INDUCTION OF DISEASE RESISTANCE TO ANTHRACNOSE IN CUCUMBER

by

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PETER R MILLS: Induction of disease resistance to anthracnose in cucumber

ABSTRACT

Systemic protection against Colletotrichum lagenarium was induced in first or second leaves of cucumber by inoculation of cotyledons or first true leaves with C. lagenarium, tobacco necrosis virus or Pseudomonas lachrymans. Heat-killed conidia of C. lagenarium, avirulent Colletotrichum sp., avirulent bacteria, fungal cell wall material or fungal nucleic acid did not induce similar resistance nor did physical or chemical damage.

The time course and direction of induced resistance were investigated as were the levels of infection on the inducer leaf and the effects of concentration of spores in the challenge inocula.

The effect of temperature, non-ionising radiation, light, metabolic inhibitors and water status of host tissues on induction of resistance was also investigated.

It was found that 6-benzylaminopurine sprayed on to cucumber leaves could reduce lesion size but not lesion number whereas polyacrylic acid, acetylsalicylic acid and several other related compounds caused both reduction of lesion size and number when injected into cucumber cotyledons four days prior to challenge inoculation. Other methods of application such as spraying and uptake through roots were not so effective.

Enzymes produced by C. lagenarium in liquid shake culture included pectic enzymes, xylanase and arabanase. These enzymes degraded equally well cell wall material extracted from protected and unprotected cucumber plants.

Enzymes produced in systemically protected second leaves of cucumber were compared with those from healthy unprotected leaves. RNase, peroxidase and chitinase remained at control levels whereas there were increases in β-(1-3) glucanase and phenylalanine-ammonia lyase. Their importance is discussed.

The histology of infection shows that the initial stages of infection are the same in both protected and unprotected leaves. Formation of appressoria and penetration occur at the same time, although penetration of protected leaves is much reduced. Lignin-staining haloes were found in protected tissue 5 days after challenge inoculation. Their importance is discussed.

LIST OF ABBREVIATIONS

A	-	absorbance
c	-	about
C^o	-	Centigrade degrees
cm	-	centimeter(s)
Co	-	company
d	-	day(s)
DNA	-	deoxyribonucleic acid
Fig	-	figure
g	-	gram(s)
gdw	-	glass distilled water
>	-	greater than
h	-	hours
LANS	-	Long Ashton Nutrient Solution
ℓ	-	litre(s)
Ltd	-	limited
m	-	metre(s)
mg	-	milligram(s)
mℓ	-	millilitre(s)
mm	-	millimeter(s)
M	-	molar
mM	-	millimolar
µM	-	micromolar
µg	-	microgram(s)
µm	-	micrometer(s)
µℓ	-	microlitre
min	-	minute(s)
M wt	-	molecular weight

nm	-	nanometer(s)
No	-	number
OD	-	optical density
PAL	-	phenylalanine-ammonia lyase
PG	-	polygalacturonase
ppm	-	parts per million
psi	-	pounds per square inch
RNA	-	ribonucleic acid
Rf	-	rate of flow
Rg	-	rate of flow relative to glucose
rpm	-	revolutions per minute
RVU	-	relative viscosity units
s	-	second(s)
spp	-	species
tlc	-	thin layer chromatography
UV	-	ultra violet (light)
V/V	-	volume per volume
W/V	-	weight per volume
Var	-	variety
vol	-	volume
wt	-	weight

CONTENTS

INTRODUCTION

It is sometimes possible to protect a susceptible plant against a particular pathogen by first inoculating that plant with an avirulent pathogen. Normally, the pathogen must be applied to the same site as the avirulent pathogen following a delay of several hours.

Most examples of this type of "cross-protection" are dependent on the production by the host of antifungal substances termed phytoalexins and this phenomenom was the basis of the phytoalexin concept introduced by Müller and Börger (1941).

Recently, Hammerschmidt, Acres and Kuć (1976) have reported that cucumber can be protected against the anthracnose disease caused by Colletotrichum lagenarium by prior inoculation with the same fungus or Cladosporium cucumerinum. Resistance to the virulent pathogen may be induced in upper leaves of susceptible plants by infection of the lower leaves with one of the above fungi.

Due to the failure of Deverall (1977) to detect phytoalexins in the Cucurbitaceae, it seems unlikely that this remote cross protection is mediated via such compounds.

This research was undertaken to clarify the response of cucumber to infection by C. lagenarium. To survey the types of infection of a leaf that cause resistance to develop. To determine the pattern of distribution of induced resistance. To assess the possibility of replacement of the inducer organism with fungal cell free extracts, metabolic products or chemical inducers and to analyse the nature of induced resistance by microscopy.

It was hoped that a better understanding of induction of resistance in genetically susceptible cucumber might stimulate a search for similar mechanisms in other plants.

B. REVIEW OF THE LITERATURE

A plant can be said to be "immune" to a certain pathogen if, under ideal conditions for development of that pathogen, no disease can be detected.

There are various degrees of resistance, from almost immune to total susceptibility. A change in response of a plant to a pathogen, induced by the same or a different pathogen and leading to resistance, has been termed "induced resistance", "acquired immunity", "acquired resistance" or "cross protection".

That plants have the ability to acquire some degree of resistance to infection has been known for almost one hundred years. Early work was summarised by Chester (1933) who put forward many instances of such induced protection, similar to the long accepted induced immunity of animals. Chester criticised this work, however, for the lack of proper scientific approach which had led to equivocal results. Despite this, some of the original work gives possible examples of induced resistance.

The concept of acquired immunity was strengthened seven years after Chester's review by Müller and Börger (1940) who described the protection of potato tubers from a virulent race of Phytophthora infestans following a previous inoculation with an avirulent race. This led to speculation of an induced antimicrobial compound present in plant tissue. This concept has dominated plant pathology since that time.

Contained in the literature are numerous reports of the effects of "external" factors such as temperature, light, soil pH, soil type, and

physical damage on resistance and susceptibility of a plant to a pathogen. It is not therefore suprising that the changes induced in a plant's metabolism by infection can lead to a change in this response. It is difficult, however, to find examples whereby induced resistance caused by previous infection by the same or a different pathogen can be replaced by physical or chemical damage. This suggests that induced resistance depends on the activation of a mechanism or mechanisms whose specific function is the protection of a plant against infection.

Examples of induced resistance can be found in the literature for most types of host/pathogen combinations. The following section will deal with some of these examples, but no attempt will be made either to cover the literature completely (although it is hoped that no major work has been excluded) or, at this stage, to discuss the underlying mechanisms.

Diseases will be categorised according to the apparent effect on the host, not on the type of pathogen.

A. Root-rots and damping-off diseases

Tu (1977), investigating severe Phytophthora root-rot of soybean, reported that the application of the bacterium Rhizobium japonicum to the soil immediately after planting lessened the severity of the root-rot. Paxton and Chamberlain (1967), also working with soybean, showed that plants could be locally cross-protected with Phytophthora cactorum against subsequent infection by P. magasperma var. sojae (now called glycinea).

Experiments on the root disease of sweet potato caused by certain

isolates of Ceratocystis fimbriata showed that inoculation of cut surfaces of susceptible tissue with non-virulent isolates induced resistance or immunity to subsequent challenge by pathogenic isolates. This induced immunity was limited to a thin layer adjacent to the surface inoculated with the non-pathogen (Weber and Stahmann, 1966). Similarly, Heale and Sharman (1976) found that harvested carrot root tissue under certain conditions is susceptible to attack by Botrytis cinerea. This susceptibility is lessened by the pretreatment of root slices of carrot with heat-killed conidia of B. cinerea and also cell free germination fluids.

Stem rot of squash caused by Fusarium solani was reduced by infection of plants with squash mosaic virus, watermelon mosaic virus and, to a lesser extent, cucumber mosaic virus. Increasing the inoculum potential of the Fusarium sp. diminished the protective effect of the various viruses however (Diaz-Polanco, Smith and Hancock, 1969).

Wong (1975) found that in glasshouse and growth chamber experiments, Gaeumanomyces graminis var. graminis, a fungus closely related to the wheat and oat take-all fungus, but not pathogenic to temperate cereals, reduced take-all infection along the roots. Cross-protected wheat plants produced grain yields significantly greater than those of unprotected plants but not significantly different from those of healthy wheat plants. A Phialophora - like fungus from grass roots did not confer the same degree of protection, indicating some form of specificity in the induced protection. This is at odds with the results of Balis (1970), Scott (1970) and Deacon (1973) who obtained good control of take-all fungi with Phialophora radicicola.

B. Wilt diseases

Wilt diseases have been the focus of much attention and there are many reports for claims of induced resistance. Schnathorst and Mathre (1966) could reduce infection of cotton by Verticillium albo-atrum by up to 66% when conidia of a mild strain were applied one week before the severe one. Mathre and Garber (1967) induced resistance by using high inoculum densities of Thielaviopsis basicola, a root-rot pathogen of cotton.

Matta and Garibaldi (1977), using tomato as the host, found that pre-inoculation with avirulent Verticillium albo-atrum protected the plant by 34-88% against V. dahliae according to the temperature regime employed.

Peppermint and spearmint were cross-protected against disease induced by a virulent isolate of V. dahliae when inoculated first with the weak pathogen V. nigrescens (Melouk and Horner, 1975). Resistance to Fusarium wilt has also been recorded.

Long (1963) reported protection of cowpea following prior inoculation with isolates of Cephalosporium sp. Likewise, Davis (1967, 1968) showed that test-tube grown tomato, flax, carnation, cabbage and watermelon seedlings inoculated with formae speciales of F. oxysporum other than those to which they are susceptible were subsequently more resistant to those virulent formae speciales.

Phillips, Leben and Allison (1967) treating wounded roots of tomato seedlings with cultures of a Cephalosporium sp. prior to inoculation with F. oxysporum f. sp. lycopersici, reduced the number of Fusarium colonies isolated from the stems.

Bacterial wilt of tobacco caused by Pseudomonas solanacearum has also been shown to be reduced by mixing different proportions of avirulent and virulent cells (Averre and Kelman, 1964).

C. Powdery-mildew diseases

Induced resistance to powdery mildew has also been noted. In 1969, King, Hampton and Diachuns reported that the development of Erysiphe polygoni on leaves of red clover was retarded by previous infection with bean yellow mosaic virus.

Cohen, Reuveni and Kenneth (1975) completely protected tobacco plants from powdery mildew by previous inoculation with a downy mildew, and Ouchi, Oku and Hibino (1976) protected barley from virulent races of Erysiphe graminis by prior inoculation with avirulent forms.

Goheen and Schnathorst (1961) found that vines infected with leafroll (a disease caused by a virus) were more than 90% resistant to vine powdery mildew, Uncinula necator.

D. Rust diseases

Yarwood (1954) made one of the first experimental studies of induced resistance to a rust disease (although Giddings had apparently suggested in 1918 that resistance to cedar-apple rust could be induced in juniper). Yarwood demonstrated that localised infection of Phaseolus vulgaris by Uromyces phaseoli greatly reduced the susceptibility of adjacent areas of the leaf to subsequent inoculation with the same pathogen. He later demonstrated induced resistance to bean rust by

prior inoculation with Puccinia helianthi and the reciprocal protection of sunflower leaves to P. helianthi by Puccinia phaseoli.

Johnston and Huffman (1958) reduced infection of wheat by a virulent race of P. recondita by prior inoculation with an avirulent race, and in the same year Wilson (1958) protected beans against Uromyces phaseoli by inoculation with tobacco mosaic virus (TMV) (he also showed the reciprocal protection from TMV by U. phaseoli). By using aqueous diffusates of uredospores he achieved 81 and 68% protection against rust and TMV respectively.

Schwenk (1964) induced resistance in flax to a virulent race of Melampsora lini, again by prior inoculation with an avirulent race. Littlefield (1969) later expanded this work (see later reference to this work). More recently, Cheung and Barber (1972) showed that previous contact of wheat with an avirulent race of Puccinia graminis f. sp. tritici, before inoculation with the virulent race, caused a reduction of about 80% of the number of pustules per cm^2 leaf area.

E. Anthracnose diseases

I. On Phaseolus vulgaris

Colletotrichum lindemuthianum exists as several physiological races and a given cultivar of French bean may be resistant or susceptible to the various races.

Interactions between various races and cultivars have led workers to believe that there are two distinct forms of induced resistance - localised and systemic.

Localised resistance was demonstrated (Rahe, et al. 1969) by application of a cultivar non-pathogenic race as inducer and a cultivar pathogenic-race as challenge, to the same site on a bean hypocotyl. These could be applied together, or with a lag period between inducer and challenge. The cells of the bean hypocotyl did not appear to be penetrated by the cultivar non-pathogenic-race. The cultivar pathogenic-race challenge inoculation penetrated but its development was then severely restricted. Germination and penetration were as normal so the inducer inoculum had no apparent effect at this stage.

Systemic induced resistance (Elliston, Kuć and Williams, 1971, 1976) was demonstrated by the application of cultivar non-pathogenic-races at 0.5 and 1.5 cm intervals along an hypocotyl. Challenge inoculation of the cultivar pathogenic-race at sites between the inducer inoculation 12 hours later led to a reduction in normal lesion development.

Localised induced resistance can no doubt be explained by the accumulation of antifungal substances but, as no such substances have as yet been shown to move significant distances, protection 5 mm away from the induction site must involve the translocation of a factor which in some way 'sensitises' the cells. This must induce a change in the physiology of those cells, a change from a susceptible to a resistant response to a subsequent challenge.

It should be noted that Skipp and Deverall (1973) were unable to achieve similar induced resistance at a distance from the inducer inoculation in green hypocotyls.

II. In Cucurbits

The interactions between cucurbits and Colletotrichum lagenarium, the causal agent of anthracnose, have provoked a great deal of work following the report of Hammerschmidt, Acres and Kuć (1976) that cucumber plants could be systemically protected from C. lagenarium by prior inoculation of lower leaves with either the same pathogen or with Cladosporium cucumerinum.

Subsequent investigations (Kuć, Shockley and Kearney, 1975; Jenns and Kuć, 1977; Kuć and Richmond, 1977; Caruso and Kuć, 1977) have revealed that eight cultivars of cucumber, watermelon and, to a certain extent, muskmelon could all be protected in a similar way. Inoculation of a single leaf of young cucumber plants with a suspension of C. lagenarium spores gave protection, evident as a delay in symptom expression and a reduction in the number and size of lesions, to the leaf above when challenge inoculated with the same pathogen. Repeated inoculation of such protected plants ensured continual protection throughout the 10 week duration of the experiment.

In a more detailed investigation, it was found that inoculation of the first true leaf with 40 5 $\mu\ell$ drops of C. lagenarium (5×10^5 spores $m\ell^{-1}$) protected plants for 4-5 weeks against 10^5 spores $m\ell^{-1}$ challenge. Protection was lost after 5-6 weeks in the entire plant. A second 'booster' inoculation 3 weeks after the first extended the length of protection into the fruiting period. Experiments using so called resistant cultivars showed the response to be the same, with a slight delay in lesion appearance.

The spore concentration of the inducer inoculum was found to effect the degree of protection achieved. A spore concentration of 10^3 spores $m\ell^{-1}$ inoculated on the first leaf protected leaf two against 10^5 spores $m\ell^{-1}$, giving rise to 12 lesions per leaf (compared to 38 in control plants). As the concentration of the inducer inoculum increased to 10^5 spores $m\ell^{-1}$ the number of lesions on leaf two dropped to an average of 6 lesions per leaf.

Protection was detectable in leaf two 96 hours after inoculation of leaf one, and removing leaf one 96 hours after inoculation had no effect on the protection in leaf two. Similarly, removal of the protected leaf after 120 hours did not reduce the protection when the leaf was subsequently challenged.

Protection can also be elicited under field conditions. Cucumber, watermelon and muskmelon plants were grown and inoculated in the glasshouse. These plants were then transferred to the field one week after inoculation of the first true leaf. Ten days later the inoculated leaf was given a booster inoculation and, seven days after that, challenge inoculated by spraying both surfaces of the leaves until run-off. The plants were kept in high humidity for 2 days. Two weeks later 47 of 69 control plants were dead whereas only one out of 69 protected plants was.

Induction of resistance to anthracnose has not been restricted to fungi alone. Infection of one cotyledon with tobacco necrosis virus (TNV) has been shown to protect the other cotyledon and the first true leaf from infection by C. lagenarium.

So it seems that necrosis caused by an infectious agent leads to induced resistance whereas attempts to give similar protection by mechanical injury and dry ice, both of which lead to necrosis, have so far been unsuccessful.

F. Other leaf diseases

Sinha and Trivedi (1972) inoculated susceptible rice plants with live suspensions of avirulent Helminthosporium oryzae before the virulent race. This led to 83-85% reduction in symptoms. They followed this up (Trivedi and Sinha, 1976) by showing that rice seeds soaked in germination fluids of avirulent or virulent races were also protected.

Spurr (1977) achieved 60% reduction in tobacco brown spot disease caused by Alternaria alternata by application of a non-virulent isolate of Alternaria sp.

Pretreatment of tobacco leaf material with heat killed suspensions of various Pseudomonas spp. protected the tissue against wild fire disease caused by Pseudomonas tabaci (Lovrekovich and Farkas, 1965).

McIntyre and Miller (1978), also using tobacco, were able to protect against cultivar pathogenic races of Phytophthora parasitica var. nicotianae by cultivar non-pathogenic races, cell free sonicates and also Pratylenchus penetrans.

G. Miscellaneous diseases

A translocated resistance phenomenon in Prunus domestica induced by

artificial infection at two week intervals has been shown to act up to 18 cm away, reducing the expansion of subsequent infections of Cytospora cincta (Hubert and Helton, 1967).

Injection of 5 x 10^6 cells of avirulent isolates of Erwinia amylovora and Pseudomonas tabaci into apple shoots, midway between the apex and the first node, protected the shoots against subsequent injections at precisely the same place 30 minutes later, with virulent strains of E. amylovora (Goodman, 1967).

Garrett and Crosse (1975), in mixed infection experiments with Pseudomonas morsprunorum and other Pseudomonads pathogenic to plum and French bean, reduced the leaf scar infection of the cherry cultivar Napolean by up to 58%.

In similar experiments, New and Kerr (1972) prevented crown gall induction in tomato and peach seedlings by simultaneous inoculation of non-pathogenic and pathogenic biotypes of Agrobacterium tumefaciens.

Protection of "Bartlett" pear against fire blight with avirulent Erwinia sp. has also been reported (McIntyre and Williams, 1972), and protection was also shown to be induced when cell-free sonicates of virulent and avirulent E. amylovora were used (McIntyre, Kuč and Williams, 1973). This investigation was taken a stage further when the sonicates were shown to lose their protective properties once the nucleic acid had been precipitated by protamine sulphate. Deoxyribose nucleic acid (DNA) from virulent E. amylovora, E. herbicola and Xanthomonas campestris all protected "Bartlett" pear from fire blight infection.

H. Virus diseases

A strain of virus with the ability to move systemically in certain hosts can cause severe symptoms both in the inoculated and uninoculated leaves. Eventually, new growth of a plant infected in such a fashion may show no systemic symptoms, although the virus may still be present in these leaves. This is sometimes called 'acquired tolerance' or 'recovery'. When these normal looking leaves are inoculated with either the same virus or a closely related one, no symptoms develop, whereas control plants of the same age show severe symptoms. If, however, the 'recovered' leaves are inoculated with a local lesion forming unrelated challenge virus, normal symptoms develop. This phenomenon is used by virologists as an indication of the relatedness of two strains of virus.

Wingard (1928) was the first to report that symptomless leaves in 'recovered' plants infected with tobacco ringspot virus could not be induced to produce ringspot symptoms by reinoculation with sap from severely infected leaves. Price (1936) reported that the virus concentration markedly decreased in such plants and recovery was followed by an acquired immunity.

McKinney (1929), Thung (1931) and Salaman (1933) later reported that systemically infected plants did not produce additional symptoms when inoculated with another strain of the same virus. The presence of a virus in a plant was regarded as a prerequisite for protection against subsequent infection by the same virus or by one of its strains. Salaman (1938) showed that protection in tobacco, induced by avirulent forms of potato virus X to virulent forms of the same virus, depended upon the extent to which the former multiplied and systemically

infected the plants. This would be borne out by the work of Benda and Naylor (1958) who found that when shoots were cured of tobacco ringspot virus infection by high temperature treatment, they reverted to their former susceptibility to reinfection.

Wallace (1944) suggested from his studies of sugar beet curly top in tobacco and tomato that "protective substances" and "plant antibodies" may be the cause of immunity. This was because resistance was passed between plants by grafting an infected plant to a healthy one, but no such resistance was seen following transmission by the leaf hopper vector.

Gilpatrick and Weintraub (1952) observed systemic acquired resistance to carnation mosaic virus in plants whose lower leaves had been infected, but no virus was demonstratable in the upper uninoculated leaves. These upper leaves were, however, resistant to a challenge inoculation. Unfortunately the "protective mechanism" could not be demonstrated 2½ years later (Weintraub and Kemp, 1961).

Yarwood (1953, 1960) observed localised acquired immunity to tobacco mosaic virus in the virus free area immediately adjacent to necrotic areas induced by the same virus in Phaseolus vulgaris var. Pinto. Similar results were obtained by Ross (1961a) using Nicotiana tabacum var. Samsun NN. Ross (1961b) further proved development of systemic acquired resistance in virus-free parts of Samsun NN tobacco after inoculation of other parts of the same plant with a virus that induced necrotic lesions. He suggested that the infection by a necrosis - inducing virus was essential for the development of this kind of acquired resistance.

Loebenstein (1962) however was able to induce partial protection in Nicotiana glutinosa to tobacco mosaic virus (TMV) with dilute native TMV protein solutions. Four days was needed for the induction, eliminating possible direct inhibitory effects, and some systemic protection was afforded to upper untreated leaves in N. glutinosa and Datura stramonium, despite the immobility of the inducing protein.

Ross (1966) induced systemic resistance in Phaseolus vulgaris var. Pinto, and cowpea using various combinations of different viruses as inducer and challenge.

The following are more detailed reviews of virus cross-protection:

Bennett (1953); Matthews (1970); de Zoeten and Fulton (1975); Gibbs and Harrison (1976) and Zailtin (1976).

I. Chemical Induction of Resistance

Certain types of chemicals are known to induce resistance in plants against various diseases. Kuć et al. (1959) found that soaking apple leaves in D or DL-phenylalanine prevented the occurrence of scab infection and that the effect was greater than the naturally occurring resistance of the plant.

Homma, Shida and Misato (1973) reported that rice seedlings which had been sprayed with or soaked in solutions of various amino-acid derivatives were resistant to rice blast. This work was confirmed by Arimoto, Homma, Ohtsu and Misato (1976) who induced resistance in rice plants for up to 55 days by soaking rice seeds in dodecyl DL-alaninate hydrochloride for 72 hours.

Polyacrylic acid (PA), an interferon inducer in animals, has been shown to induce resistance to tobacco mosaic virus in Nicotiana tabacum cv. Xanthi-nc (Gianinazzi and Kassanis, 1974), to tobacco ringspot virus in Nicotiana tabacum - White Burley, and to pelargonium leaf curl virus in Datura stramonium (Reported in Cassells, Barnett and Barlass, 1978). White (1979), again using tobacco cv Xanthi-nc, showed that injection with acetyl-salicylic acid (aspirin), benzoic acid or salicylic acid had the same effect as PA, inducing new proteins and resistance to tobacco mosaic virus.

