROBEL, GA and HESS, WM, 1974. Evidence for the presence of the toxin binding pretein on the plasma membrane of sugar cane cells. Proc. Natl. Acad. Sci. U.S.A. 71, 1413 - 1417.

SÜLE, S and KLEMENT, Z, 1971. Effect of high temperature and the age of bacteria on the hypersensitive reaction of tobacco. Acta. Phytopath. Acad. Aci. Hung. 6, 119 - 122.

TAKEBE, I, 1975. The use of protoplasts in plant virology. Ann. Rev. Phytopath., 13, 105 - 125.

THATCHER, FS, 1939. Osmotic and permeability relations in the nutrition of fungus parasites. Amer. J. Bot., 26, 449 - 458

THYR, BD, 1968. Bacterial canker of tomato: inoculum level needed for infection. Plant Dis. Reptr., 52, 741 - 743.

TOMIYAMA, K; TAKAKUWA, M and TAKASE, N 1958. The metabolic activit in healthy tissue neighbouring the infected cells in relatio to resistance to Phytophthora infestans (Mont) De Bary in potatoes. Phytopath., 2, 237 - 250.

ToMIYAMA, K, 1963. Physiology and biochemistry of disease resistance of plants. Ann. Rev. Phytopath., 1, 295 - 324.

TOPROVER, Y and GLINKA, Z 1976. Calcium ions protect beet root cel membranes against thermally induced changes. Physiol. Plant., 37, 131 - 134.

TURNER, JG and NOVACKY, A, 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. Phytopath., 64, 885 - 890.

WAITZ, L and SCHWARTZ, W,1965. Untersuchungen über die von Pseudomonas phaseolicola (Burkh.) hervorgenifene Fettfleckrankheit der Bohme II Untersuchingen zur pathogenese. Phytopathol. Z., 26, 297 - 312.

WARD, HH, 1902. On the relations between host and parasite in the bromes and their brown rust Puccinia graminis.Ann. Bot. 16, 233.

WOOD, RKS and TVEIT, M , 1955. Control of plant diseases by use of antagonistic organisms. Bot. Rev., 21, 441 - 492.

YODER, OC and WHALEN, ML, 1975. Variation in susceptibility of stored cabbage tissues to infection by Botrytis cinerea. Can. J. Bot., 53, 1972 - 1977.

YOUNG, JM, 1974a Development of bacterial populations in vivo in relation to plant pathogenicity. N.Z.J.Agric. Res. 17 105 - 113.

YOUNG, JM, 1974bEffect of water on bacterial multiplication in plant tissue. N.Z.J.Agric. Res., 17, 115 - 119.

ZAUMEYER, WJ, 1932. Comparative pathogenical histology of three bacterial diseases of bean. J. Agr. Res., 44, 605 - 632.

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