J. Modes of Action

I. Protectant organism acting directly on pathogen

a. Physical antagonism

Physical antagonism was the method proposed by Johnston and Huffman (1958) in which wheat leaves were locally protected against Puccinia recondita by prior inoculation with the oat rust P. coronata f. sp. avenae. The suggestion was that the reduction in the number of possible infection courts was caused by blockage of the stomatal entry sites.

Kochman and Brown (1975) proposed a similar mechanism on oat leaves using wheat rusts as the protectants.

Littlefield (1969), however, using avirulent races of flax rust to protect against virulent races, concluded that induced resistance could only be partially explained by stomatal blockage.

Similarly Mathre et al. (1967) have shown that the severity of Verticillium wilt diseases is greatly reduced following infection of cotton by Thielaviopsis basicola root rot. They suggest that chlamydospores of T. basicola plug the conducting elements sufficiently to prevent, or at least slow down Verticillium colonisation.

Lippincott and Lippincott (1969) postulated that the inhibition of a virulent strain by a non-virulent strain of Agrobacterium tumefaciens was due to exclusion of the virulent strain from specific attachment sites within the host tissue. This has not been generally accepted as the explanation.

Direct action of a bacterium against a fungus was exhibited by the interaction between Phytophthora megasperma and Rhizobium japonicum, where it was shown by Tu (1977) that R. japonicum colonised the surface of the P. megasperma mycelium to an extent which may have prevented contact between pathogen and host.

b. Chemical antagonism

Johnston and Huffman (1958), in an alternative explanation of stomatal blockage of wheat against rust, suggest that induced resistance may be due to contact inhibition by the action of substances produced by the latent mycelium of the avirulent organism which was not able to establish itself parasitically.

It is well established that many rust fungi produce germination inhibitors. Allen (1955) showed that the rate of germination of

uredospores of Puccinia graminis f. sp. tritici was inversely proportional to the quantity of spores present under given conditions. By floating the spores on water for 8-24 hours this was no longer the case. As a result of this he proposed the existence of at least one substance which can prevent uredospore germination.

Yarwood (1954) showed that volatile substances released from rust infected bean leaves inhibited germination of uredospores of other rust fungi. Yarwood (1956) explained his cross-protection of sunflower by Uromyces phaseoli against Puccinia helianthi by the release of Trimethylethylene or other chemicals by the spores and mycelium of the rusts.

Doubt was cast by Deverall (1977) on this work as he points out that unless inhibition is much stronger between uredospores of different species than between those of the same species, it is difficult to understand why there is no self-limiting effect of increasing the spore concentration, which there does not appear to be.

Other self-inhibitors have been characterised. Macko et al. (1972) found that sunflower rust, corn rust and snapdragon rust all produced methyl-3, 4 dimethoxycinnamate.

Not all cases of induced resistance to rust can be explained by toxic gas production. Littlefield (1969) found no evidence for this method with avirulent and virulent flax rust.

The inhibitory effects of bacteria on pathogens has been well documented from as far back as 1939 when Gordon and Haenseler showed that Rhizoctonia solani could be inhibited by Bacillus simplex. Since then there have been many reports of the activity of bacteria, mainly in the genus Bacillus, on diseases of the below ground parts of crop plants such as damping-off and seedling blights.

Dunleavy (1955) controlled damping-off by adding Bacillus subtilis to sterile soil inoculated with a Rhizoctonia sp. from beet. He attributed this to antibiotic production by the bacterium. Gregory et al. (1952) obtained similar control of damping-off in alfalfa, as did Vasudeva (1952) with wilt and Weinhold and Bowman (1968) in potato scab.

On aerial parts of plants, Bacillus spp. have been equally efficient (Levine, Bamberg and Atkinson, 1936; Morgan, 1963).

Recently, two unidentified bacteria have been shown to be antagonistic to Colletotrichum gloeosporoides and also to stimulate appressoria formation in vitro (Lenne and Parberry, 1976). Swinburne and Brown (1976) have shown that B. subtilis affects infection of apples with Nectria galligena.

Doherty and Preece (1977) have shown that living cells of B. cereus completely inhibit the germination of the uredospores of P. allii on agar and markedly reduce development of rust infection of leek leaves when sprayed on to leaves before inoculation with the rust.

Similarly, spores of Botrytis cinerea exhibited poor germination

and growth in water drops on the surface of chrysanthemum leaves. This was thought to be due to bacteria sedimenting on to the leaves from the air (Blakeman and Fraser, 1971).

Production of a chemical antagonist has been shown by Kerr (1972) and New and Kerr (1972) to prevent crown gall caused by Agrobacterium tumefaciens by the prior inoculation with the avirulent A. radiobacter. In this particular case, production of a bacteriocin by A. radiobacter is claimed to be the mechanism, not competition for attachment sites as claimed by Lippincott and Lippincott (1969).

Chemical antagonism may also be the mechanism by which tobacco foliage can be protected against Peronspora tabacina by prior infection with the fungus (Pont, 1959; Cruikshank and Mandryk, 1960), as a strong germination inhibitor of the blue mold pathogen was isolated from infected tobacco leaves (Leppik, Holloman and Bottomley, 1972).

Fravel and Spurr (1977), also using tobacco, were able to control brown spot disease caused by Alternaria alternata by prior exposure to Bacillus mycoides.

II. Induced changes in the host plant

a. Phytoalexins

The literature concerning phytoalexins has been reviewed many times (Cruickshank, 1963; Ingham, 1972; Kuč, 1976 and Deverall, 1977) and will therefore not be covered here.

Their relevance to this work was thought to be minimal but their importance as a defence mechanism in many of the examples of induced resistance in this thesis must be stressed.

b. Protein changes

Mechanical injury to plants has been shown to result in changes in protein metabolism (Kahl, 1973; Birecka, Briber and Catalfano, 1973). It is therefore not surprising that injury as a result of infection should produce similar metabolic changes.

Increases in the respiration rate following infection are common, (Farkas and Király, 1955; Toyoda and Suzuki, 1960; Millerd and Scott, 1962; Wood, 1967 and Daly, 1976), as too are increases in mitochondria (Asahi, Honda and Uritani, 1966). The stimulation of respiration is normally more pronounced in resistant plants when attacked by potential pathogens (Wood, 1967). Similarly, Tomiyama et al. (1955) reported marked increases in respiration in potato tuber tissue infected with incompatible strains of Phytophthora infestans, but not with compatible ones. Contradicting this was work done by Brenneman and Black (1979) who, again with P. infestans, but with tomato, found that respiration of resistant tissue, though increased over the control, was less than that of susceptible tissue.

Recently, Zazzerini (1976) has shown that, on the third day following inoculation of tomato seedlings with the non-pathogenic fungus Fusarium oxysporum f. sp. dianthi, there are quantitative alterations in respiration and an increase of aromatic amino-acids

as well as some changes in protein metabolism. He considered these changes to be related to the transient immunisation effect produced by F. oxysporum f. sp. dianthi against the virulent fungus F. oxysporum f. sp. lycopersici.

Peroxidase

Although the precise metabolic function of peroxidase (PA) in higher plants is obscure, the synthesis or activity of a phenol-oxidising enzyme such as PA in disease resistant plants is known to be generally higher in the infected tissues of resistant cultivars than in infected susceptible ones. There are several reports showing evidence of increased enzyme activity following infection (Farkas and Stahmann, 1966; Jennings, Brannaman and Zschule, 1969; Kawashima and Uritani, 1965; Lovrekovich, Low and Stahmann, 1967; Novacky and Hampton, 1968). These have led to the idea that changes in peroxidase activity may be involved in the defence mechanisms of plants.

Benedict (1972) showed that peroxidase activity in tomato cultivars could be altered by various intensities and wavelengths of light and could be related to resistance to Septoria lycopersici. Increased peroxidase activity led to increased resistance.

Keder (1959) used peroxidase levels as a test in the selection of potato varieties resistant to blight. When crude saps of leaves of healthy potato plants were tested for relative peroxidase activity, a positive correlation was found between peroxidase activity and the degree of resistance to Phytophthora infestans. This was suggested as a quick method for screening for resistance genes.

Simons and Ross (1971) found that induced systemic resistance in tobacco to tobacco mosaic virus coincided with increased peroxidase activity in upper leaves. Similarly in half leaf experiments where half was inoculated with tobacco mosaic virus, enzyme activity increased in the opposite half leaf.

However, Rathmell and Sequiera (1975) were unable to induce resistance in tobacco by infiltrating leaves with horseradish peroxidase despite the increase in peroxidase activity following infiltration with heat-killed cells of an avirulent form of Pseudomonas solanacearum.

Van Loon (1976a) was able to show that systemic acquired resistance of Nicotiana tabacum cv. Samsun NN to tobacco mosaic virus, expressed primarily as a decrease in lesion size, was associated with increased initial peroxidase and catalase activity. These increases were positively correlated with lesion density on inoculated leaves. Artificial aging, trimming of plants, root inundation, drought or salt stress all stimulated peroxidase activity but enhanced, rather than deminished, subsequent lesion enlargement. Van Loon concluded that peroxidase activity was a reflection of the physiological state of the plant rather than responsible for regulating the rate of lesion enlargement.

Further evidence supporting the non-involvement of peroxidase was presented by Nadolny and Sequiera (1980) who induced high levels of peroxidase activity in tobacco using the non-pathogen Bacillus subtilis, a bacterium which had been shown earlier not to induce a protective response in tobacco (Lozano and Sequiera, 1970).

It seems likely that peroxidase increases are not directly involved in disease resistance and that increases are due to injury to host cells as a result of pathogen activity.

Phenylalanine ammonia-lyase

The role of phenylalanine ammonia-lyase (PAL) in infected tissue is well known but not completely understood. There are many examples of changes in PAL activity following infection by fungi, bacteria, viruses and nematodes (Fritig, et al. 1973; Rathmell, 1973; Brueske, 1980).

Involvement in the increased production of phenolic compounds derived from phenylalanine, intermediates in the production of antifungal metabolites, is well documented (Creasy and Zucker, 1974). PAL is the first enzyme of the phenylpropanoid pathway and is therefore considered to be the key enzyme in the regulation of phenylpropanoid compounds and their derivatives (Camm and Towers, 1973). It has been suggested (Loebentein, 1972 and Tanguy and Martin, 1972) that increases in phenylalanine derived metabolites are a consequence of wounding and a secondary event induced by necrogenesis, but there is much evidence that this is not the case and that PAL is of paramount importance in the defence mechanisms of many hosts against a variety of pathogens.

The part played by PAL in systemic resistance is unclear. Fritig et al. (1973) found stimulated PAL activity located around tobacco mosaic virus lesions in tobacco, but in uninoculated systemically resistant leaves, PAL remained the same as in control plants. Challenge inoculation of these leaves led to less PAL than in

non-protected ones. This, however, is in direct contrast to results reported by Simons and Ross (1971) who again found no increases in PAL activity in upper leaves of tobacco systemically protected but, on challenging, PAL activity was greater and phenol build-up faster than in control plants.

Soluble proteins

In various pathogen/host combinations the appearance of several new proteins has been reported. In a hypersensitive host, localisation of tobacco mosaic virus in the living tissue surrounding necrotic lesions - that is in tissue that shows resistance to secondary infection (Ross, 1961b), is accompanied by host synthesis of new protein components that have been designated I-IV (Van Loon and Van Kammer, 1970). Spread of this induced resistance to other uninoculated leaves also results in the appearance of these new proteins according to Kassanis, Gianinazzi and White (1974), although Van Loon pointed out that systemic acquired resistance was also expressed in the upper parts of tobacco plants in which tissue necroses were induced in the lower leaves by spraying with mercuric chloride instead of tobacco mosaic virus inoculation. In this case no new protein components were induced.

The association of these new, or so called 'b' proteins, with virus localisation is well documented (Antoniw and Pierpoint, 1978; Gianinazzi and Martin, 1975b; Rohloff and Learch, 1977; Van Loon, 1976b).

Much less work has been done to date on the detection of novel proteins in response to fungal or bacterial infections.

De Wit and Bakker (1980) reported finding such a protein in tomato in response to infection by Cladosporium fulvum, but they found that it had no inhibitory effect on the growth of C. fulvum in the tomato leaf.

Gianinazzi et al. (1980), however, link the appearance of 'b' proteins in tobacco following infection with Thielaviopsis basicola directly with resistance. They found 'b' proteins only in T. basicola resistant cultivars of tobacco but they did not postulate a role for such a protein.

Similarly, Ahl et al. (1980), using tobacco, showed that the 'b' proteins are formed after injection of high concentrations of the incompatible phytopathogenic bacterium Pseudomonas syringae.

Andebrhan et al. (1980), using cucumber, stimulated novel protein production following infection with Colletotrichum lagenarium. This protein had the same Rf. value as novel protein induced after infection of cucumber with tobacco necrosis virus (Coutts, personal communication).

Novel proteins have also been induced chemically, together with induced resistance to viral infection by injection with either polyacrylic acid (Gianinazzi and Kassanis, 1974) or Nocardia asteroides extracts (Gianinazzi and Martin, 1975a).

So far it has been shown that certain hosts will produce novel proteins as a result of infection or chemical induction. In tobacco, the same proteins are formed in response to viral, fungal

or bacterial infection and also chemicals such as acetylsalicylic acid and salicylic acid. No definite role has yet been proposed, although it has been suggested that novel proteins may be involved in lesion restriction, and it is still possible that they may be an effect not the cause of resistance.

Other host enzymes

Host enzymes capable of causing lysis of fungal cell walls have been detected in tissues of higher plants. Abeles et al. (1971) found that prolonged gassing of detached Phaseolus vulgaris leaves with 10 ppm ethylene led to a 50 fold increase in chitinase activity. Pegg and Vessey (1973) showed healthy and Verticillium infected tomato plants to contain the same enzyme.

Wargo (1975) suggested that the presence of lytic enzymes as a protective mechanism may account for the resistance of healthy tissues to invasion by Armillaria mellea.

The role of lytic enzymes in reducing infection by fungal pathogens has also been emphasised by Blackhurst and Wood (1973) and Dixon and Pegg (1969), both working with tomato and Verticillium.

Rabenantoandro, Auriol and Touzé (1976), looking for a similar mechanism, were unable to implicate β-(1-3) glucanase, of host origin, in a defensive role in melon anthracnose.

Netzer, Kritzman and Chet (1979), however, concluded that the increase in activity of the host muskmelon β-(1-3) glucanase in

resistant cultivars provided a potential defence mechanism against Fusarium oxysporum f. sp. melonis induced wilt.

That lytic enzymes are produced by several hosts is now certain. That these enzymes can be stimulated to a level that would totally repel an invading fungus is less certain, but the evidence is such that the possibility exists that induced host polysaccharidases, capable of degrading hyphal wall components, may provide a defence mechanism against certain pathogens.

Lignification

Lignin is one of the major wall components of woody plants (Fergus et al. 1969). The monomeric subunits of lignin are the oxidation products of sinapyl, coniferyl and p-hydroxycinnamyl alcohols. Lignin can be covalently linked to other polymeric wall components (Cowling and Brown, 1969) and, once lignified, polysaccharide constituents in the cell wall are highly resistant to enzymatic degradation (Dehority, Johnson and Conrad, 1962; Ride, 1975, 1980; Ride and Pearce, 1979).

Lignification as a defence mechanism has been suggested by several investigators. Hijwegen (1963) using cucumber and Cladosporium cucumerinum provided evidence that lignification was probably at least part of the mechanism, as lignification occurred in resistant but not susceptible cucumber cultivars. Henderson and Friend (1978) found that deposition of lignin-like material occurred more rapidly and initially to a greater extent in resistant potato tubers than in susceptible ones and that this was in fact specific to pathogenic races of Phytophthora infestans.

Ride (1975), using wheat, suggested that rapid lignification in response to non-pathogens but not to wounding, and its close association with the approach of fungal hyphae, may play some part in the restriction of these fungi. The rate of lignification was much slower in response to pathogenic Septoria nodorum and S. tritici.

However, as Ride (1980) points out, it is difficult to assess the importance of lignification in resistance until more is known of the ways in which the process might hinder fungal progress and the quantities of lignin required to prevent growth completely. Ride suggests the following six ways in which lignification might hinder a fungus. It may provide resistance to cell wall degrading enzymes; provide a barrier to mechanical pressure; restrict diffusion of nutrients and water from plant to fungus or toxins from fungus to plants; accumulation of phenolic precursors may be toxic to the fungus and it may be possible that the fungal hyphae themselves become lignified.

Protection of reed canary grass by prior inoculation with a non-pathogen coincides with induced activity of enzymes involved in lignification (Vance and Sherwood, 1977). These enzymes (peroxidase, phenylalanine ammonia-lyase and tyrosine ammonia-lyase) are common to both lignin and phytoalexin production but, in the case of reed canary grass, attempts to find phytoalexins were unsuccessful.

It is also worth noting that although phytoalexins can be induced by a variety of factors including fungal products (Daniels and

Hadwiger, 1976), antimetabolites (Bailey, 1969), plant growth substances (Chalutz and Stahmann, 1969), ultra-violet irradiation (Hadwiger and Schwochau, 1971), heavy metal ions (Perrin and Cruickshank, 1965), metabolic inhibitors, oxidising and reducing agents, and DNA intercalating compounds (Hadwiger and Schwochau, 1971), Pearce and Ride (1980) found that apart from filamentous fungi, amongst all the above abiotic factors, only mercuric ions elicited the formation of lignin. This suggests mechanisms much more specific for lignin than for phytoalexins.

Direct evidence for the containment of pathogens in vivo by lignin is still lacking but its involvement as a defence mechanism in certain host/pathogen situations is now almost certain.

Plant hormones

The effect of hormones on the growth and development of plants is well known but not always well understood. The involvement of hormones in certain disease responses is well documented. Cytokinins are involved in the Green Island effect (Király, Hammady and Pozsár, 1967), the so-called fasciation disease of pea caused by the release of a cytokinin by the pathogen Corynebacterium fascians (Klämbt, Thies and Skoog, 1966), false broom-rape (Hamilton, Lowe and Skoog, 1972), club root in crucifers caused by Plasmodiophora brassicae (Katsura, et al. 1966), rust gall formation of pines caused by Cronartium fusiforme (Rowan, 1970), and root knot galls caused by certain nematodes (Krupasagar and Barker, 1966).

An equally impressive list of the involvement of auxins,

gibberellins, abscisic acid and ethylene could be presented to exemplify the changes, caused in hosts by pathogenesis, in plant hormone balance. These changes could conceivably alter the response of a plant to further infection. Cytokinins could be especially important here due to their ability to retard senescence and prevent loss of chlorophyll (Jacoky and Dagon, 1970), which could easily lead to the masking of chlorotic symptoms and thereby an underestimation of disease severity. This was the conclusion reached by Balázs, Sziraki and Király (1977) following the lack of tobacco mosaic virus symptoms in protected tissue coinciding with increased cytokinin levels despite no actual decrease in virus content in challenged leaves.

Miscellaneous mechanisms

I. Agglutination

Susceptible Bottom special tobacco cuttings pretreated by stem uptake inoculation with suspensions of avirulent mutants of Pseudomonas solanacearum developed a protection against wilting when subsequently challenged with virulent bacteria. Onset of protection required at least 24 hours of pretreatment and the degree increased up to 4 days (Main, 1968). Associated with this protection was the presence of an agglutinating factor isolated from the cuttings which also increased with time. Virulent cells suspended in cold sterile phosphate buffer extracts of protected cuttings lost their motility quickly, agglutinated and failed to multiply. No such effects were observed in water pretreated susceptible control cuttings. Main concluded that the increased

protection with time was related to the increased agglutinating titre.

II. Proteinase inhibitor

Wounding of the leaves of potato or tomato plants by adult Colorado potato beetles, or their larvae, induced a rapid accumulation of a proteinase inhibitor throughout the plant's tissues that were exposed to the air. This effect could be stimulated by mechanically wounding the leaves. The transport of a factor out of damaged leaves took place rapidly after the wound was inflicted and the levels of proteinase inhibitor in both damaged and adjacent leaves rose strikingly within a few hours (Green and Ryan, 1972).

III. Heat induction

Sugarcane clones genetically susceptible to eyespot disease, Helminthosporium sacchari, became resistant to the pathogen or to helminthosporoside activity during warm summer months (Byther and Steiner, 1975). Exposure of plants to 30 to 35°C, preincubation of leaves in a N_2 atmosphere, or preincubation in the presence of protein synthesis inhibitors also induced resistance. Hot water treated leaves reverted to a susceptible condition. The authors suggest a possible mechanism operating may be a change in the status of a toxin-binding protein or in other proteins involved with the action of helminthosporoside.

Other membrane proteins or enzymes have been thought to be inactivated by mild heat treatment (Ordin et al. 1974).

IV. Osmotic change

Goheen and Schnathorst (1961), in an attempt to explain the resistance of vines to Uncinula necator following infection with a leaf-roll virus, suggested that the effect was almost certainly due to the accumulation in leaves of carbohydrate and that changes in osmotic values due to this accumulation prevented colonisation by the powdery mildew fungus.

This follows earlier work of Schnathorst (1959) in which he put forward the same mechanism for the resistance of certain lettuce varieties to Erysiphe cichoracearum.

K. Specificity

It is normal that resistance, induced either by an incompatible reaction, mild pathogenesis or severe localised pathogenesis elsewhere on a plant, is effective against a range of pathogens which would normally give a compatible reaction. It is also normal for several different incompatible reactions to lead to resistance to a pathogen.

Induction of resistance in cucumber to Colletotrichum lagenarium can be achieved by prior inoculation with C. lagenarium, Cladosporium cucumerinum (Hammerschmidt, Acres and Kuč, 1976) or tobacco necrosis virus (Jenns and Kuč, 1977).

Inoculation of cut surfaces of susceptible sweet potato root tissue with certain non-pathogenic isolates of Ceratocystis fimbriata induced resistance not only to pathogenic C. fimbriata but also to several other pathogens tested (Weber and Stahmann, 1966).

Recently, McIntyre, Dodds and Hare (1981) have reported systemic resistance to tobacco mosaic virus, Phytophthora parasitica var. nicotianae, Pseudomonas tabaci, Peronospora tabacina and, remarkably, increased resistance to an aphid, Myzus persicae, by prior inoculation with tobacco mosaic virus. In this case it seems unlikely that tobacco has a single defence mechanism against viral, bacterial, fungal and insect pathogens. Such diversity of protection adds weight to the hypothesis that initial infection causes a signal or message to be passed to other parts of the plant which sensitises cells to react quickly activating different defence mechanismson secondary invasion by a variety of pathogens.

There are many examples of non-specific induction of resistance to rusts by rusts. Littlefield (1969) protected flax against rust infection by prior inoculation with Puccinia graminis tritici and Puccinia recondita tritici as well as with race 1 Melampsora lini.

As expected, however, not all incompatible reactions induce resistance. Goodman (1967) protected apple stem tissue against Erwinia amylovora by prior injection of avirulent E. amylovora, Pseudomonas tabaci and with a saprophytic bacteria but was unable to protect with Xanthomonas pruni, Bacillus subtilis or B. cereus.

Some degree of specificity has been claimed for the inducer organism in the case of protection of "Bartlett" pear against fire-blight by DNA from the fire-blight pathogen (McIntyre, Kuč and Williams, 1975). DNA from virulent and avirulent E. amylovora, E. herbicola and Xanthomonas campentris elicited protection, but DNA from other sources did not.

Specificity of induction of resistance was also shown by Berard, Kuč and Williams (1973) who reported that diffusates obtained from incompatible reactions of cultivar non-pathogenic races of Colletotrichum lindemuthianum on Phaseolus vulgaris protected cultivars having the same disease reaction to such a race.

Induction of lignification in wheat leaves with non-pathogens appears to be restricted to filamentous fungi (Pearce and Ride, 1980).

There is no evidence to suggest that resistance can be induced in a plant against one and only one pathogen.

L. Timing

Comparison of the interval required from an induction reaction to appearance of resistance between different host/pathogen combinations must be viewed with considerable caution. Indeed, it is quite probable that differences encountered are due to the different mechanisms of resistance operating in a particular host.

Skipp and Deverall (1973) were able to show some protection of bean against compatible Colletotrichum lindemuthianum races by applying the incompatible race one day later.

Paxton and Chamberlain (1967) protected Harosoy soybean locally by prior inoculation with Phytophthora cactorum 24 hours prior to subsequent inoculation with P. megasperma, and Carroll and Lukezic (1972) reported resistance to virulent isolates of Corynebacterium insidiosum when susceptible alfalfa cultivars were infiltrated with a suspension of avirulent mutants 6 hours earlier.

In these three cases, phytoalexin production was the suggested mechanism of induced resistance.

Resistance mechanisms that rely on the establishment on the host of the inducer organism require days rather than hours to be effectual.

Protection of cotton from severe strains of Verticillium albo-atrum requires exposure of the host to mild strains of V. albo-atrum for one week (Schnathorst and Mathre, 1966), allowing time for the mild strain to colonise the xylem vessels.

Later work by Bell and Presley (1969), however, using heat-killed conidia of V. albo-atrum to induce resistance, indicated that protection was due to gossypol related compounds, induction also taking one week.

Littlefield (1969) needed only 4 hours between inoculation of avirulent and virulent flax rust races, but resistance increased when the time interval was 24 hours and induced resistance was effective for 7 days. Kochman and Brown (1975) found that 4 days was required for maximum protection of oats against rust.

For powdery mildews, Ouchi, Oku and Hibino (1976) showed the necessary delay for barley protection to be 6 hours.

Systemic protection, as would be expected, requires several days. Kuć and Richmond (1977) reported systemic protection to Colletotrichum lagenarium in cucumber needed a minimum of 4 days to develop, lasting 4-5 weeks and as much as 11 weeks if a booster inoculation was given 3 weeks after the first.

Similar findings were reported by Hecht and Bateman (1964) where induced resistance to tobacco mosaic virus in tobacco by prior inoculation of lower leaves with Thielaviopsis basicola was detectable within 3 days, reaching a maximum at 6-8 days and persisting for at least 14 days.

M. Induced susceptibility

A great deal of research spanning 50 years has recorded the incidence of induced resistance in plants to infectious agents. In comparison, very little work has been done on induced susceptibility, a situation in which a virulent strain of a pathogen on infection can predispose a plant to infection by a normally non-virulent strain. Such an effect appears to negate host resistance mechanisms with obvious consequences.

There are several reports in the literature in which viral infections have been associated with increased susceptibility of some plants to a number of fungal pathogens.

Bateman (1961), using cucumber, a host which has been shown many times to be associated with induced resistance (see earlier sections), was able to increase post-emergence damping-off caused by Rhizoctonia spp. from 10-15% to 60-87% by prior inoculation with cucumber mosaic virus.

Watson and Guthrie (1964) found that severe root-rot caused by Fusarium oxysporum or F. roseum on red clover only developed when the plants were infected with clover yellow mosaic virus.

Further examples of increased susceptibility to root-rots have included peas (Farley and Lockwood, 1964) susceptible to F. solani f. sp. pisi

when inoculated with pea mosaic virus or bean yellow mosaic virus; corn susceptible to F. moniliforme (Futtrell and Scott, 1969) when infected with maize dwarf mosaic virus, and also to Pythium graminicola, Diplodia zeae, F. oxysporum and F. roseum (Mwanza and Williams, 1966). Tu and Ford (1971) found that corn predisposition by viral infection was associated with an increase in carbohydrate and ninhydrin positive substances in the host rhizosphere. This root leakage may well be increasing the inoculum potential of root infecting fungi.

Such a mechanism cannot readily explain instances of induced susceptibility to shoot diseases.

Lamey and Everette (1967) noted that rice plants infected with the hoja blanca virus showed large coalescing lesions when subsequently inoculated with Cochliobolus miyabeanus (brown spot), compared with pinpoint lesions on non-virus infected plants. No effect could be found on lesion development of rice blast (Pyricularia oryzae). Such specificity was not found by Beute (1970) who reported that infection of Gladiolus hortulansus with cucumber mosaic virus or tobacco ringspot virus increased severity of root-rots, storage rot of corns and leaf spot caused by Curvularia.

Beniwal and Gudauskas (1974), using corn and sorghum infected with maize dwarf mosaic virus, were able to show increased susceptibility to Helminthosporium maydis. The fungus produced large necrotic lesions and sporulated earlier on virus infected plants. This change in host response of sorghum appeared to be host dependent since only 5 of the 16 hybrids tested showed a positive association. Beniwal and Gudauskas suggested that the vigour of maize dwarf mosaic virus infected plants

may be sufficiently diminished rendering them susceptible to other pathogens.

Increased susceptibility induced by a fungal infection is a much rarer recorded occurrence, but Russell (1966), noted that in a field experiment, symptoms of Erysiphe betae were significantly more severe on sugar beet plants which were infected with Peronospora farinosa than on plants which were not thus infected. No explanation was given.

Ouchi, Oku and Hibino (1976) suggested that barley became more susceptible to Sphaerotheca fuligenea after infection of the same leaves with Erysiphe graminis f. sp. hordei and Moseman, Scharen and Greeley (1965) reported that small numbers of conidia of a non-virulent strain of E. graminis were produced on barley after prior inoculation with virulent strains.

Although only relatively few cases of induced susceptibility have been reported, its importance should not be underestimated. Many of the cited examples have been increased susceptibility to root diseases, a factor which should be considered when using mild viral infections as commercial protectants (see next section).

N. Importance

Though the concept of acquired physiological immunity was reviewed by Chester (1933) almost 50 years ago, few investigators have tested its validity in the greenhouse or field.

In the same year, Salaman (1933) suggested that cross-protection between

related viruses may be of economic importance. Since then many examples of induced resistance have been put forward but few have been adopted on a wide scale. There are notable exceptions to this, one being the protection of tomato plants in the field from virulent strains of tobacco mosaic virus by hypovirulent strains in Japan (Komochi, Goto and Oshima, 1966) and also in Europe, especially in glasshouse tomatoes in Holland (Rast, 1975). In the United Kingdom, glasshouse trials since 1970 have shown an overall increase in tomato yield of 7% using the Dutch mutant of tobacco mosaic virus as protectant (Upstone, 1974).

Grente (1969 and 1971) working in France has shown that European chestnut forests may be protected from blight by the introduction of hypovirulent strains of Endothia parasitica, and protection of the American chestnut, Casterea dentata in the same way was shown by Anagnostakis and Vaynes (1973).

Italian prune trees have been protected against Cytospora cincta for a whole growing season following inoculation with avirulent C. cincta, (Randall and Helton, 1976).

Caruso and Kuć (1977), in three separate field trials, partially protected cucumber, watermelon and to some extent muskmelon against Colletotrichum lagenarium but McLean (1967) reported that anthracnose in melons was very dependent on environmental conditions, a factor which may override the induction of resistance under field conditions.

Commercial application of the phenomenon of cross-protection or induced resistance has not yet been exploited.

Points to be considered before undertaking such measures (assuming that a successful protectant can be found) are numerous. Crop losses must be severe enough to warrant use of mild strains of pathogens causing smaller consistent losses. The protectant must be screened for pathogenicity to other hosts and also possible mutation. Reliable safe methods of application must be adopted and adequate supplies of protectants should be maintained.

Lastly, and possibly of overriding importance, the probable reluctance of growers to apply pathogens, however mild, to a valuable crop.

C. MATERIALS AND METHODS

A. Plant Material

1. Source of plant material

Cucumber (Cucumis sativus) seeds of cultivar Baton Vert 6662 were bought from Suttons and Sons Ltd, Reading.

2. Growth of plants

Cucumber seeds were planted in John Innes No. 2 compost in plastic trays (34 x 20 x 5 cm), twenty seeds per tray, in the glasshouse under Philips 400 W mercury vapour lamps (4000 lumens per sq m) as light and heat source. Temperature was maintained at 25°C ± 5° with a 14 hour/day photoperiod. Seeds were transplanted into 7.5 cm diameter pots after 7-10 days and grown in the glasshouse until reaching the required size.

B. Fungal Material

1. Source of fungi

a. Colletotrichum lagenarium (Dass) Ell and Halst (causative agent of cucumber anthracnose) cultures were supplied by Imperial College stock culture collection.

b. Colletotrichum lindemuthianum (Sacc and Magn) Briosi and Cav (causative agent of French bean anthracnose) race δ was supplied by Imperial College stock culture collection.

2. Growth of fungi

a. Throughout the research, acidic vegetable juice agar was used to maintain the fungi as follows:-

Vegetable juice (Campbell's V8)	200 mℓ
Agar	20 g
Distilled water	800 mℓ

Cultures were grown at 25°C in the dark.

b. For large quantities of fungus, liquid medium was used, prepared as follows:-

Carbon source (usually sucrose)	15 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Acid casein hydrolysate	4.6 g
Trace element solution	1 mℓ
Glass distilled water	1000 mℓ

The trace element solution was made up as follows:-

Salt	Stock solution (mg 100 $m\ell^{-1}$)
$FeSO_4.7H_2O$	249
$CuSO_4.5H_2O$	40
$ZnSO_4.7H_2O$	44
$MnSO_4.4H_2O$	41
$Na_2Mo_4.2H_2O$	51

250 mℓ and 2000 mℓ Erlenmeyer flasks had 100 and 1000 mℓ of media respectively and were seeded with a dense spore suspension made up in sterile distilled water. Flasks were placed on an orbital shaker at 80 rpm at 25°C for various periods.

3. Preparation of inoculum

a. Colletotrichum lagenarium cultures were used between 5-8 days after sub-culturing. These were rinsed with sterile distilled water and spores were agitated into suspension with a sterile loop. This was then filtered through four layers of muslin and washed by two centrifugations at 3,000 rpm for 10 minutes in an MSE medium centrifuge, Cat No. 8900. Spore concentration was adjusted after counting using a haemocytometer.

b. Colletotrichum lindemuthianum cultures were used between 9-10 days after sub-culturing. Inoculum preparation was as above.

4. Application of inoculum

C. lagenarium and C. lindemuthianum were applied to cucumber and French bean by either an Agla Micrometer Syringe (Burroughs Wellcome Ltd, London) dispensing 3 μℓ drops, or injected directly into leaf tissue using a hypodermic syringe (Orwell 30G).

Tissue was kept in a humid atmosphere for 48 hours following inoculation. Inoculum drops were then allowed to dry and tissue was kept at c 25°C until symptoms developed.

5. Re-isolation

To ensure continued pathogenicity, cultures were re-isolated by surface sterilising an infected leaf, cutting a small segment with a sterile scalpel and placing on growth medium. Resulting cultures were tested for pathogenicity by reinoculation of plants.

C. Bacterial Material

1. Source of bacteria

a. Pseudomonas lachrymans (causal agent of Angular Leaf Spot of cucumber) B5375 from melon, Rhodesia (Zimbabwe) and B6574 from cucumber, Canada were kindly supplied by the Commonwealth Mycological Institute, Surrey.

b. Pseudomonas phaseolicola (causal agent of Halo Blight of beans) was kindly supplied by A. Slusarenko, Imperial College.

2. Growth of bacteria

Cultures were maintained on Nutrient agar

Nutrient broth No. 2	13 g
Agar	13 g
Distilled water	1000 mℓ

3. Preparation of inoculum

Cultures were used after two days growth at 25°C. Sterile distilled

water was added and a suspension made by agitating with a glass rod. Concentrations were adjusted after counts using a nephelometer.

4. Application of bacteria

Bacteria were injected into cotyledons or leaves using a hypodermic syringe (Orwell 30G).

D. Virus Material

1. Source of virus

Tobacco necrosis virus (TNV) d-strain was kindly supplied by Dr R H A Coutts of Imperial College.

2. Storage of virus material

Infected leaf tissue was kept at $-20^{o}C$ until use.

3. Preparation and application

1 g of infected tissue was ground in a mortar with 5 mℓ of 0.05 M phosphate buffer pH 7. The resultant homogenate was rubbed onto either cotyledons or leaves after previously dusting with carborundum.

E. Chemical Assays

1. Anthrone assay

The anthrone assay (Plummer, 1971) was used to estimate the carbohydrate

content of test solutions. Four mℓ of anthrone reagent (0.2% W/V anthrone in 67% V/V H_2SO_4, freshly prepared) was added to 1 mℓ of the test solution in a boiling tube (3 replicates) mixed rapidly and placed in a boiling water bath for 10 mins. After cooling, the optical density was recorded at 620 nm against a reagent blank (Beckman model 35). A standard curve was prepared using glucose (10 to 100 μg $m\ell^{-1}$) and the carbohydrate content of test solutions determined in terms of glucose equivalents.

2. Lowry test

The protein content of test solutions was determined using the method of Lowry et al. (1950). Reagents prepared were:-

A. 2% (W/V) Na_2CO_3 in 0.1 N NaOH.

B. 1% (W/V) $CuSO_4.5H_2O$ and 2% (W/V) Na K tartrate mixed in equal volumes immediately prior to use.

C. Alkaline copper reagent : 50 parts of A to 1 part of B.

D. Folin-Ciocalteu reagent diluted to 1 N.

One mℓ of protein sample and 5 mℓ of reagent C were mixed and kept at room temperature for 30 mins. 0.5 mℓ reagent D was added and mixed. After a further 30 mins the absorbance of the solution was estimated at 750 nm against a reaction blank (Beckman model 35). A calibration curve was prepared with solutions of crystalline bovine serum albumen containing between 10 and 100 μg $m\ell^{-1}$.

F. Extraction of Fungal Material

1. Fungal cell-walls

Fungal cell-wall material was extracted by a slightly modified version of the method described by Ayers et al. (1976).

C. lagenarium or C. lindemuthianum were harvested from liquid grown cultures by suction filtration through a Buchner funnel. For each 10 g fresh weight of fungus, the following procedure was used:-

The mycelium was washed on muslin with 400 mℓ of distilled water, 400 mℓ of 100 mM K_2HPO_4 buffer, pH 7.2 and then 400 mℓ of 500 mM K_2HPO_4 buffer pH 7.2. This was followed by homogenisation in a Sorvall omnimixer in 25 mℓ of the 500 mM buffer. The homogenised mycelium was washed 8 times with 50 mℓ aliquots each of the 500 mM buffer and distilled water. The remaining cell membrane and wall material was homogenised and washed in 200 mℓ each of chloroform-methanol (1:1) and finally acetone. The extracted material was then air-dried, crushed and kept in sealed containers at -20°C until required. 200 mℓ of water were added to 2 g of the dried powder and autoclaved at 15 psi for 1½ hours. This was filtered, millipore filtered and dialysed overnight at 4°C in 200 times its volume. A small sample was used for the anthrone assay, the remainder rotary evaporated to the required concentration.

2. Culture filtrate components

Culture filtrates from liquid cultures of C. lagenarium grown at 23-25° in 100 mℓ of medium in 250 mℓ Erlenmeyer flasks were passed through a

Buchner funnel, centrifuged at 5000 rpm for 15 mins and membrane - sterilised (0.45 μ filter). The filtrate was extensively dialysed and reduced to 10% of its original volume by rotary evaporation at 40°C. The final preparation is referred to as culture filtrate and was used immediately or stored at -20°C.

3. Extraction of fungal nucleic acids

Nucleic acids were extracted using a method based on that described by Marmur (1961). C. lagenarium was grown in 100 mℓ of medium in 250 mℓ Erlenmeyer flasks. After 7 days, cultures were filtered through two layers of muslin and suspended in 1% SDS at 65°C for 1 hour. Perchlorate was added to a final concentration of 1 M and the whole mixture shaken with an equal volume of chloroform/isoamyl alcohol (24:1) in a stoppered flask for 30 mins. The resulting emulsion was separated into 3 layers by a 5 minute centrifugation at 7,500 rpm. The upper aqueous phase containing the nucleic acids was carefully pipetted off into a tube. The nucleic acids were then precipitated by addition of 2 volumes cold (-20°C) 95% ethanol. This was left overnight at -20°C then centrifuged at -10°C at 12000 g for 30 mins. The precipitate was dried thoroughly and taken up into 10 mℓ of saline-citrate (0.015 M NaCl plus 0.0015 M trisodium citrate). Concentration of nucleic acids was estimated, after dilution, by the 260/280 ratio of absorbance (Beckman model 25).

G. Spore Germination Test

The method of Purkayastha and Deverall (1965) was modified slightly. The use of cleaned microscope slides was preferred to new ones. These were obtained by cleansing in warm water and detergent followed by rinsing

with several changes of tap water. The washed slides were then immersed in distilled water for 48 hours and then in acetone for several hours. The slides were dried in an oven at 60°C. These slides were thought to be biologically clean and did not allow test drops to spread. Test solutions were applied to the clean slides as 3 10 µl drops dispensed from an Agla Micrometer Syringe. One 3 µl drop of a suspension of C. lagenarium spores was added to each test drop. If any of the drops spread the slide was disgarded. Slides were placed on moistened filter paper in sandwich boxes and incubated at 24°C in the dark for the appropriate period. Germination was stopped and spores stained by the addition of a drop of aniline blue in lactophenol (Amann's Medium) to each test drop.

Amann's Medium

Aniline blue (water soluble)	1 g
Glycerol	25 mℓ
Lactic acid	25 mℓ
Phenol	25 g
Distilled water	25 mℓ

H. Preparation of Plant Extracts

1. Isoflavonoid compounds

Tissue was homogenised in ethanol in a Sorvall omnimixer (1 g tissue/ 10 mℓ ethanol). The homogenate was filtered under suction through Whatman No. 1 filter paper. The filtrate was retained and the solid residue re-extracted twice more as above. The clear filtrates were pooled and evaporated to dryness in a rotary evaporator at 40°C. The

residue was taken up in 10 mℓ of distilled water, centrifuged at 1400 g for 15 minutes, the supernatant removed, residue re-washed with 10 mℓ of distilled water, centrifuged and the supernatants pooled. These were partitioned 3 times with equal volumes of ethylacetate. Excess water was removed from the organic phase by the addition of anhydrous Na_2SO_4. After decanting, the solution was evaporated to dryness. The residue was taken up in a small volume of ethylacetate and used for thin layer chromatography (t.l.c.).

2. Soluble plant proteins

Protein extracts from test materialwere prepared by grinding tissue in a mortar with extraction buffer (1 mℓ/g).

Extraction buffer:-

1 M Tris/HCl pH 8

0.5 M sucrose

3% mercaptoethanol

The homogenate was centrifuged at 40000 g for 30 mins and the supernatant retained for analysis on 10% acrylamide gels.

3. Water soluble carbohydrate (WSC)

Five 12 mm leaf disks cut with a cork borer from test material were homogenised in a mortar in 15 mℓ of distilled water. The homogenate was centrifuged at 12000 g for 10 mins and the supernatant used to determine the WSC by the Anthrone assay (See section E1).

4. Amino-acids

Three 4 mm leaf disks were taken from test material and left overnight in 5 mℓ of methanol at 4°C. One mℓ of this extract was used for amino acid determination by the method described by Yemm and Cocking (1955). This method also detects the presence of ammonia, but since this is only one-third as reactive as ∝-amino nitrogen compounds, its influence on the determination was assumed to be negligible.

5. Chlorophyll

Three 4 mm leaf disks were taken from test material and left overnight in 5 mℓ of ethanol at 4°C. Relative amounts of chlorophyll were determined using a Beckman model 35 spectrophotometer by absorbance at 670 nm using water as a blank.

6. Cell wall material

I. Healthy and protected plants were placed in the dark for 24 h to destarch. Leaves 2 and 3 were detached (leaf 1 had been inoculated with C. lagenarium or water 9 days previously), and weighed. Between 400-500 g fresh weight of material was used. Leaves were homogenised with approximately 10 volumes of ethanol (precooled to -20°C) in a "Sorvall" omnimixer for 5 min. The homogenate was filtered through Whatman No. 1 filter paper. The residue was mixed with 3 volumes of cold acetone and filtered. Acetone washes were continued until the residue was pigment free. The residue was then filtered through 2 layers of miracloth and homogenised with 10 volumes of 0.1 M potassium phosphate buffer

pH 7.0. Filtration and homogenisation was repeated a further 4 times. The residue was then washed with distilled water until the filtrate was clear. The residue was homogenised with 10 volumes of chloroform-methanol (1:1 V/V), filtered, washed with chloroform-methanol and washed finally with acetone. The residue was dried for 24 h at 50°C, then ground in a pestle and mortar to a fine powder. This powder is regarded as the crude cell-wall extract.

Cell-wall material from protected and unprotected cucumber plants was used as carbon source in liquid shake culture for C. lagenarium.

II. Pectic substances

Dried cell-wall materials extracted from protected and unprotected cucumber plants as above were boiled with 50 volumes of 0.1 M citrate-oxalate buffer pH 4 for 15 min. The mixture was centrifuged at 12000 g for 15 min, supernatant decanted and filtered through Whatman No. 1 filter paper. The residue was dried and retained for further extractions (III). The supernatant was then mixed gradually with 2 volumes of ethanol. Pectic substances precipitated and were removed with a glass rod and by centrifugation at 12000 g for 15 min. The fibrous pectic material was dried for 24 hours at 50°C, weighed and ground to a fine powder in a pestle and mortar.

III. Xylan rich fraction

The residual cell-wall material from II was soaked in 0.1 M KOH for 12 h. After filtration the alkaline solution was acidified with acetic acid to pH 4.8 giving a precipitate which was discarded.

The polysaccharides of the supernatant were precipitated by 3 volumes of ethanol and collected by centrifugation at 12000 g for 15 min. The pellet was resuspended in 4.3 M KOH for 24 h. The xylan-rich fraction was precipitated by the addition of acetic acid to pH 4.8 and 3 volumes of ethanol. The pellet was collected by centrifugation, washed with acetone and dried for 24 h at 50°C.

All the above fractions were stored at 4°C until use.

7. Tissue diffusates

First leaves of cucumber plants were inoculated with c twenty 3 µℓ drops of a suspension of C. lagenarium spores (1×10^7 mℓ$^{-1}$) as described earlier. Seven days later the developed lesions were cut from inoculated leaves with a cork borer and placed in an Erlenmeyer flask with 100 mℓ of sterile distilled water. The areas immediately surrounding these lesions were removed and placed in a separate flask, as were disks from the protected second leaves. Disks from water inoculated plants were cut as control. Tissue was shaken on an orbital shaker at c 100 rpm for 24 hours. The liquid obtained, referred to as tissue diffusate, was centrifuged (2000 g for 20 min), millipore filtered and reduced to 1/10 of the original volume by rotary evaporation at 40°C.

Cotyledons of 15 day old cucumber plants were injected with tissue diffusates using a hypodermic syringe (Orwell 30G). Seven days later the first true leaves were challenge inoculated with a suspension of C. lagenarium spores (1×10^5 mℓ$^{-1}$).

I. Treatments

1. Application of cytokinin

a. To plants

A stock solution of 6-benzylamino purine (6-BAP) was prepared by dissolving 6-BAP in a small volume of ethanol and diluting with distilled water. Control treatments containing equivalent volumes of ethanol were used. First leaves of cucumber plants were sprayed daily with either 10, 1, 0.1 or 0 mg ℓ^{-1} 6-BAP using a Shandon spray gun from 15-20 cm until run-off for 11, 6 or 3 days.

b. To liquid cultures

C. lagenarium was grown in 100 mℓ of medium in 250 mℓ Erlenmeyer flasks (section B26). 6-BAP was added to a final concentration of 10, 1, 0.1 or 0 mg ℓ^{-1}. Fungal dry weights were assessed after various periods by drying in an oven at 50°C.

2. Application of abscisic acid (ABA)

A stock solution of 10 mg ℓ^{-1} of ABA was prepared. First leaves of cucumber plants were sprayed daily with either 10, 1, 0.1 or 0 mg ℓ^{-1} using a Shandon spray gun from 15-20 cm until run-off, for either 11, 6 or 3 days.

3. Non-ionizing radiation

Leaf disks (12 mm) cut from protected or unprotected cucumber plants

were placed on moist filter paper in 9 cm petri dishes and irradiated with ultra violet (UV) light from a "Phillips TUV 30 watt" germicidal single tube with output of 8 watts at 253.7 nm wavelength. Disks were 140 cm from the UV source.

4. Temperature

Leaf disks (12 mm) taken from protected and unprotected cucumber plants were immersed in water at 5, 15, 25, 35, 45 and 55°C for periods between 5-300 seconds. Disks were air-dried and inoculated as described earlier (section A4).

5. Polyacrylic acid

Polyacrylic acid was kindly supplied to Dr R H A Coutts by Allied Colloids Ltd, Bradford as Dispex N40.

Dispex N40

Nature	sodium salt
Appearance	Pale yellow
Active content	40 ± 1%
Viscosity	15-25 cS at 25%
pH	7.0-7.5
Specific gravity	1.3
Molecular weight	c 1500-2000

Dispex N40 was diluted to 4 or 2 mg $m\ell^{-1}$ with phosphate buffer pH 6.8.

a. Leaf injection

Solutions were injected into cucumber cotyledons using an hypodermic syringe (Orwell 30G). Complete water soaking of cotyledons could usually be achieved with one or two penetrations per cotyledon.

b. Systemic uptake

I. Cut petioles were placed in solutions of Dispex N40 in phosphate buffer pH 6.8 in vials for various periods of time. Leaves were kept at 25°C in a growth cabinet.

II. Cucumber seeds were placed in seed trays containing vermiculite and watered with Long Ashton Nutrient Solution (LANS) containing Dispex N40.

6. Acetylsalicylic acid and related compounds

Acetylsalicylic acid, salicylic acid, 4-hydroxybenzoic acid, 4-aminobenzoic acid, 4-aminosalicylic acid, gentisic acid, resorcinol and protocatechuic acid were all dissolved in phosphate buffer pH 6.8 (some substances needed gentle warming). Methods of application were those described for Dispex N40.

7. Polyethylene glycol 6000 (PEG) and mannitol

A range of concentrations between 10-25% were prepared by dissolving PEG in distilled water. Whole cotyledons or leaf disks (12 mm) cut from protected or unprotected cucumber plants were floated on these solutions

in 9 cm petri dishes for various lengths of time in a growth cabinet at c 24°C. Leaf material was then blotted dry, placed on distilled water and challenged.

The Relative Water Content (RWC) was determined according to the following formula:-

$$RWC = \frac{\text{(fresh weight) - (oven-dry weight)}}{\text{(fully turgid weight) - (oven dry weight)}} \times 100$$

Disks were weighed after floating on PEG solutions and again after becoming fully turgid on distilled water. Dry weight was determined when material reached constant weight in an oven at 60°C.

Mannitol was dissolved in distilled water to give final concentrations of 0.5, 0.25 and 0.05 M solutions. Procedure was then as above for PEG.

8. Actinomycin D and cycloheximide

Actinomycin D (AD) and cycloheximide (CH) were obtained from Sigma (London) Chemicals Co, Kingston-upon-Thames, Surrey. Solutions at 20, 10 and 2 ppm were prepared for both chemicals. Leaf disks (15 mm) were cut from protected and unprotected cucumber leaves and placed on solutions of the inhibitors in 9 cm petri dishes, 24 hours prior to, at the same time as, and 24 hours after challenge inoculation by C. lagenarium. Following treatment with inhibitors, disks were removed and placed on moist filter paper in 9 cm petri dishes.

J. Histology

Leaf disks (4 mm) were cut from inoculated sites of protected and unprotected leaves. These were cleared using chloroform-lactic acid-methanol (1:1:1) at room temperature for 2 days, or in 90% ethanol in a boiling water bath for 2-3 mins.

Such disks were used subsequently.

1. Fungal structures

Leaf disks were placed on a clean microscope slide and covered by 1 or 2 drops of lactophenol cotton blue. The slide was warmed gently for 1-2 mins after which the disks were placed in clear lactophenol for several hours to destain.

2. Lignin

a. Leaf disks were placed in 0.1% toluidine blue in 0.1 M phosphate buffer pH 6.5 for 2 mins. Disks were then rinsed and mounted in water or buffer.

This test detects polyphenolic material.

b. Leaf disks were placed in phloroglucinol (0.5 g phloroglucin plus 100 mℓ 75% ethanol) for 4 mins. One drop of concentrated HCl was added and the disks mounted.

This test detects cinnamaldehyde end groups.

3. Callose (β1-3 D glucan)

Leaf disks were placed in 0.1% aniline blue in 0.07 M tri-potassium orthophosphate buffer at pH 12 for 24 hours at 20°C. Disks were viewed using epifluorescence optics.

K. Light Microscopy

Cleared leaf disks, stained or unstained, were observed for reaction and penetration of cells and other features.

Photographs were taken with an Exacta VX 1000 camera mounted on the microscope. The camera was loaded with Kodak high speed Ektachrome (tungsten) reversal film (ASA 125).

L. Chromatography

1. Ascending thin-layer-chromatography (tlc)

Tlc plates were 20 cm square plastic or glass backed silica gel (0.25 mm) coated, (Griffin and George Ltd).

a. Isoflavonoid compounds

Extracts from plant material (section Ha) were taken up in c 200 $\mu\ell$ of ethyl acetate and applied in bands to the tlc plate origin using a drawn-out Pasteur pipette. This was applied at a loading equivalent to approximately 1 g extracted tissue per cm.

Chromatograms were routinely run at c 24°C in a Shandon Chromatank after equilibration in a solvent-saturated atmosphere for 30 min. The solvent used was chloroform-methanol (25:1).

Detection of substances was by either ultra violet absorbing and/or fluorescing bands using a UV light source at 254 and 366 nm respectively, or by development of inhibitory zones produced in C. lagenarium bioassays which were carried out as follows. Developed tlc plates were assayed for antifungal compounds by spraying the entire surface with a heavy suspension of C. lagenarium (not Cladosporium cucumerinum) conidia in a sucrose liquid medium. Zones of inhibition appeared white against a dark background of C. lagenarium mycelium 2 to 3 days after incubation in high humidity chambers at room temperature.

b. Enzyme digestion products

Enzyme reaction mixtures were set up as described (section N3). 50 µl aliquots were taken from the mixtures at various time intervals, boiled for 5 min and spotted onto the origin of a tlc plate. Reference standards (0.2%) were dissolved in 10% isopropanol and 10 µl applied to tlc plates. Plates were run in a Shandon Chromatank after equilibration in a solvent saturated atmosphere for 30 min. The solvent used was Butanol-acetic acid-water (20:5:11). Chromatograms were run for 3-4 hours. After drying, enzyme digestion products were developed by spraying plates with aniline-xylose reagent (Xylose, 1 g in 3 ml; add aniline, 1 ml and make up to 100 ml with methanol), allowed to dry then heated to 105°C for 5 min. Organic compounds appear as brown spots on a white background.

2. Paper chromatography

80 μℓ of reaction mixtures of enzyme digestions were spotted onto Whatman No. 3 paper. Reference standards were prepared as above. Chromatograms were run by descent with the solvent ethylacetate-pyridine-acetic acid-water (5:5:1:3) for c 20 h. After drying, products were developed by spraying the paper with silver nitrate in acetone (prepared by diluting 0.1 mℓ saturated silver nitrate to 20 mℓ with acetone, then adding water dropwise until the silver nitrate redissolves). After drying the plate was sprayed with 0.5 N NaOH in aqueous ethanol. Brown silver oxide is produced, reducing sugars forming dense black spots against a brown background.

M. Gel Electrophoresis

Gel electrophoresis was used for the resolution of proteins in a LKB 2117 Multiphor apparatus. Cylindrical 10% acrylamide gels were prepared as follows:-

a.

1. Gel buffer

1 N HCl	48.0 mℓ	) disolved in 100 mℓ
Tris	36.3 g	) of water pH 8.9

2. Acrylamide stock

Acrylamide	30 g	) in 100 mℓ of
Methylene Bis-acrylamide	0.8 g	) distilled water

3. Ammonium persulphate

Ammonium persulphate 0.1 g in 1 mℓ H_2O

This was made up fresh each time.

4. TEMED

(NNNN - tetramethylethylene diamine)

b. 10% acrylamide gels were prepared as follows:

3.75 mℓ of 1

9.99 mℓ of 2

0.015 mℓ of 4

16.095 mℓ of H_2O

This mixture was degassed before addition of 0.15 mℓ of 3.

The above mixture was poured into glass gel tubes (c 100 mm x 6 mm) immediately after the addition of the ammonium persulphate. Polymerisation occurred at room temperature.

Running buffer was as follows:-

Tris	6 g)	made up)	
	)	)	pH 8.3
Glycine	28.8 g)	to 1000 mℓ)	

Samples were loaded onto the gels in sucrose with bromophenol blue (0.12 g/100 mℓ) as marker. Gels were run at 4 mA per gel until the marker was within 1 cm of the bottom of the tube. After removal from the tubes, gels were stained with Coomassie blue (0.03% in

methanol-water-acetic acid, 5:5:1) overnight and destained in methanol-water-acetic acid (5:5:1).

N. Enzymology

1. Substrates for cell-wall degrading enzymes, sodium polypectate (NaPP) and Pectin from citrus (Sunkist Growers Inc., Ontario, California, USA) were washed before use in enzyme assays. 50 g was added to 300 mℓ 70% ethanol containing 0.05 M HCl and mixed for 60 mins. Substrates were then collected on Whatman No. 41 filter paper and washed several times with 70% ethanol until no traces of chloride were detected in the washings by the 'silver-nitrate' test. The residue was then washed with 95% ethanol and dried in an oven at 70°C to constant weight.

To prepare solutions of pectic substances, substrates were added slowly to water stirred vigorously on a magnetic stirrer. Solutions were made to twice the final concentration required and brought to the required pH by adding an equal volume of double strength buffer. Substrates were clarified by low speed centrifugation (5000 g for 10 mins) before use and prepared freshly each day.

Xylan (ex larch) and araban (ex larch) (Koch-Light Laboratories, Colnbrook, Bucks) were used in cell-wall degrading enzyme assays. Araban was washed in a large volume of 95% ethanol containing 0.05 M HCl until no reducing sugars could be detected in the supernatant using the Nelson/Somogyi assay (Nelson, 1944). The substrate was then collected on Whatman No. 41 filter paper, washed with 95% ethanol to remove chloride, washed with absolute alcohol and dried to constant weight at 70°C.

2. Other substrates

a. Laminarin (a β-(1-3) glucan) (kindly supplied by Dr S. A. Archer) was dissolved in citrate buffer 0.1 M pH 4.7 (8 mg/mℓ) at 90°C in a water bath.

b. Chitin (N-acetyl-D-Glucosamine) (BDH Chemicals Ltd, Poole, Dorset) was dispersed in citrate buffer 0.1 M pH 5.8 (20 mg/mℓ).

3. Measurement of enzymatic activities

a. Release of reducing groups (Nelson, 1944).

Copper reagent A:-

Na_2CO_3 (anhydrous)	25 g
potassium sodium tartrate	25 g
$NaHCO_3$	20 g
Na_2SO_4 (anhydrous)	200 g

These were dissolved in c 800 mℓ of distilled water, diluted to 1ℓ, filtered and stored at room temperature.

Copper reagent B:-

$CuSO_4.5H_2O$	15 g	) diluted to 100 mℓ)
H_2SO_4 (concentrated)	1 or 2 drops	) water

Arsenomolybdate reagent:-

Ammonium molybdate (25 g) was dissolved in 450 mℓ water and 21 mℓ

conc H_2SO_4 added; 3 g of sodium arsenate in 25 mℓ water was added and the solution incubated at 37°C for 48 h. The reagent was stored in a brown glass bottle at room temperature.

Copper reagents were mixed on the day of use, 25 parts A to 1 part B. The test sample (1 mℓ or smaller volume made up to 1 mℓ) was added to 1 mℓ copper reagent and heated in a boiling water bath for exactly 30 mins. After cooling, 1 mℓ of arsenomolybdate reagent was added and thoroughly mixed on a "Whirlimixer" until CO_2 evolution had ceased. The blue colour produced was estimated at 660 nm against a reagent blank in a Beckman model 35 spectrophotometer.

Calibration curves were obtained with a series of xylose, arabinose and glucose solutions and enzyme activity expressed as μg $m\ell^{-1}$ or μmoles released.

b. Viscosity reducing activity

Viscometry was carried out in Cannon-Fenske Viscometers (size 200) in a water bath at 30°C. Reaction mixtures contained:-

8.0 - 8.9 mℓ	1.2% pectic substance or carboxymethyl cellulose (CMC)
1.0 mℓ	$CaCl_2$ (to give final concentration between 0.001 - 0.1 M)
	or
1.0 mℓ	H_2O
0.1 - 1.0 mℓ	enzyme preparation

Assays for polygalacturonase (PG) used pectic substrate in 0.1 M sodium citrate buffer pH 5 with no $CaCl_2$. For trans-eliminase (TE) assays, $CaCl_2$ was mixed with the enzyme before addition to substrate to prevent substrate gelling. Reaction was buffered in 0.1 M glycine-NaOH pH 9.0.

Assay for CMCase activity was 0.21% CMC in 0.1 M sodium citrate buffer at pH 5.0 with water.

On addition of enzyme, the reaction mixture was mixed by blowing through the viscometer and then viscosity measured at intervals. Enzyme activity is expressed as $(1/t) \times 1000$ where t is the time (min) for a 50% reduction in substrate viscosity. All viscometers were calibrated against water, the flow time for 10 mℓ representing 100% reduction in viscosity.

c. Thiobarbituric acid test (Ayers et al. 1966)

The reaction mixtures were as described for viscometry with NaPP or pectin as substrates. These were incubated at 30°C for 30 min. 1 mℓ of sample was taken out and added to 5 mℓ of thiobarbituric acid (0.04 M) and 2.5 mℓ of HCl (M) in a test tube and mixed well. The tube was covered with a metal cap and placed in a boiling water bath for 30 min. After cooling, the red colour produced was read at 515 nm (PG) or 550 nm (TE) against a reaction blank in a Beckman model 35 spectrophotometer.

4. Determination of trans-eliminative activity

When pectic substrates are degraded by trans-elimination there is an

increase in the appearance of 4, 5-unsaturated bonds which absorb strongly at 232 nm.

Reaction mixture:-

NaPP or Pectin (0.6-1.2%)	1.9 mℓ
$CaCl_2$ (final concentration 0.001-0.1 M)	0.1 mℓ
Enzyme preparation	1.0 mℓ

Matching cuvettes were placed in a Beckman model 35 spectrophotometer and zeroed with water. Substrate, $CaCl_2$ and water (reference) or enzyme (reaction) were placed in the cuvettes and increase in absorbance at 232 nm noted at various time intervals. Trans-eliminative activity is expressed as increase in absorbance per min at 232 nm.

5. Extraction and assay of phenylalanine ammonia-lyase (PAL)

One g of appropriate tissue was homogenised in a pestle and mortar in 5 mℓ of 0.025 M borate buffer pH 8.8, containing 50 μM of mercaptoethanol. The crude homogenate was clarified by centrifugation (14000 g for 20 min) and the cleared supernatant assayed for PAL according to the method of Zucker (1968). Extraction was done at $4^{o}C$.

Reaction mixture:-

60 mM L-phenylalanine	1 mℓ
(in 0.05 M borate buffer)	
0.05 M borate buffer pH 8.8	1.5 mℓ
Enzyme preparation	0.5 mℓ

After mixing tubes were incubated for 2 h at 37°C. Change in optical density at 290 nm was read as a measure of enzyme activity. Hadwiger (1971b) has calculated that the liberation of 3.3 n moles of cinnamic acid will cause an increase of 0.01 in the OD.

6. Extraction and assay of peroxidase

After weighing, 20 leaf disks (4 mm) from appropriate tissue were ground with a pestle and mortar in 10 mℓ of phosphate buffer, 0.01 M pH 6.0. The homegenate was centrifuged at 10000 g for 15 min and the supernatant used as the enzyme preparation. Enzyme activity was assayed according to the method of Lobenstein and Lindsey (1963).

Reaction mixture:-

Pyrogallol reagent	2.5 mℓ
Enzyme preparation	0.5 mℓ
Hydrogen peroxide	0.5 mℓ

Enzyme preparation was added to pyrogallol reagent in a cuvette and zeroed against a reaction blank in a Beckman model 35 spectrophotometer at 404 nm. The reaction tube was removed and hydrogen peroxide added. The tube was inverted once, immediately re-inserted into the spectrophotometer and the time taken for an increase in optical density of 0.2 units recorded. As there is likely to be no appreciable decomposition of the peroxide in such a short period of time, enzyme activity can be assumed to be inversely proportional to the time required for the change in OD. Absolute peroxidase activity values are therefore expressed as sec mg fresh wt^{-1}.

7. Extraction and assay of chitinase

Ten g of appropriate tissue were ground in a "Sorvall" omnimixer in 10 mℓ of 0.1 M sodium citrate buffer pH 5.8. The suspension was centrifuged at 12000 g for 20 min, the pellet resuspended in the same buffer and recentrifuged. The two supernatant fluids were combined and dialysed overnight against 200 times the volume in the same buffer. A typical reaction mixture consisted of:-

20 mg chitin dispersed in 0.8 mℓ, pH 5.8 citrate buffer

0.2 mℓ enzyme preparation

Tubes were incubated at 25°C for 2 h and assayed by the Nelson/Somogyi method.

8. Extraction and assay of β-(1-3) glucanase

Ten g of appropriate tissue were ground in a "Sorval" omnimixer in 10 mℓ of 0.1 M sodium citrate buffer pH 4.7 supplemented with 0.1 mℓ of Triton X-100. The suspension was centrifuged at 12000 g for 20 min. The pellet was resuspended in 10 mℓ of the same buffer and recentrifuged. The two supernatant fluids were combined and dialysed against the buffer overnight. All preparations were done at 4°C.

β-(1-3) glucanase activity was determined in cell-free extracts in the following reaction mixture:-

Laminarin in 0.1 M Na citrate buffer pH 4.7	8 mg in 0.8 mℓ
Enzyme preparation	0.2 mℓ

Tubes were incubated for 2 h in a water bath at 25°C after which reducing groups were assayed by the Nelson/Somogyi method. Control mixtures were identical but stopped at zero time.

9. Extraction and assay of RNase (Chakravorty and Scott, 1979)

Ten g of appropriate leaf material were homogenised in 100 mℓ of ice-cold 50 mM potassium phosphate buffer, pH 6.8 containing 10 g polyvinylpolypyrrolidine. The slurry was passed through two layers of Miracloth and the filtrate was centrifuged at 10000 g for 30 min in a refrigerated centrifuge (MSE Hi-spin 21). The pellets were discarded and the pH of the supernatant fraction was adjusted to 5.0 with 1 N HCl. After standing overnight at 0°C, the precipitate formed was collected by centrifugation as above. The supernatant is regarded as "soluble RNase". The pellets were redissolved in 50 mM potassium phosphate buffer, pH 6.8 and clarified by centrifugation. This fraction is regarded as "pH 5 insoluble RNase".

RNase activity was assayed using high molecular weight yeast RNA.

Reaction mixture was, in a final volume of 1 mℓ:-

Sodium acetate buffer pH 5.8	40 mM
Yeast RNA	0.6 mg
Enzyme preparation	50-100 µg protein

Samples were incubated for 20 min at 33°C. The incubation mixtures were then chilled quickly in an ice bath and 2.0 mℓ of a precipitating reagent (1 N HCl in 76% ethanol containing 0.5% $LaCl_3$) was added to each

tube. The contents of the tube were mixed thoroughly, allowed to stand for 10 min and centrifuged until the supernatant was clear. The absorbance of the supernatant fraction was read at 260 nm and corrections were made for enzyme blanks.

One unit of RNase activity is defined as the amount of enzyme catalysing an increase in the absorbance at 260 nm of 1.0 under the standard conditions of assay with yeast RNA as substrate.

10. Determination of the mechanism of bond lysis by pectolytic enzymes

The time required for a 50% reduction in viscosity was determined as described earlier (NcII) for substrate concentrations of 1.2% and 0.6%. In a similar series, samples of the reaction mixture were removed at times corresponding to the appropriate t_{50}, determined as above, and the reaction stopped by addition of the Nelson/Somogyi reagent and reducing groups determined.

From the data, % hydrolysis of the substrate can be calculated thus:-

$$\% \text{ hydrolysis} = \frac{\text{mg anhydrogalacturonic acid liberated}}{\text{mg anhydrogalacturonic acid in original substrate}} \times 100$$

This was calculated on the basis of 78% (W/W) galacturonide content of substrates.

11. Isoelectric focusing

This method was used for analytical separation of proteins according to their isoelectric point.

The principle of the method depends on the establishment of a pH gradient between two electrodes, the proteins in the sample migrating along this gradient to points corresponding to their individual isoelectric points. The pH gradient is produced by the use of low molecular weight 'Ampholine' carrier ampholytes which give gradients between pH 3-10 or fractions of this range. These ampholytes are mixtures of a large number of different aliphatic polyaminopolycarboxylic acids and were used at a final concentration of 1%.

Using an LKB 'Ampholine' electrofocusing column (110 mℓ) solutions for the isoelectric focusing were prepared as follows:-

Cathode solution (light)

NaOH	0.1 g
Water	10 mℓ

Anode solution (dense)

Phosphoric acid	0.2 mℓ
Water	14 mℓ
Sucrose	12 g

Gradient solution (light)

Carrier ampholytes	0.75 mℓ
Culture filtrate	45 mℓ
Water	14.25 mℓ

Gradient solution (dense)

Carrier ampholytes	1.75 mℓ
Culture filtrate	15 mℓ

Water	25.25 mℓ
then add 28 g of sucrose	

The column has an inner and outer water jacket through which externally-cooled water, maintained at 4°C by a water-bath thermostat, is circulated.

To set up the column, the outlet was closed and the dense electrode solution poured in carefully in sufficient quantity to cover the lower electrode. The electrode solutions are included to prevent contact of the carrier ampholytes with the electrodes. The dense and light gradient solutions were then added by means of a gradient mixer. The flow was kept at the constant rate of 4 mℓ min^{-1} by gravity feed to ensure even mixing and to avoid discontinuities in the sucrose gradient. Using this method, the sample is evenly distributed throughout the gradient. Filling of the column continued until just below the upper electrode. The light electrode solution was then layered carefully on top of the gradient solution to cover the upper electrode. The column was then connected to an LKB DC Power supply and the voltage adjusted to give maximum power of not more than 2-3 W. During the run the voltage is adjusted to higher values up to c 1000 v until the current falls to a steady low level at which time the run is completed. Running time was about 48 h. To empty the column, water was pumped through the column using a peristaltic pump and 5 mℓ fractions were collected. The pH values of the fractions were measured using a pH meter. Fractions were then assayed for enzyme activity or stored at -20°C.

12. Determination of the Michaelis constant for trans-eliminative enzymes

The Michaelis constant (km), which is normally expressed in units of

concentration (mole litre^{-1}), is the substrate concentration which gives half the maximum velocity of a chemical reaction. Although the km cannot show the affinity of an enzyme for a particular substrate directly, since this is given by the dissociation or substrate constant of an enzyme substrate complex, km approximates to this value when the velocity constant K_{-1} is greater than constant K_2 in the following reaction.

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES \xrightarrow{K_2} E + \text{products}$$

E - enzyme; S - substrate; ES - enzyme-substrate complex

K_{+1}, K_{-1} and K_2 are velocity constants

The Michaelis constant for <u>trans</u>-eliminase was determined as follows:-

The enzyme was incubated with the substrate at final concentrations of 0.01% - 0.5% with $CaCl_2$ as described earlier. Enzyme activity was assayed by the change of absorbance at 232 nm over a given time period. Graphs of the reciprocal of activity against the reciprocal of substrate concentration (Lineweaver-Burk plot) were plotted. Km is the reciprocal of the intercept of the graph at the 'x' axis (substrate concentration).

O. Sterilization

Glassware and media were sterilised by autoclaving at 15 psi for 15 min. Pipettes were sterilised in metal cannisters in an oven for 8 h at 300^{o}C. Solutions of substances which would have been altered by autoclaving were sterilised by passage through a membrane filter (0.45 μ).

P. pH measurements

The pH of large volumes was measured with a 'Pye 290' (Pye Unicam, Cambridge) pH meter. For smaller volumes, BDH narrow range indicator papers were used.

Q. Spectrophotometry

A 'Beckman' model 35 spectrophotometer with a 'Beckman' potentiometric recorder was used for all absorption measurements in conjunction with fused silica cuvettes of 1 cm pathlength.

R. Chemicals

Unless otherwise stated, chemicals were of 'Analar' grade and were obtained from the British Drug House (BDH), Hopkin and Williams, Koch Light and Sigma Chemicals. Methanol and ethanol were obtained from James Burroughs Ltd; Davis standard agar (Davis Gelatine Ltd) was used throughout this work. Glass distilled water was used whenever 'water' is referred to in the text.

S. Long Ashton nutrient solution

The following stock solutions were prepared:-

	Salt	g ℓ^{-1}
A.	KNO_3	101.0
B.	$Ca(NO_3)_2.4H_2O$	236.0
C.	$MgSO_4.7H_2O$	92.0

D.	$NaH_2PO_4.2H_2O$	104.0	
E.	$MnSO_4.4H_2O$	11.15	Combined in 1 ℓ with a small amount of EDTA solution.
	$CuSO_4.5H_2O$	1.25	
	$ZnSO_4.7H_2O$	1.45	
	H_3BO_3	15.5	
	$Na_2MoO_4.2H_2O$	0.605	
	$CoSO_4.7H_2O$	0.285	
F.	FeNa EDTA / $FeSO_4.7H_2O$		Prepared as follows:-

Dissolve 27.8 g $FeSO_4.7H_2O$ in 330 mℓ water.

Dissolve 37.8 g EDTA-$Na_2.2H_2O$ in 300 mℓ water.

Mix the two solutions, aerate and make up to 1000 mℓ.

LANS was prepared as follows:-

Stock	mℓ ℓ^{-1}
A	4
B	4
C	4
D	2
E	0.2
F	0.5

Stock solutions were kept at 4^{o}C until required.

D. EXPERIMENTAL RESULTS

Section 1. The Phenomenon

This section will deal with experiments designed firstly to confirm the occurrence of induced resistance in cucumber, and secondly to attempt to clarify the phenomenon.

1. Inoculation of cotyledons protecting first leaves.

To determine whether Cucumis sativus cv. Baton Vert could be systemically protected against the anthracnose fungus Colletotrichum lagenarium by prior inoculation with the same fungus or other cucumber pathogens, the following experiments were done:-

a. Infection of cotyledons by C. lagenarium

Cotyledons of 15 day old cucumber plants were each inoculated with six 3 $\mu\ell$ drops of spores of C. lagenarium (1×10^7 $m\ell^{-1}$) as in Materials and Methods. After 7 days the first true leaf was challenge inoculated with twenty 3 $\mu\ell$ drops of spores of the same fungus (1×10^5 $m\ell^{-1}$).

Lesion size and number on challenged first leaves were noted after a further 7 days (Table 1.1).

Table 1.1 Protection of first leaf following infection of cotyledons with Colletotrichum lagenarium

Cotyledon treatment	Lesion diameter on challenged 1st leaf/mm	% of inoculated sites producing lesions
H_2O	4.45[a]	100
C. lagenarium spores	2.11[ab*]	47

a. mean of 80 lesions on 4 plants

b. significantly different at 0.001% level

b. Infection of cotyledons with tobacco necrosis virus (TNV)

Cotyledons of 15 day old cucumber plants were inoculated with the manually transmissible soil borne tobacco necrosis virus (D serotype) by rubbing cotyledons with diluted sap (1:5 w/v) from infected plants after dusting cotyledons with carborundum powder. Seven days later the first leaf was challenge inoculated with twenty 3 $\mu\ell$ drops of a spore suspension of C. lagenarium (1 x 10^5 $m\ell^{-1}$). Lesion size and number were noted after a further 7 days (Table 1.2).

Table 1.2 Protection of first leaf following infection of cotyledons by tobacco necrosis virus

Cotyledon treatment	Lesion diameter on challenged 1st leaf/mm	% of inoculated sites producing lesions
H_2O	4.46^{a}	100
TNV	2.45^{ab}	53

a.)
) see footnotes of Table 1.1
b.)

c. Infection of cotyledons with Pseudomonas lachrymans

The bacterial pathogen, Pseudomonas lachrymans, causal agent of cucumber angular leaf spot, was either sprayed on to or injected into cotyledons of 15 day old cucumber plants at various concentrations. Seven days later the first true leaf was challenge inoculated with twenty 3 $\mu\ell$ drops (1×10^{5} $m\ell^{-1}$) of a spore suspension of C. lagenarium. Lesion size and number were noted on challenged leaves after a further 7 days (Table 1.3).

Summary

1. Infection of cucumber cotyledons with C. lagenarium, TNV or Pseudomonas lachrymans causes a reduction in lesion size and number when subsequently challenged with C. lagenarium on the first leaf.

Table 1.3 Effects of Pseudomonas lachrymans on development of lesions caused by Colletotrichum lagenarium

Concentration of P. lachrymans	Cotyledons sprayed with P. lachrymans		Cotyledons injected with P. lachrymans	
	Lesion diameter on challenged 1st leaf/mm	% of inoculated sites producing lesions	Lesion diameter on challenged 1st leaf/mm	% of inoculated sites producing lesions
0	3.36	100	3.36	100
5×10^1	3.40	100	3.37	100
5×10^2	3.25	100	2.80[a]	100
5×10^3	2.92[b]	95	2.39[a]	86
5×10^4	2.80[b]	83	3.00[c]	81
5×10^5	-[e]	-	1.89[ad]	75

means of 80 lesions on 4 plants/treatment (except d)

a Significantly different at 0.001% level from control treatment

b Significantly different at 0.01% level from control treatment

c Significantly different at 0.02% level from control treatment

d means of 40 lesions on 2 plants.

e No result

2. This reduction was greater than 50% when C. lagenarium was the inducer pathogen.

3. C. lagenarium was a better inducer of resistance than TNV, which in turn was better than P. lachrymans.

2. Timecourse of induction of resistance.

a. Inducer organism, C. lagenarium

The effect of the time interval between inoculation of leaf one and challenge inoculation of leaf two was studied.

The first true leaf of 15 day old cucumber plants was inoculated with forty 3 $\mu\ell$ drops of C. lagenarium as in Materials and Methods. Groups of plants then had their first leaf removed on 3, 5, 7, 9, or 11 days after inoculation.

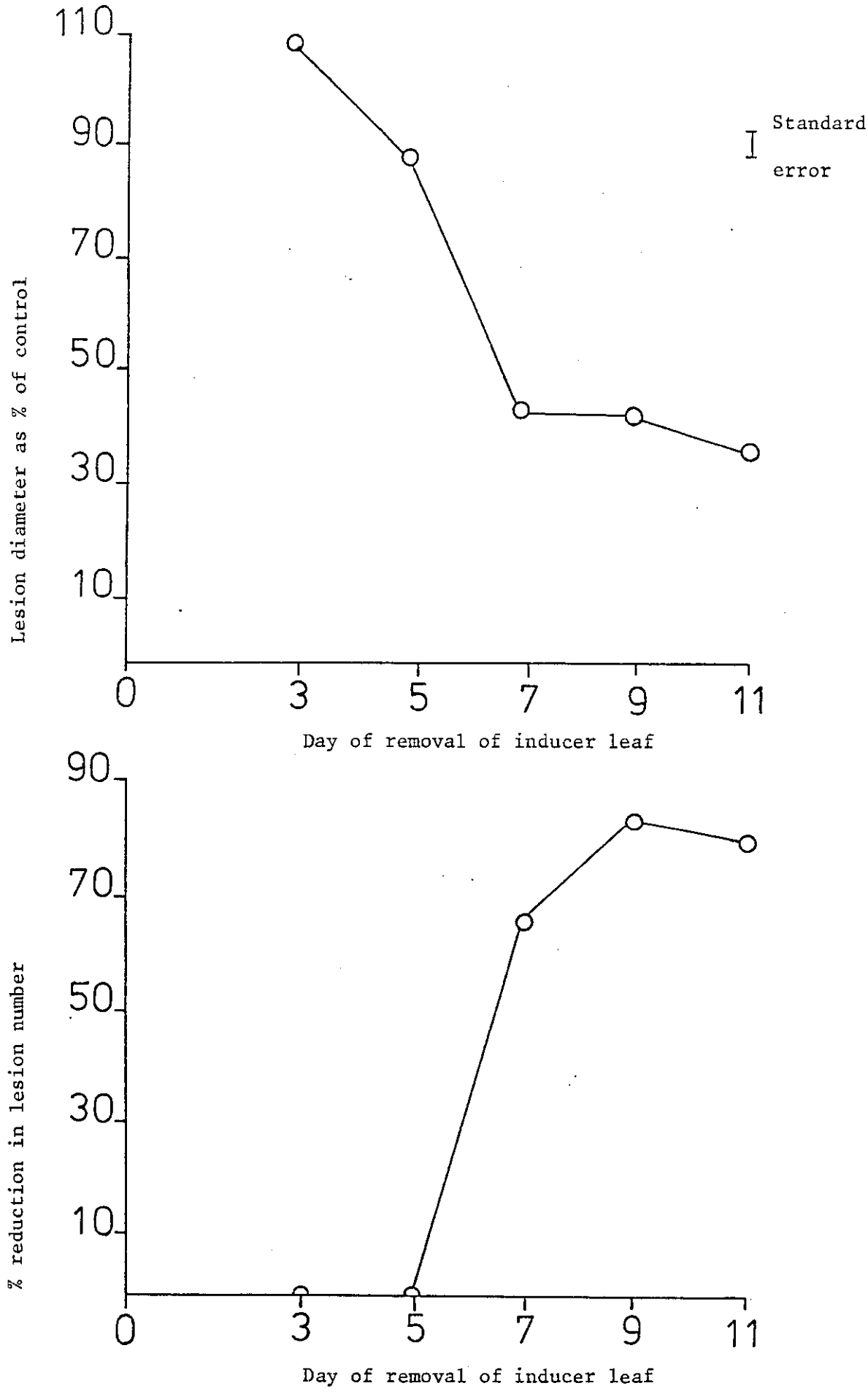
After removal, 12 mm diameter disks were cut from second leaves when plants were 26 days old and placed on moist filter paper in 9 cm petri dishes. These disks were then challenge inoculated with a spore suspension of C. lagenarium (1×10^5 $m\ell^{-1}$).

Lesion size and number were noted after a further 7 days (FIGURE 1.1)

b. Inducer agent, tobacco necrosis virus

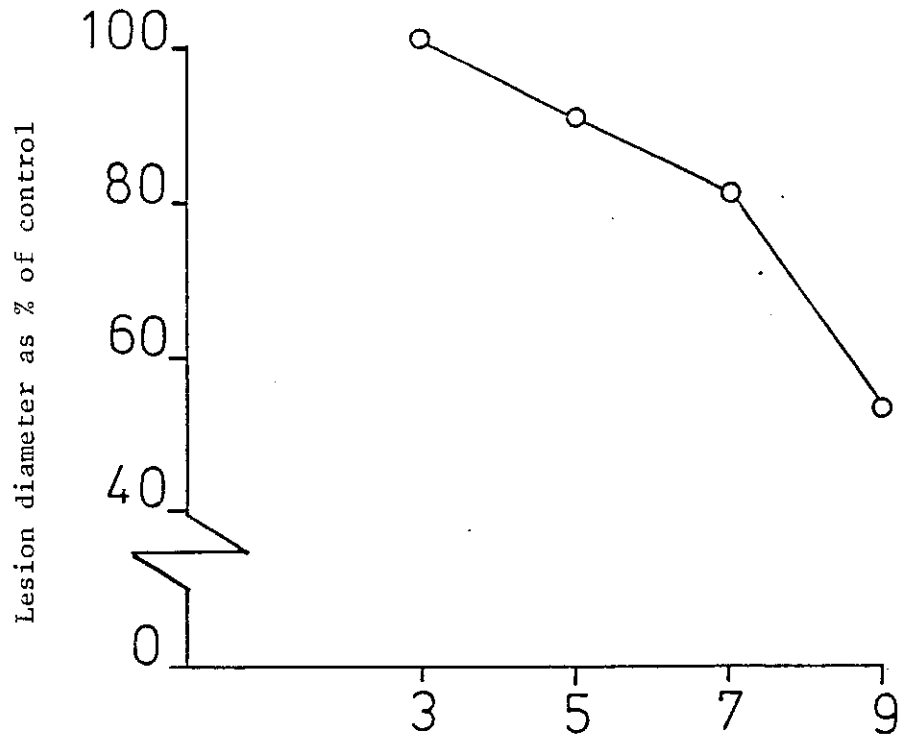
The first true leaf of 15 day old cucumber plants was inoculated with tobacco necrosis virus as in Materials and Methods. Removal

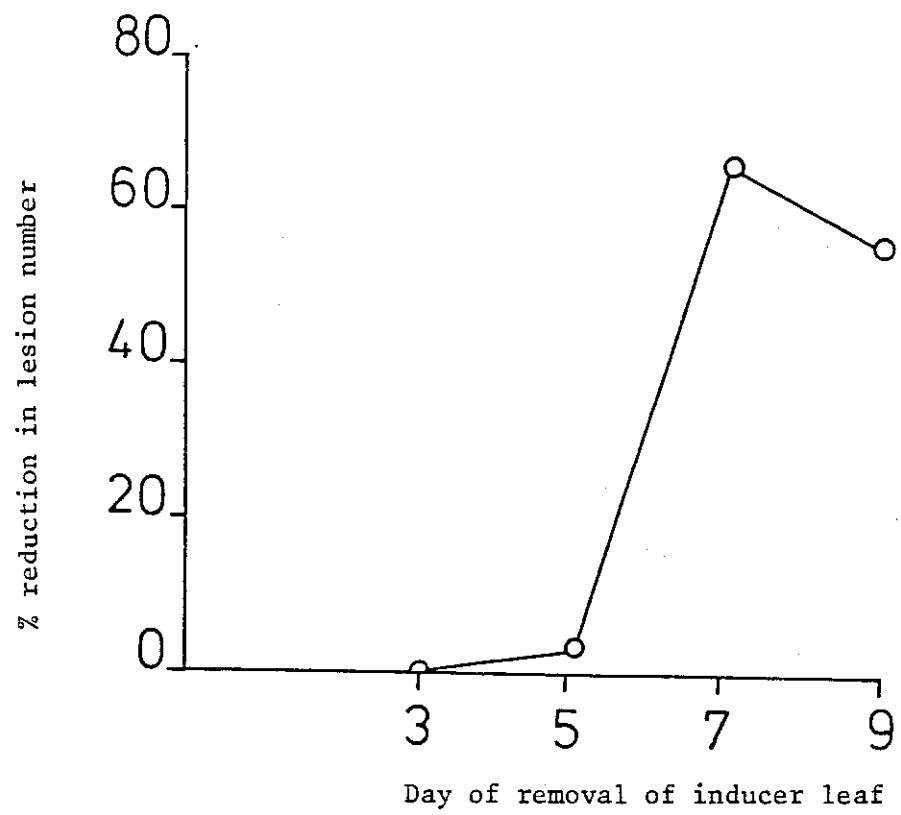
FIGURE 1.1 Timecourse of induction of resistance in second leaves, induced by Colletotrichum lagenarium



Results means of 80 disks/4 plants/treatment

FIGURE 1.2 Timecourse of induction of resistance in second leaves induced by tobacco necrosis virus





Results means of 60 disks/3 plants/treatment

of first leaves, disk taking and challenge inoculation of second leaf was as above.

Lesion size and number were noted after a further 7 days (FIGURE 1.2)

Summary

1. Reduction in lesion diameter did not occur until the infected inducer first leaf had remained attached for 5 days.

2. Reduction in lesion number was very small or none at all at 5 days after removal but increased markedly at 7 days to between 60-70% reduction over control treatments.

3. For fungal infection as inducer, the effect did not increase significantly after 9 days ie 80% reduction in lesion number, 40% in lesion diameter.

4. For virus infection as inducer, the effect was not quite as great as for fungal infection, but lesion diameter and number were both reduced by approximately 50%.

5. The infected first leaf is not necessary for maintenance of protection of leaf 2 after the induction period of 5 days.

3. Direction of induction of resistance.

The previous experiments have shown that infection of either cotyledons or first leaves by C. lagenarium, TNV or Pseudomonas lachrymans leads

to reduced symptoms caused by C. lagenarium on leaf tissue above the site of induction. The following experiments were designed to determine whether resistance could be induced in other directions.

a. Movement from a higher to a lower point.

Cucumber plants with two fully expanded leaves were used. The second leaf was inoculated with a spore suspension of C. lagenarium (1×10^7 mℓ^{-1}) as in Materials and Methods. Leaf disks were taken from the first leaf 9 days later, placed on moist filter paper in 9 cm petri dishes and challenge inoculated with a spore suspension of C. lagenarium (1×10^5 mℓ^{-1}).

Lesion size and number were noted (Table 1.4).

Table 1.4 shows that lesion diameters on challenged first leaves were significantly smaller than control treatments. Reduction was only 35% for lesion size and a maximum of 6.8% reduction in lesion number.

b. Movement from tip to base and base to tip within a single leaf.

To determine the direction in which induced resistance appears to move in a leaf, the tip or basal quarter of the first fully expanded leaf of a cucumber plant was inoculated with a spore suspension of C. lagenarium (1×10^7 mℓ^{-1}). Three 12 mm disks were taken adjacent to the mid-rib and numbered 1 (nearest to infected area) to 3 (furthest from infected area) (see FIGURE 1.3). This was done on different groups of plants up to 7 days post inducer inoculation.

Table 1.4 Protection of leaf 1 following infection of leaf 2.

Treatment of Second leaf	Lesion diameter /mm			Lesion number		
	5d*	7d	9d	5d	7d	9d
H_2O	3.86	7.94	10.98	44	44	44
C. lagenarium	2.48[a]	5.10[a]	7.13[a]	41	44	44
	(35.5)[b]	(35.8)	(35.1)	(6.8)	(0)	(0)

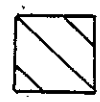
* Days after challenge inoculation

a Significantly different at 0.05% level from control treatment

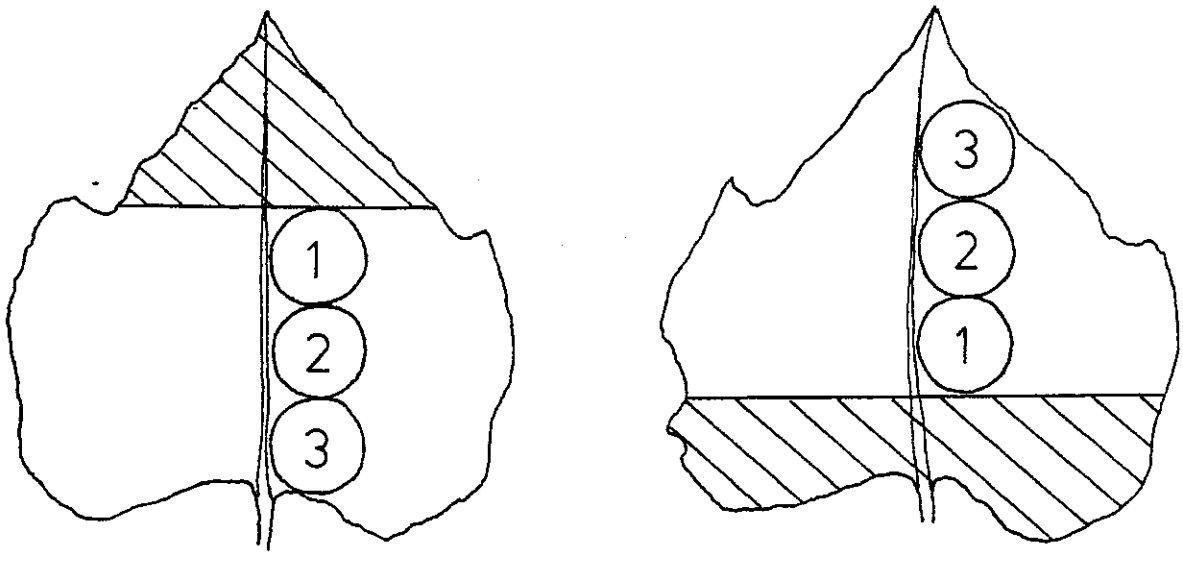
b % reduction over control treatments

Results means of 44 lesions/4 plants/treatment

FIGURE 1.3



Area treated first with H_2O or *C. lagenarium*

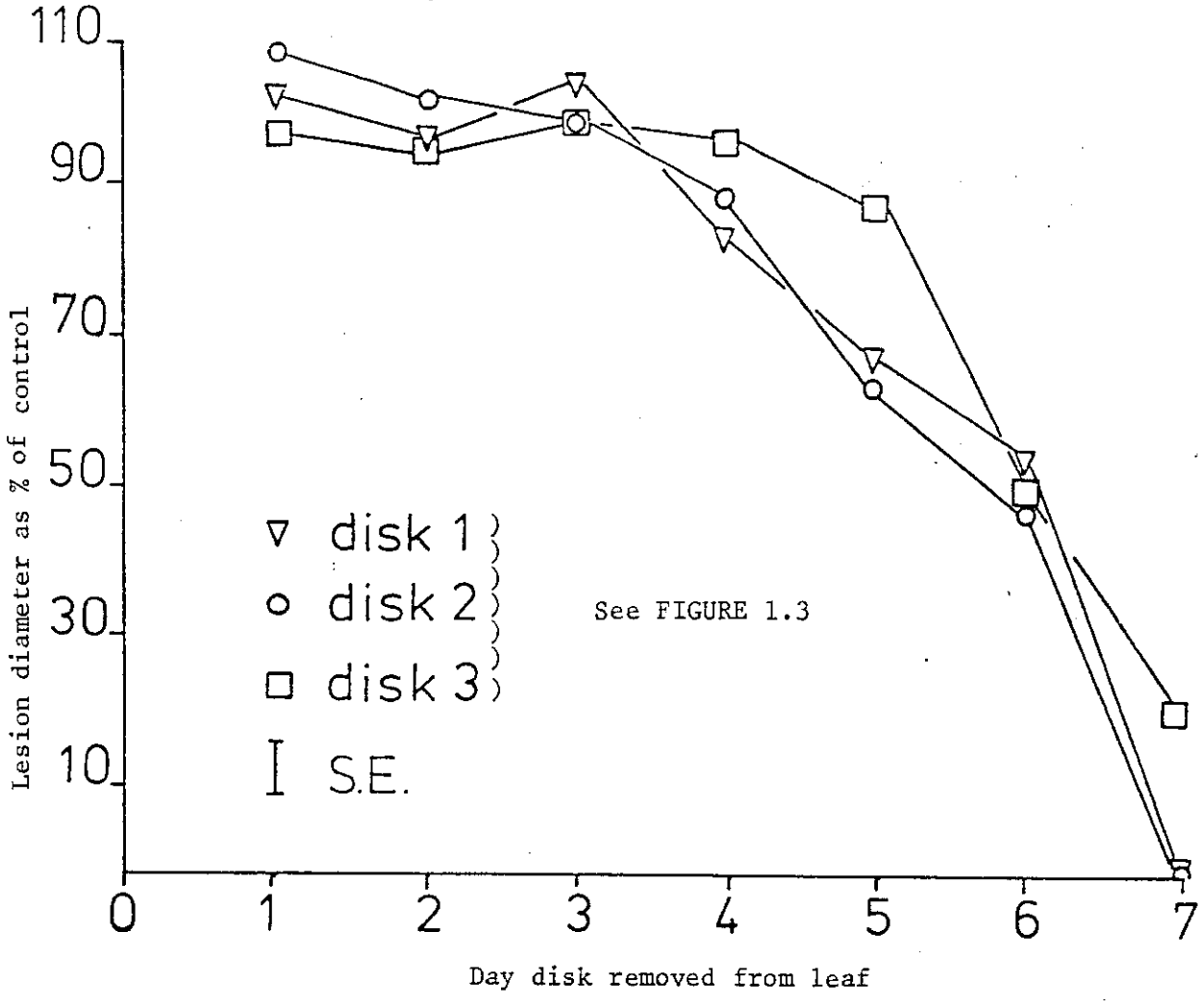


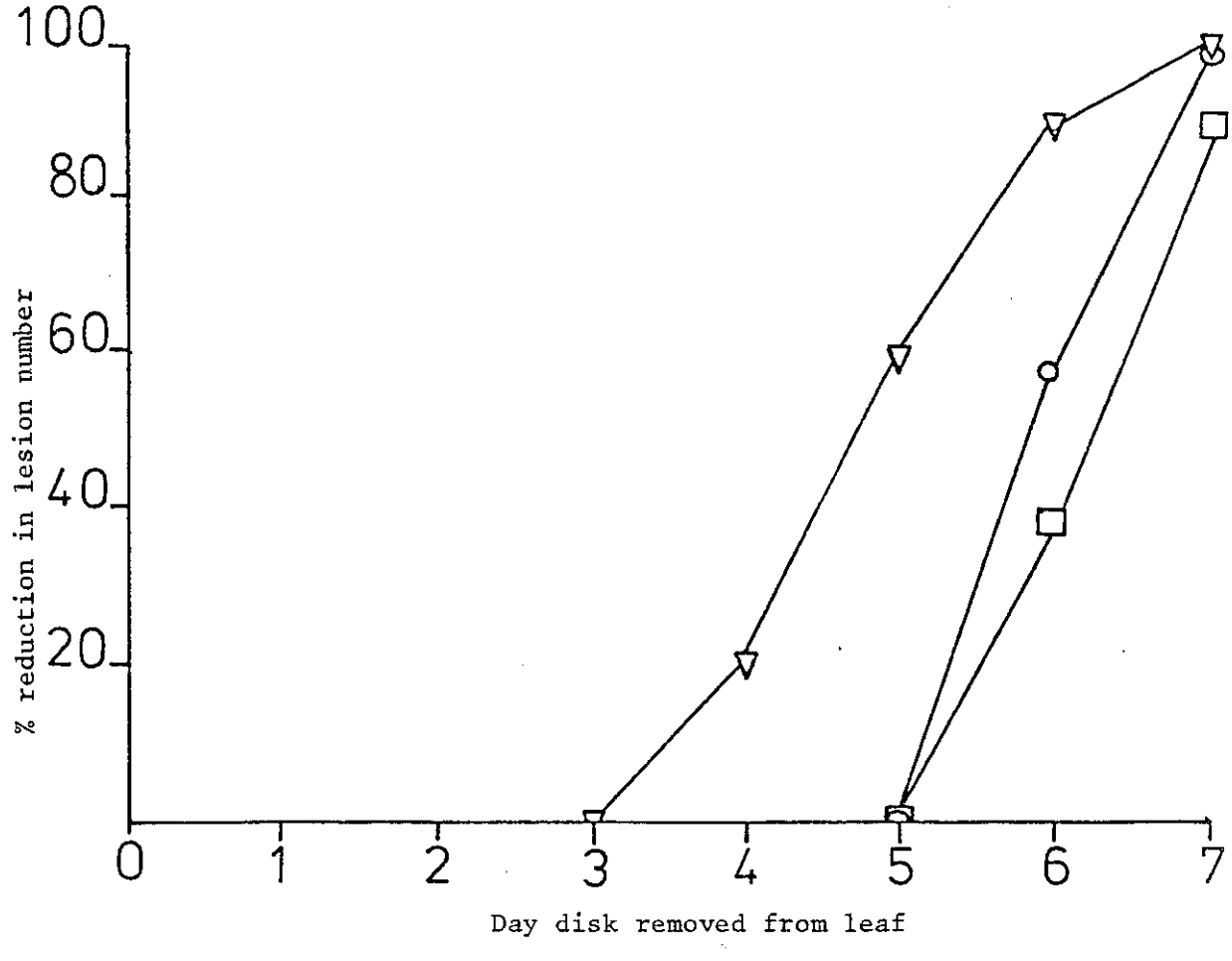
Disks were placed on moist filter paper in 9 cm petri dishes and challenge inoculated with a spore suspension of *C. lagenarium* (1×10^5 mℓ^{-1}).

Lesion size and number were noted after a further 5, 7 and 9 days. (FIGURES 1.4, 1.5 and 1.6 for tip inoculated, 1.7, for base inoculated).

FIGURES 1.4, 1.5 and 1.6 show the reduction in lesion diameter and number on disks taken from tip inoculated leaves. Disks scored 5 days after removal from leaf showed that resistance was not apparent until the disks had remained in contact with the infected tip for 4 days. Thereafter resistance increased significantly until, after 7 days of attachment to infected tip, disks 1 and 2 had no lesions at all, and disk 3 only very few.

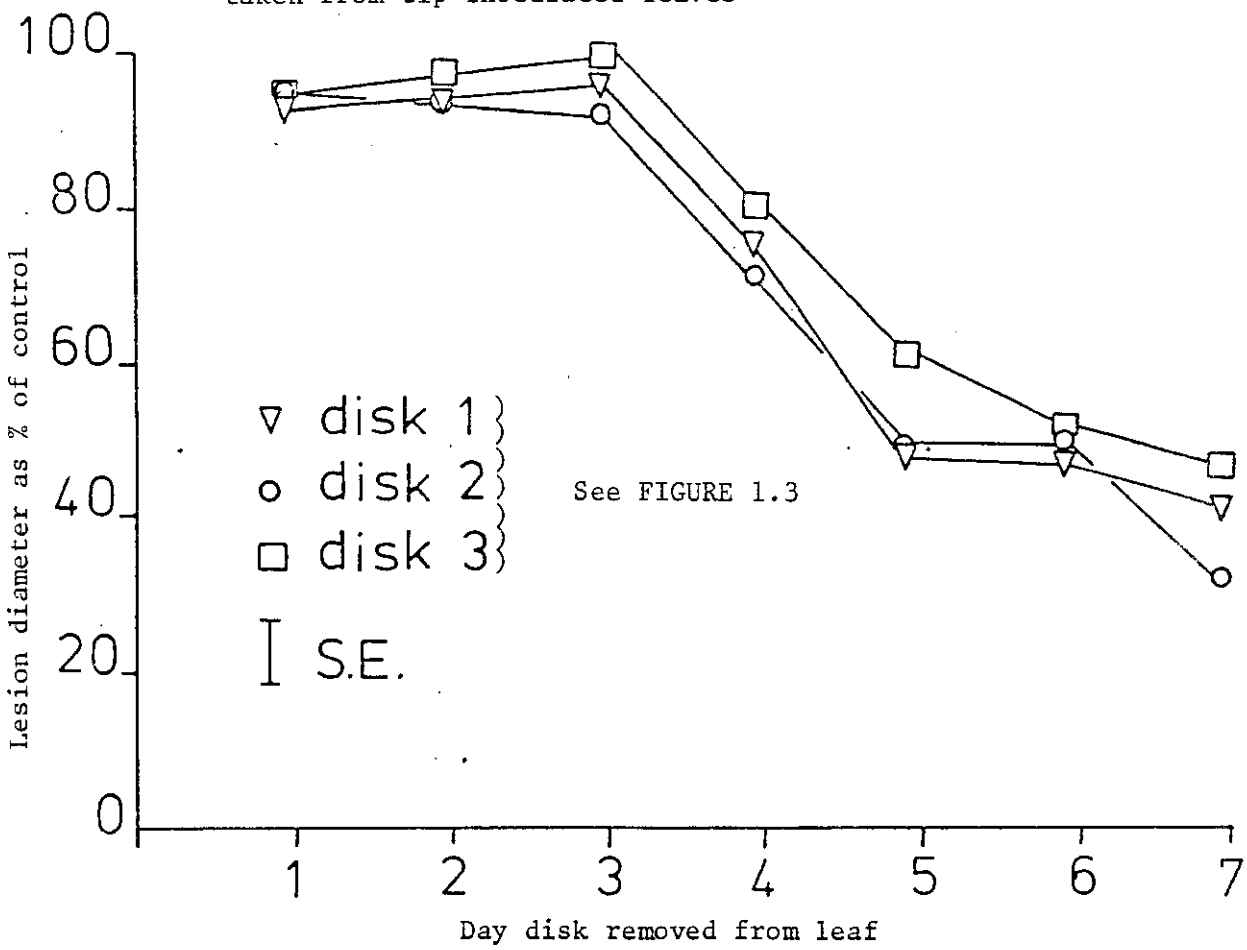
FIGURE 1.4 Lesion size and number 5 days after challenge on disks taken from tip inoculated leaves

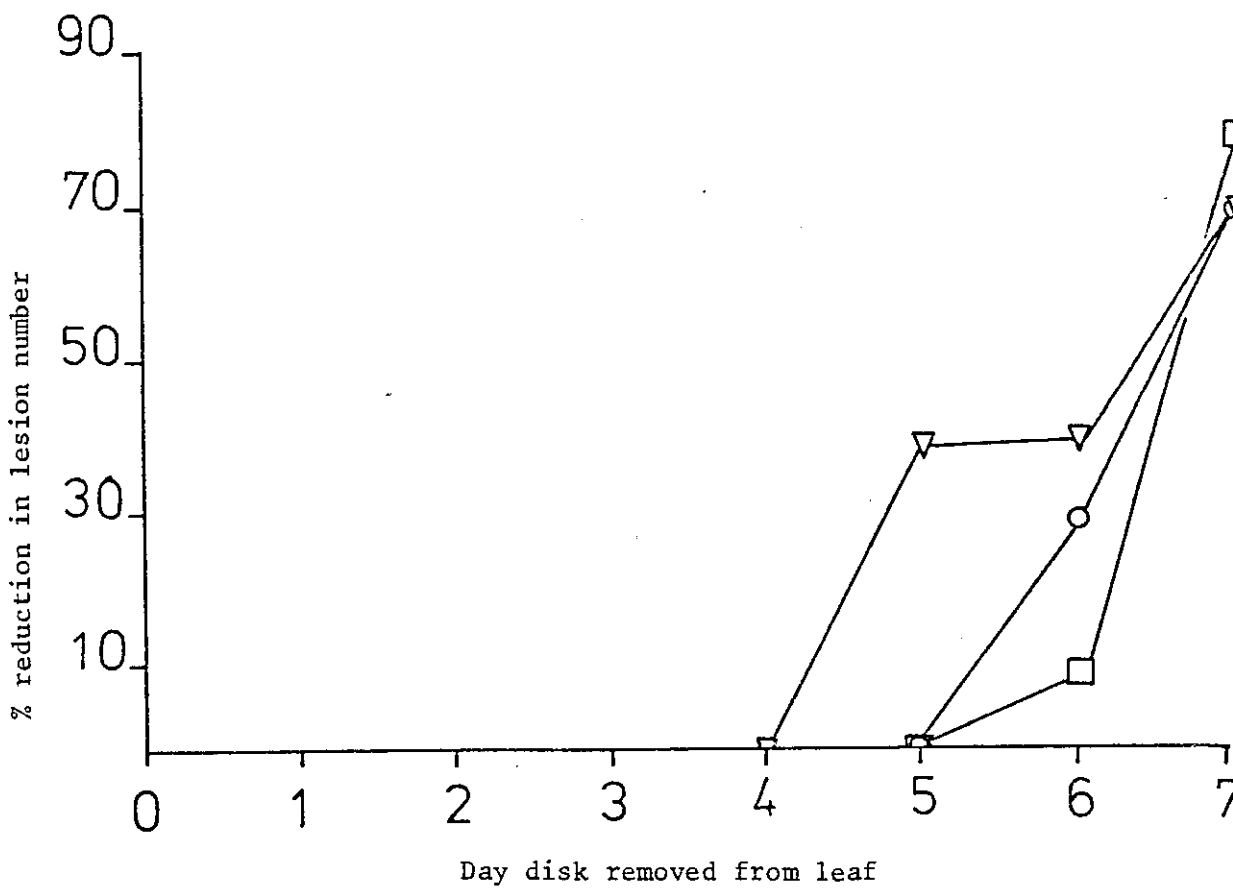




Results means of 10 disks/5 plants/treatment

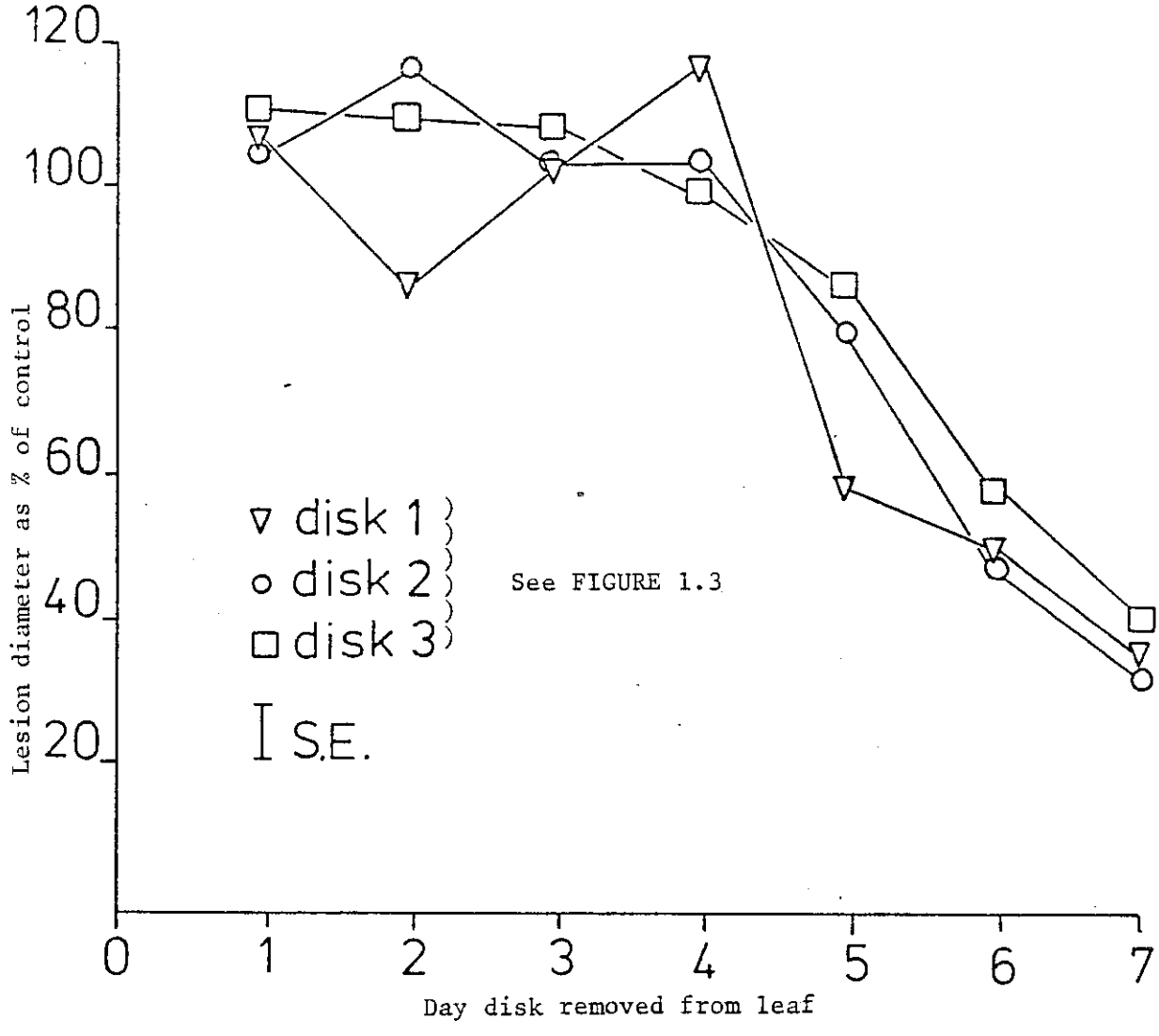
FIGURE 1.5 Lesion size and number 7 days after challenge on disks taken from tip inoculated leaves

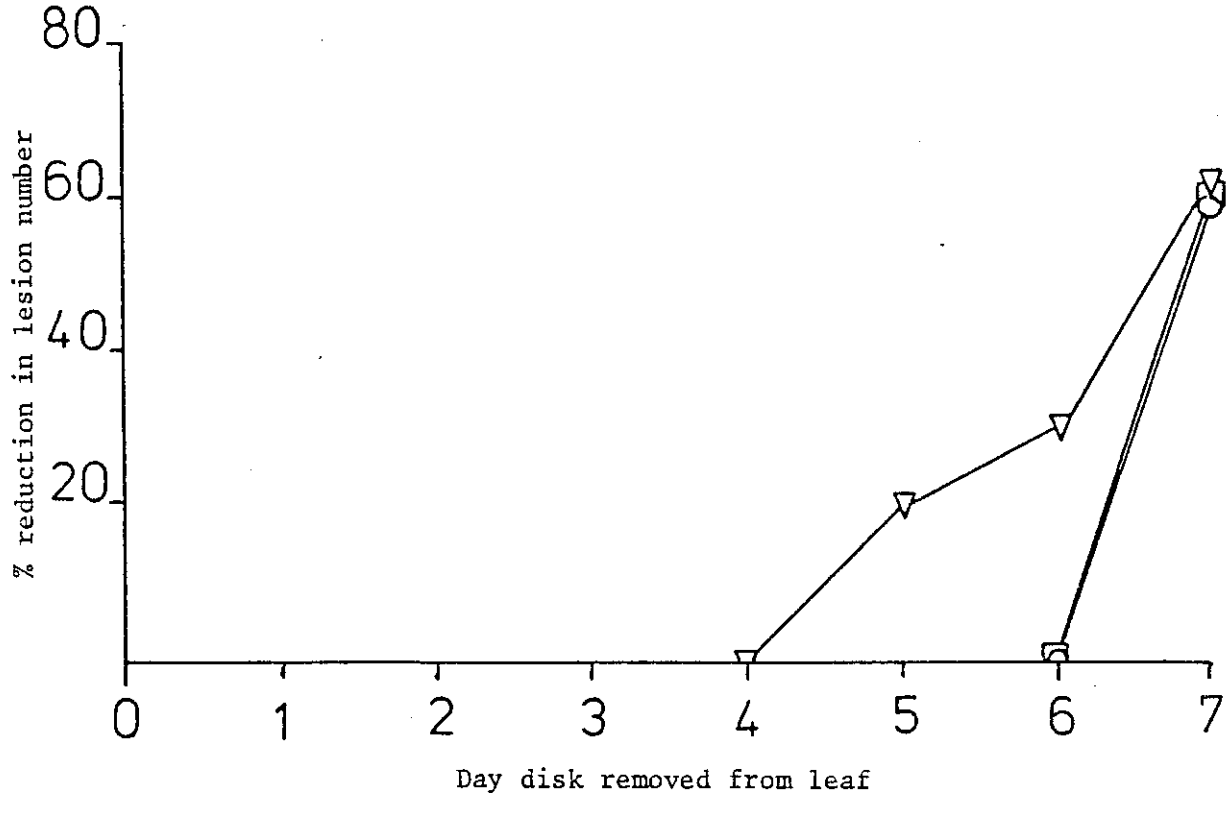




Results means of 10 disks/5 plants/treatment

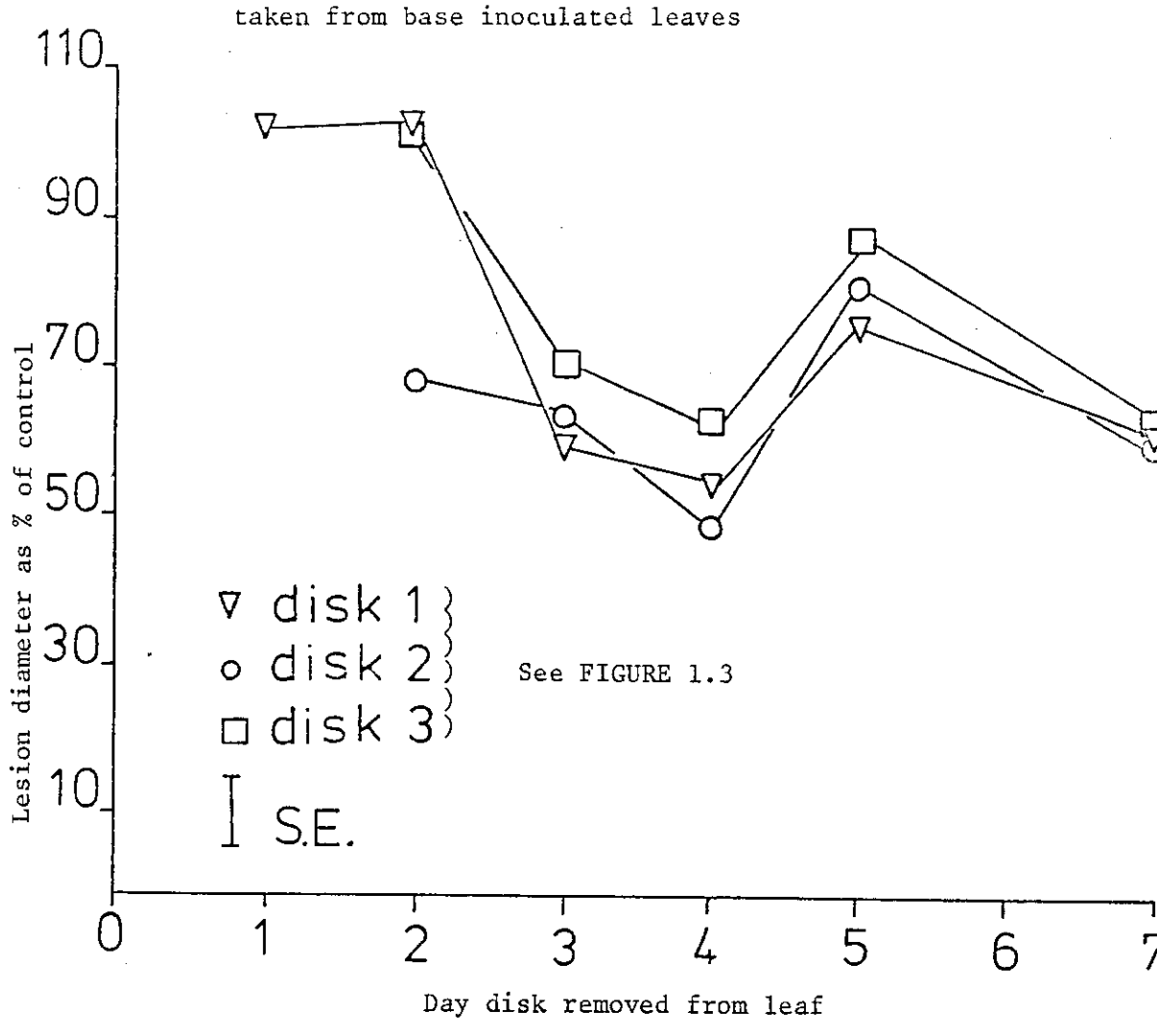
FIGURE 1.6 Lesion size and number 9 days after challenge on disks taken from tip inoculated leaves

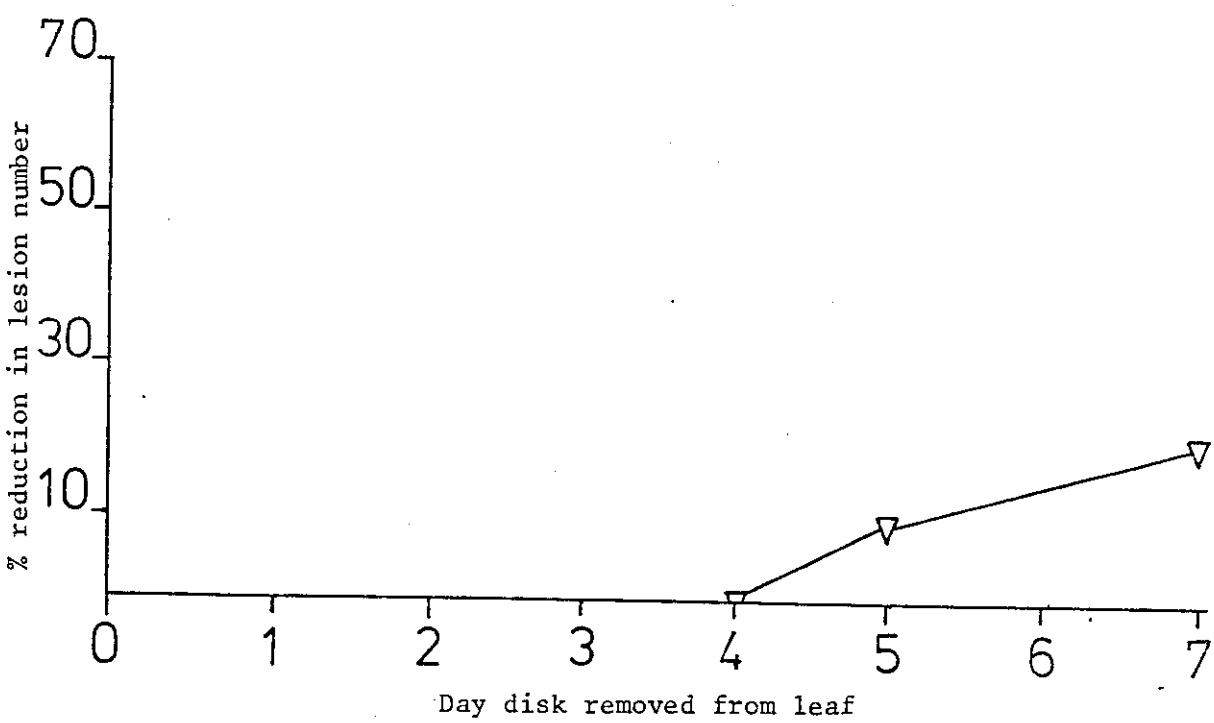




Results means of 10 disks/5 plants/treatment

FIGURE 1.7 Lesion size and number 5 days after challenge on disks taken from base inoculated leaves





Results means of 10 disks/5 plants/treatment

These same disks measured after 7 and 9 days (FIGURES 1.5 and 1.6) showed an increase in both lesion diameter and lesion number but reduction compared to control disks was still 60% and 60-70% for lesion number and diameter respectively.

As far as the position of the leaf disk on the infected leaf was concerned (disk 1, 2 or 3) there seemed to be very little difference for lesion diameter measurements, but reduction in lesion number was always apparent in disk 1 (nearest infected area) before disk 2 or 3. Disk 1 taken after 5 days of attachment showed almost 60% reduction in lesion number compared with 0% for disks 2 and 3. However, after 7 days attachment, all 3 disk positions showed similar reductions.

For base inoculated leaves, reduction in lesion size and number was much less than for tip inoculated ones. Disks scored 5 days after removal from infected leaf showed that resistance was apparent after only 2-3 days attachment when measuring lesion diameter. However, even after attachment for seven days, lesion diameter on all 3 disk positions was only 40% reduced, and reduction in lesion number only reached a maximum of less than 20% (compared to 100% in tip inoculated leaves).

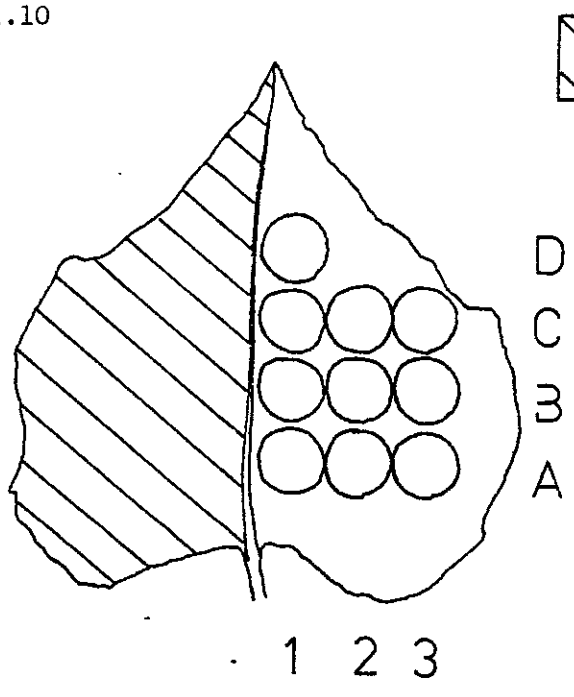
As with tip inoculated leaves, the position of the leaf disk on the infected leaf did not significantly affect the degree of reduction in lesion diameter, although the only disks showing reduced lesion number were those from position 1 (measured after 5 days), disks which later developed lesions (measured after 7 and 9 days).

c. Lateral movement within a leaf.

One side of the mid-rib of a fully expanded first leaf was inoculated with a spore suspension of C. lagenarium (1×10^7 mℓ^{-1}). Leaf disks were taken from the adjacent half leaf and challenge inoculated with a spore suspension of C. lagenarium (1×10^5 mℓ^{-1}).

Lesion size and number were noted after 5, 7 and 9 days.

FIGURE 1.10



Area treated with H_2O or C. lagenarium

Positions of disks taken from leaf (see text)

Tables 1.5 and 1.6 show the reduction in lesion size and lesion number in opposite half leaves. The tables show this reduction not to be equally distributed as would be expected if induced resistance moved equally in all directions. Taking disks A, B, C, 1, 2, 3, (see FIGURE 1.10) induced resistance should be symmetrical around disk B1, decreasing with distance. However, reduction in lesion

Table 1.5 Reduction in lesion diameter on opposite half leaf

	% Reduction in lesion diameter								
	5 days after challenge			7 days after challenge			9 days after challenge		
D*	19.1			10.7			23.5		
C	29.0	14.9	9.2	11.3	-0.5	-2.0	21.7	21.8	21.9
B	66.6	26.9	16.1	42.5	29.3	4.6	42.4	43.5	20.3
A	26.0	16.8	5	33.3	10.4	6.1	40.0	42.4	13.2
	1*	2	3	1	2	3	1	2	3

* Position of disk (see FIGURE 1.10)

Results means of 10 lesions/10 plants/leaf disk position

Table 1.6 Reduction in lesion number on opposite half leaf

	% Reduction in lesion number								
	5 days after challenge			7 days after challenge			9 days after challenge		
D*	20			10			10		
C	30	10	0	20	0	0	20	0	0
B	80	30	0	50	30	0	50	20	0
A	50	20	10	30	20	10	30	10	10
	1*	2	3	1	2	3	1	2	3

* Position of leaf disk (see FIGURE 1.10)

Results means of 10 lesions/10 plants/leaf disk position

number was biased towards row A (reduction in A1, 2, 3 always greater than C1, 2, 3) which indicated that the apparent movement of induced resistance was across the opposite half leaf but also basipetally.

Summary

1. Infection of the second leaf caused a 35% reduction in lesion size on challenged first leaf but only a small reduction in lesion number was apparent when measured after 5 days (lost after 7 days).

2. In tip inoculated leaves there was a 100% reduction in lesion number 5 days after challenge in the disks closest to the infected area, and disks adjacent to those disks.

   Comparable reduction in base inoculation leaves was only 20%.

3. Further development of the same lesions taken from tip inoculated leaves, as measured after 7 and 9 days, reduced the amount of protection to 60% and 60-70% for lesion size and number respectively.

4. Disks needed to be attached for 4 days before reduction in lesion size was apparent.

5. Lateral movement across opposite half leaves was evident, but biased towards the base of the leaf.

6. Disks taken from the furthest opposite edge showed only very slight reduction in lesion size or number despite being only a maximum of 36 mm away from the inducing infection.

7. Apparent 'movement' of induced resistance in the whole plant seems to be out of the infected inducer leaf and up the plant. Only a small amount of opposite 'movement' is evident.

4. Effects on induced resistance of:-

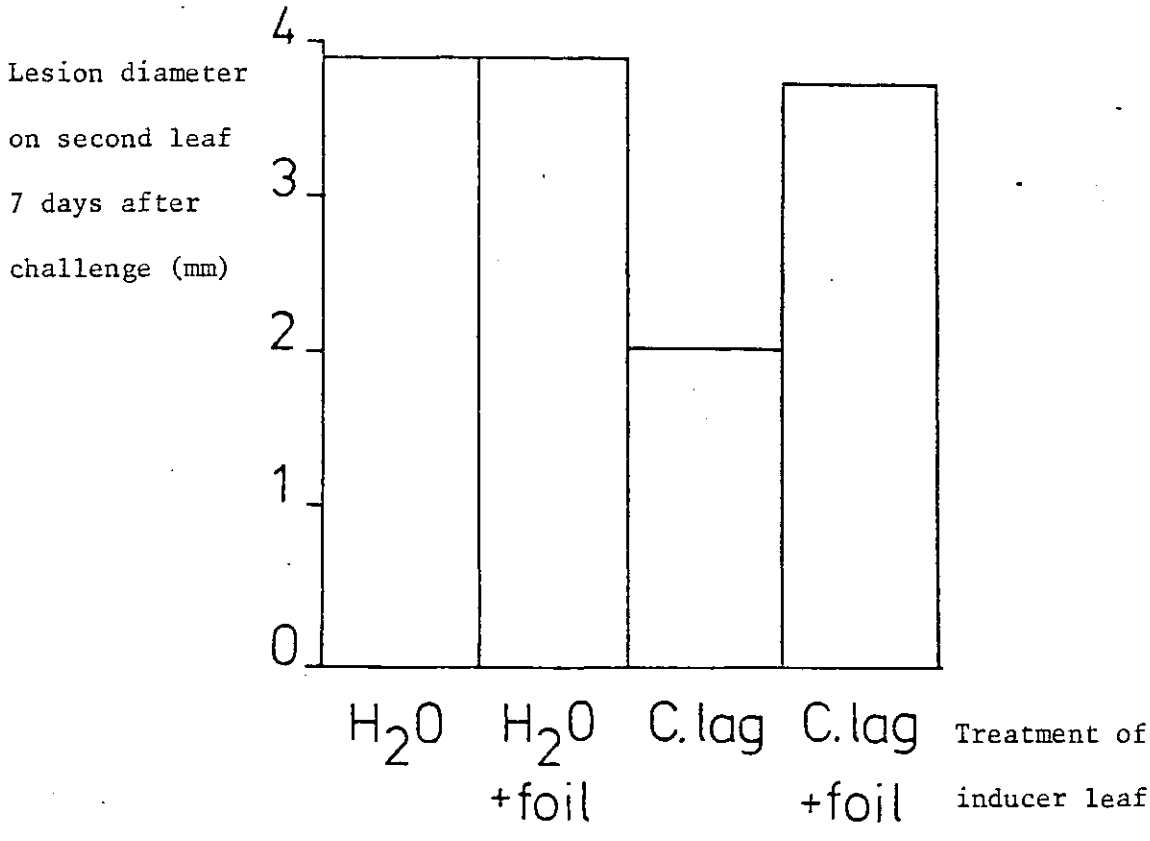
a. Light shielding infected first leaves.

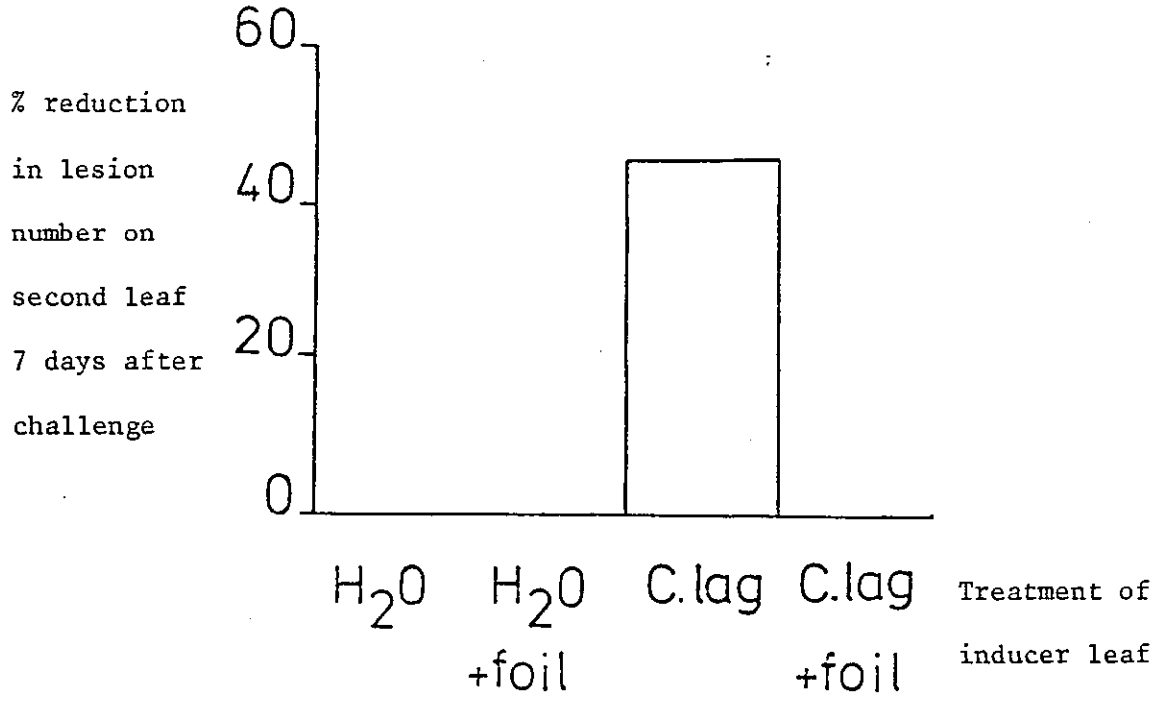
The preceding experiments have shown 'movement' of induced resistance to be generally out of an infected leaf. For further evidence of a phloem-borne stimulus for induction, infected first leaves were shielded from light to minimise movement of solutes out of the leaf.

The first true leaves of 15 day old cucumber plants were inoculated with a spore suspension of C. lagenarium (1×10^7 mℓ^{-1}) or water as in Materials and Methods. Twenty four hours later, aluminium foil was wrapped around the first leaves to prevent light reaching them. (Foil was not used immediately after inoculation to prevent dislodging inocula.) Second leaves were challenge inoculated 9 days later with a spore suspension of C. lagenarium (1×10^5 mℓ^{-1}) and lesion size and number were noted after a further 7 days. FIGURE 1.11 shows that placing the first leaf of a water inoculated plant in aluminium foil had no effect on lesion size or number on the subsequently challenged second leaves.

Plants with first leaves inoculated with C. lagenarium showed characteristic reduction of lesion size and number in subsequent challenge of second leaves.

FIGURE 1.11 Effect of light shielding inducer leaf on lesion development on second leaf





C. lag = Colletotrichum lagenarium

Results means of 20 lesions/4 plants/treatment

Plants with C. lagenarium inoculated first leaves covered with aluminium foil, however, were not systemically protected as there was no reduction in either lesion size or number. This indicates a light dependent process either in development of the necessary mobile stimulus or in the actual movement of the stimulus of the induction of resistance. The latter is more likely as any stimulus is likely to be phloem-borne. Movement from cell to cell would not presumably have been affected by keeping the first leaf in the dark.

Summary

1. Light shielding infected first leaves prevented systemic resistance developing in second leaves.

b. Number of lesions on inducer leaf.

The first true leaves of 15 day old cucumber plants were inoculated with 1, 3, 5, 10, 15 or 20 3 $\mu\ell$ drops of a spore suspension of C. lagenarium (1×10^7 $m\ell^{-1}$) or water as in Materials and Methods. Nine days later, leaf disks were taken from the second leaves and challenge inoculated with a spore suspension of C. lagenarium (1×10^5 $m\ell^{-1}$).

Lesion size and number were noted after a further 5, 7 and 9 days (Table 1.7).

Table 1.7 shows that as few as 3 lesions on leaf 1 were sufficient to induce significant resistance in leaf 2, giving almost 20% and 15% reduction in lesion diameter and number respectively. Increasing

the number of lesions on leaf 1 to 15 had little effect on reducing further the lesion diameter, but doubled the number of lesions failing to develop. Twenty lesions on leaf 1 gave almost 60% and 47% reduction in lesion diameter and number respectively.

Table 1.7 Reduction in lesion size and number with increased lesion number on inducer first leaf.

Number of lesions on first leaf	Lesion diameter as % of control			% reduction in lesion number		
	5d*	7d	9d	5d	7d	9d
1	104.5	101.5	101.2	0	0	0
3	80.8[a]	90.2[a]	92.7[a]	14.7	0	0
5	71.5[a]	80.5[a]	85.6[a]	24.0	15.1	0
10	86.8[a]	81.3[a]	84.7[a]	27.7	27.7	22.2
15	77.0[a]	91.4[a]	78.1[a]	31.4	30.0	25.0
20	59.4[a]	63.4[a]	67.5[a]	46.8	41.9	40.6

* Days after challenge inoculation

a Significantly different at 0.05% level from control

Results means of 48 disks/4 plants/treatment

Summary

1. Lesion size and number on the second leaf were significantly reduced when as few as 3 lesions were present on the first true leaf.

2. Lesion number on the second leaf decreases as lesion number on first leaf increased.

c. Spore concentration of challenge inoculation.

To determine whether induced resistance was effective against large as well as small inocula, leaf disks were cut from healthy unprotected and systemically protected cucumber plants and inoculated with one 3 $\mu\ell$ drop of a spore suspension of C. lagenarium (between 10^4-10^7 $m\ell^{-1}$) in 9 cm petri dishes.

Lesion size and number were subsequently noted (FIGURE 1.12). FIGURE 1.12a shows the effect of increasing spore concentration on lesion number. Lesions did not develop at over 80% of the sites on protected disks when challenged with a spore concentration of 10^4 spores $m\ell^{-1}$. This decreased as the concentration increased until 10^7 spores $m\ell^{-1}$, when lesions developed at 100% of the sites inoculated.

FIGURE 1.12b shows that lesion size was similarly affected. At 10^4 spores $m\ell^{-1}$ on protected disks, lesion size was only 57% of the control, but once again, increased spore concentration increased lesion size.

The fact that induced resistance can be overcome by high inoculum levels suggests that a cucumber plant does not become resistant as in the usual sense. It is possible that symptom suppression masks the disease caused by low spore concentrations, symptoms only becoming apparent at high concentrations.

FIGURE 1.12a Effect of spore concentration of challenge inocula on lesion development

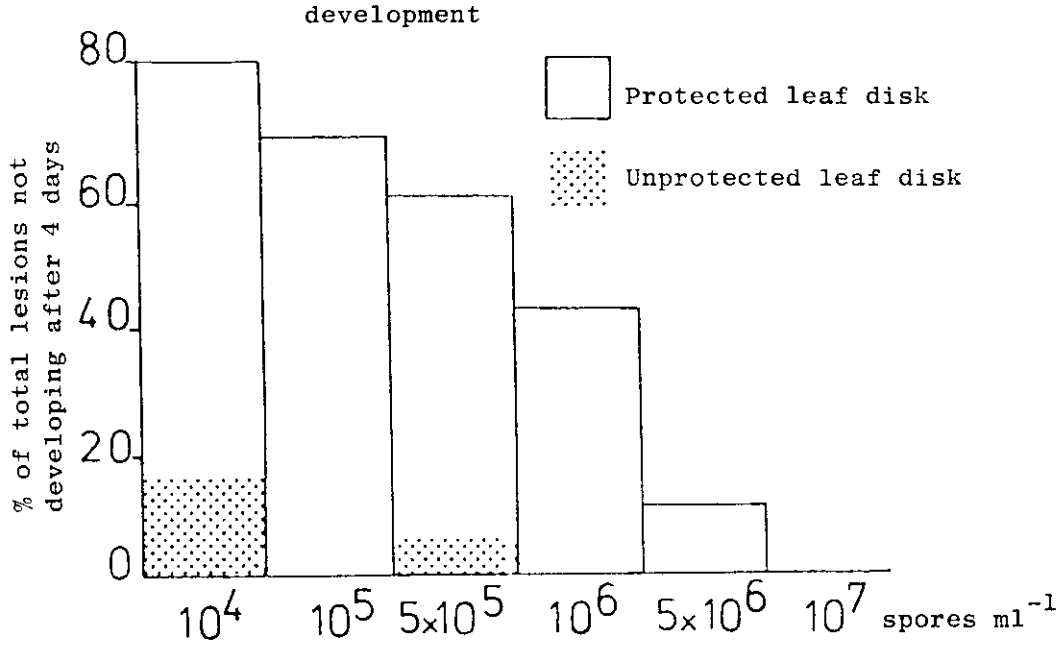
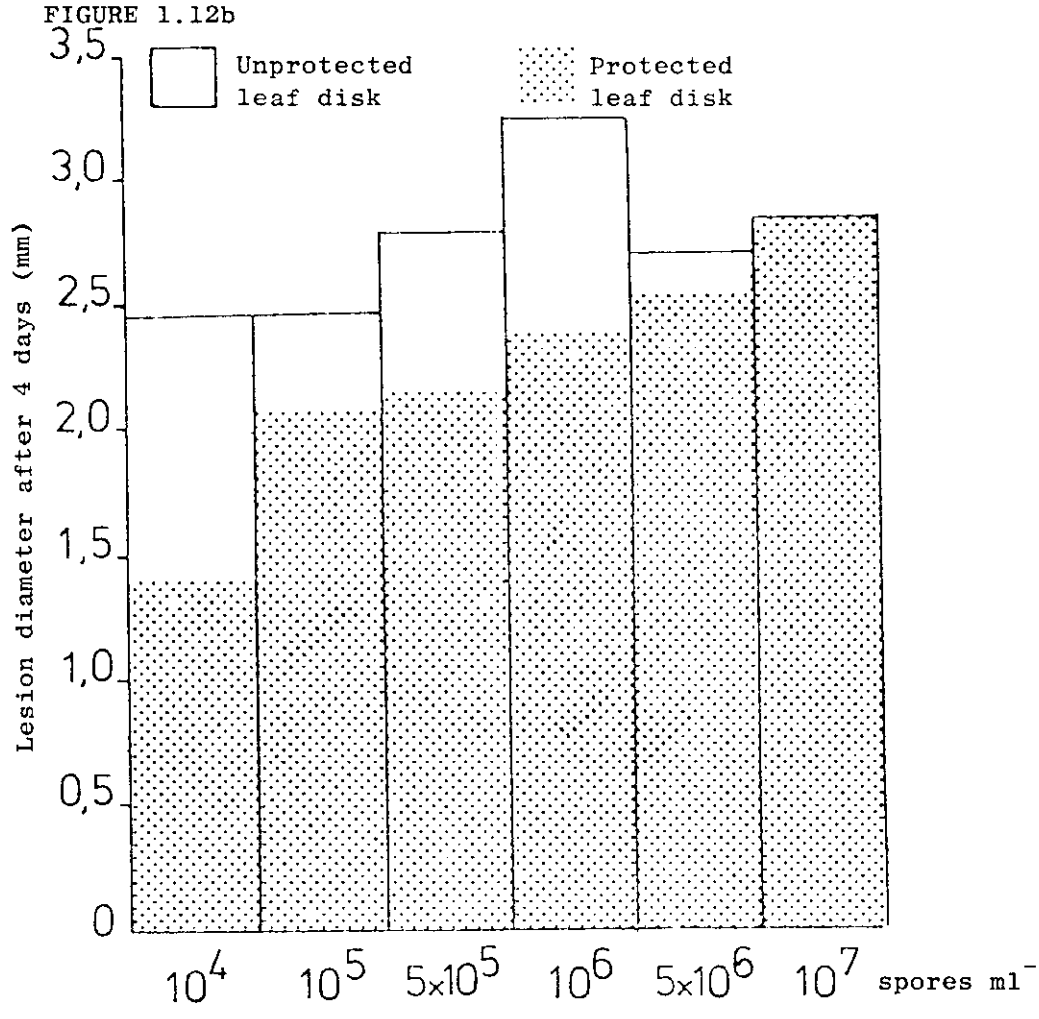


FIGURE 1.12b



Results means of 20 disks/5 plants/treatment

Summary

1. Protected disks showed an 80% reduction in lesion number and 40% reduction in lesion diameter when challenged with C. lagenarium at 10^4 spores $m\ell^{-1}$.

2. This reduction in lesion number and size was overcome by spore concentrations of C. lagenarium of 10^7 spores $m\ell^{-1}$.

d. Removal of epidermis prior to challenge.

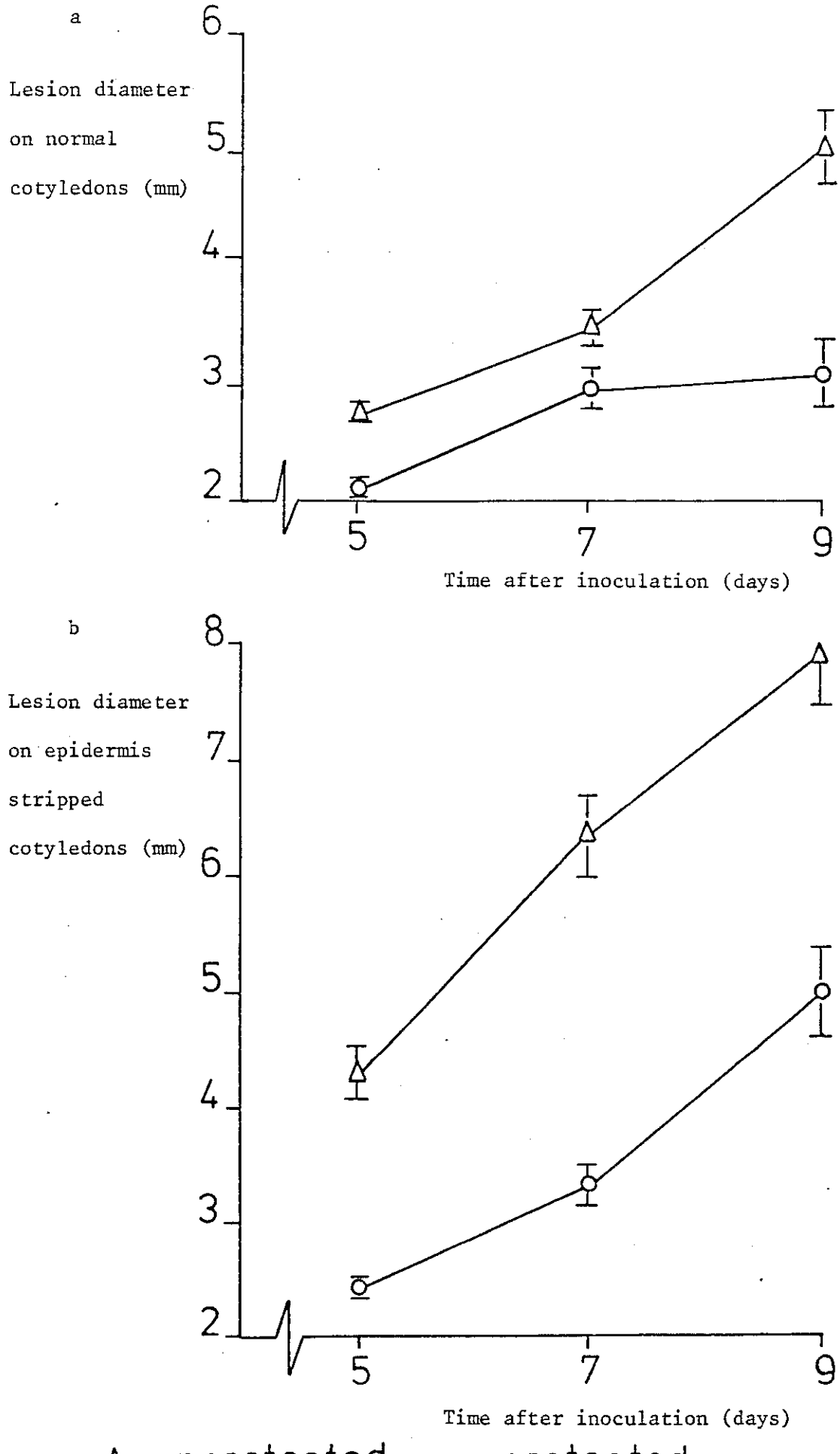
To determine what influence, if any, the epidermis has on induced resistance, cotyledons of protected and healthy unprotected plants were stripped of their lower epidermis using fine forceps. (Cotyledons were protected by inoculation of the apical quarter with C. lagenarium.) Cotyledons were then floated uppermost side down on water in 9 cm petri dishes, and inoculated with 3 $\mu\ell$ drops of a spore suspension of C. lagenarium 1 x 10^5 $m\ell^{-1}$ as in Materials and Methods.

Lesion diameter and number were noted after 5, 7 and 9 days (FIGURES 1.13 and 1.14).

FIGURE 1.13a shows the mean lesion diameter of normal protected and unprotected cotyledons. FIGURE 1.13b shows the same on epidermis stripped cotyledons.

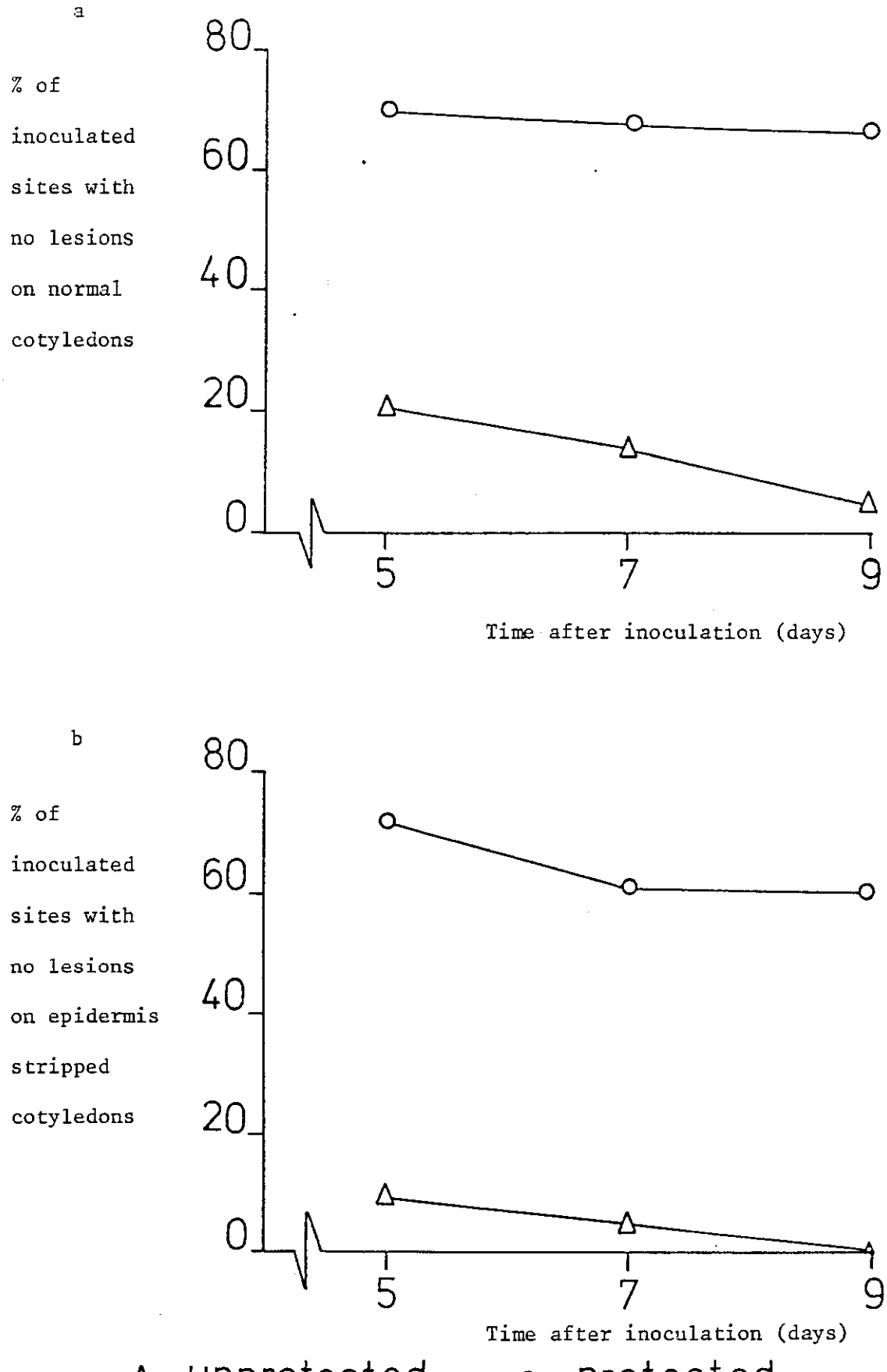
FIGURE 1.14a and b show the % of inoculated sites which subsequently develop lesions on normal and epidermis stripped cotyledons.

FIGURE 1.13 Lesion diameter on epidermis stripped and normal cotyledons



Results means of 60 lesions/10 cotyledons/treatment

FIGURE 1.14 Number of lesions developing on epidermis stripped and normal cotyledons



Results means of 60 lesions/10 cotyledons/treatment

The number of lesions developing at inoculated sites on cotyledons was not significantly altered by removing the epidermis. However, those lesions which did develop on epidermis stripped plants were larger than control (epidermis intact) plants, although there was still a significant difference between protected and unprotected treatments. Lesion diameter on protected was about 40% less than unprotected in both stripped and intact cotyledons.

Summary

1. Protection, as measured by reduction in lesion number, was not markedly affected by removal of the epidermis before challenge inoculation.

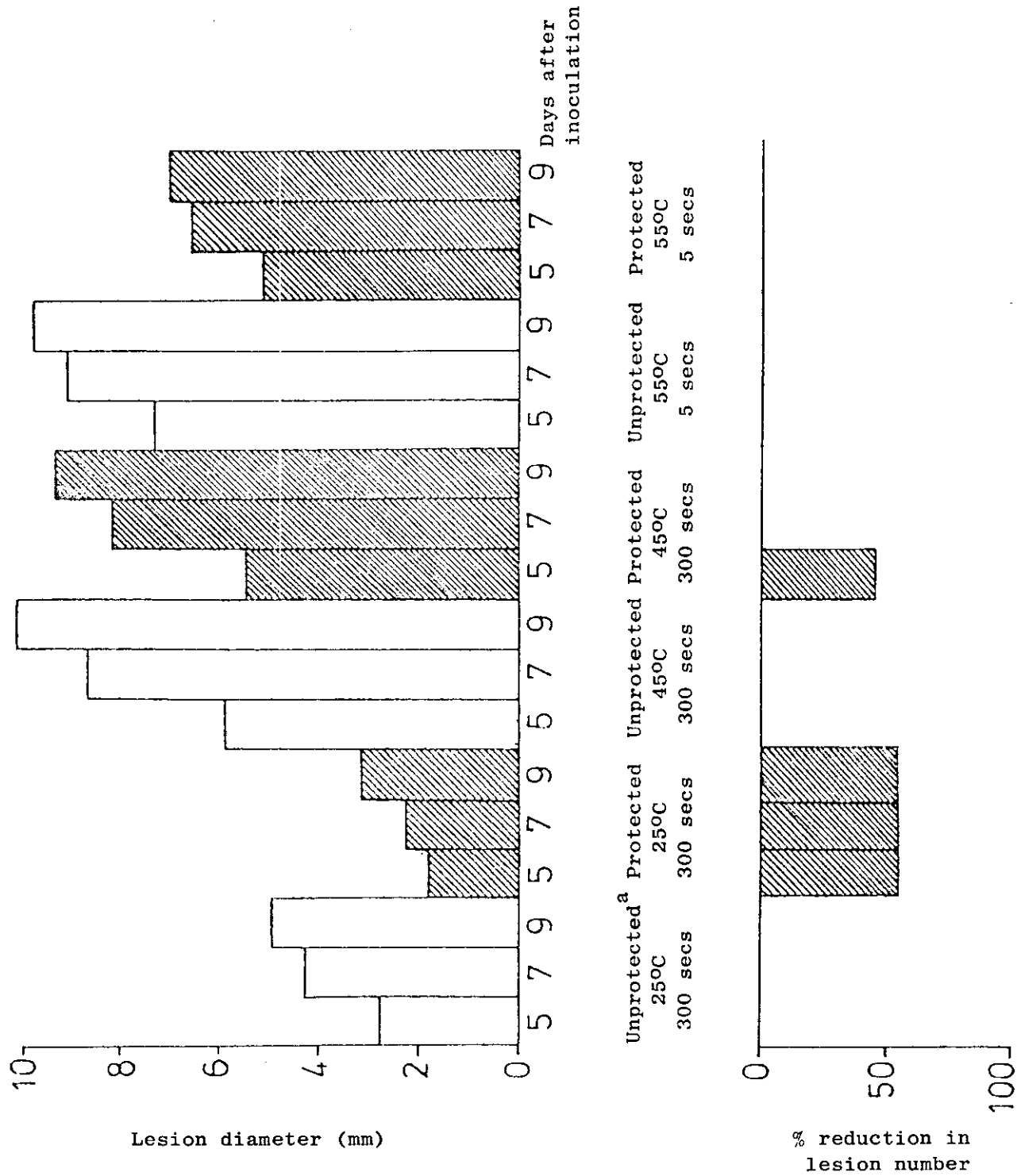
2. Lesions which did develop were larger on epidermis stripped cotyledons than intact ones although the difference in lesion diameter between protected and unprotected cotyledons was still apparent.

e. Temperature

Leaf disks from healthy unprotected or systemically protected cucumber plants were immersed in water at 5, 15, 25, 35, 45 and 55°C for periods varying from 5 to 300 seconds. Disks were air dried and placed in 9 cm petri dishes containing moist filter paper. One 3 $\mu\ell$ drop of a spore suspension of C. lagenarium (1×10^5 $m\ell^{-1}$) was placed on each disk.

Lesion size and number were noted after 5, 7 and 9 days.

FIGURE 1.15 Effect of temperature on lesion size and number



[a] Disks taken from unprotected tissue, immersed in water at 25°C for 300 secs, dried, then challenge inoculated with C. lagenarium.

Results means of 15 disks/5 plants/treatment

Pretreating unprotected disks with immersion in water at 5, 15, 25 and 35°C for the time intervals described had no effect on lesion size or number. Similarly, protected disks showed the reduction in lesion size and number as described in earlier experiments; this was not affected by immersion in water at 5-35°C.

FIGURE 1.15 shows the effect of 45°C for 300 seconds and 55°C for 5 seconds on unprotected and protected disks. Both types of tissue showed a marked increase in lesion size (up to 100%). Protected disks no longer showed a reduction in lesion number.

Heating of leaves may cause direct heat injury predisposing the host to infection. Changes induced in a plant when systemically protected by prior heat treatment or, more likely, as these results suggest, general changes in protected tissue which were also seen in healthy heat treated plants, were sufficient to overcome the effects of induced resistance.

Summary

1. Pretreating unprotected and protected disks by immersion in water at 5, 15, 25 and 35°C for intervals between 5-300 seconds had no effect on lesion size or number.

2. Immersion of unprotected disks at 45° and 55° for 300 and 5 seconds respectively markedly increased lesion size.

3. Immersion of protected disks at 45° and 55° for 300 and 5 seconds respectively increased lesion size and decreased the reduction in lesion number to zero.

f. Non-ionising radiation

The first true leaf of 15 day old cucumber plants was inoculated with a spore suspension of C. lagenarium (1×10^7 mℓ^{-1}) or water as in Materials and Methods.

After 7 days, leaf disks were taken from the second leaf and placed on moist filter paper in 9 cm petri dishes. The disks were then irradiated for various lengths of time with short-wave (253.7 nm) ultra-violet light. The disks were then challenge inoculated with a spore suspension of C. lagenarium (1×10^5 mℓ^{-1}).

Lesion size and number were noted after 5, 7 and 9 days. Table 1.8 shows that irradiation, prior to challenge inoculation, significantly increases lesion size. Unprotected disks irradiated for 20 minutes increased by about 1.5 times, and irradiated for 40 minutes increased lesion size to approximately twice the non-irradiated disks. Protected disks showed a similar increase and when measured 9 days after challenge showed no significant difference from control irradiated disks. Reduction in lesion number in protected disks was eliminated following twenty minutes treatment with U.V.

Summary

1. Unprotected leaf disks exposed to U.V. light for 20 and 40 minutes showed increase in lesion size of about 50 and 100% respectively.

2. Protected leaf disks showed similar increases.

Table 1.8 Effect of non-ionising radiation

Treatment[a] /mins	Unprotected						Protected					
	5d[b]		7d		9d		5d		7d		9d	
	LD[c]	LN[d]	LD	LN	LD	LN	LD	LN	LD	LN	LD	LN
0	100	0	100	0	100	0	70	42	59.6	40	60	40
	(2.88)[e]		(4.58)		(7.08)							
20	162	0	149	0	148	0	106	0	109	0	136	0
40	243	0	221	0	171	0	164	0	151	0	160	0

a Length of time disks irradiated/min

b Days after challenge inoculation

c Lesion diameter measured against control (= 100)

d % reduction in lesion number

e Actual diameter of control lesions (mm)

Results means of 12 lesions/6 plants/treatment

3. Reduction in lesion number on protected disks was eliminated following 20 minutes U.V. irradiation.

g. Metabolic inhibitors.

I. To determine what effect, if any, metabolic inhibitors would have on disease development in protected and unprotected cucumber tissue, cycloheximide (an inhibitor of protein synthesis in 80s ribosomes of eukaryotes) and actinomycin D (an inhibitor which acts by blocking the template function of DNA) were applied.

Leaf disks were cut from the second leaves of 22 day old cucumber plants whose first leaves had been inoculated with a spore suspension of C. lagenarium (1×10^7 mℓ^{-1}) or water 7 days previously. Disks were floated on solutions of cycloheximide (CH), actinomycin D (AD) or water for periods of 24 hours, either before, at the same time as or after a challenge inoculation of one 3 $\mu\ell$ drop of a spore suspension of C. lagenarium (1×10^5 mℓ^{-1}). Disks were removed from the inhibitors after the treatment and placed on moist filter paper in 9 cm petri dishes.

Lesion size and number were noted after 6, 8 and 10 days (Tables 1.9-1.12).

Tables 1.9 and 1.10 show that 6 days after challenge inoculation of water treated protected disks, lesion size and number were approximately 50% of those on unprotected disks.

CH, at all concentrations used, had a marked effect on lesion size of

Table 1.9 Effect of cycloheximide on lesion development of unprotected leaf disks

Treatment	Time of Application[a]	6 Days[b] LD[c]	6 Days[b] LN[d]	8 Days[b] LD	8 Days[b] LN	10 Days[b] LD	10 Days[b] LN
H_2O	A	1.55	10	2.45	0	2.60	0
	B	1.44	10	2.22	10	3.05	10
	C	1.00	20	1.61	10	3.33	10
CH 20 ppm	A	4.27	10	9.44	10	12.11	10
	B	3.63	20	9.63	10	12.22	10
	C	3.00	0	7.55	0	12.38	0
CH 10 ppm	A	3.8	0	8.35	0	12.66	0
	B	3.35	0	7.75	0	10.7	0
	C	3.00	0	6.65	0	9.65	0
CH 2 ppm	A	2.06	20	5.88	10	9.77	10
	B	1.30	0	3.75	0	6.95	0
	C	1.29	30	4.562	30	7.57	30

a = A CH applied 24 hrs before C. lagenarium
B CH applied at the same time as C. lagenarium
C CH applied 24 hrs after C. lagenarium

b = Number of days after challenge inoculation

c = Lesion diameter

d = % of lesions not developing

Results means of 10 lesions/5 plants/treatment

Table 1.10 Effect of actinomycin D on lesion development of unprotected leaf disks

Treatment	Time of Application[a]	6 Days[b]		8 Days[b]		10 Days[b]	
		LD[c]	LN[d]	LD	LN	LD	LN
H_2O	A	1.55	10	2.45	0	2.60	0
	B	1.44	10	2.22	10	3.05	10
	C	1.00	20	1.61	10	3.33	10
AD 20 ppm	A	3.36	60	7.70	50	9.33	40
	B	4.40	50	12.60	50	15.00	40
	C	3.50	0	9.80	0	15.00	0
AD 10 ppm	A	2.13	60	5.50	40	6.94	10
	B	2.50	50	7.30	50	10.30	50
	C	2.78	30	9.00	20	11.38	20
AD 2 ppm	A	1.94	20	4.14	20	9.25	20
	B	2.75	30	4.25	30	5.92	30
	C	3.25	30	7.50	30	12.50	30

a) b) c) d) See footnotes under Table 1.9

Results means of 10 lesions/5 plants/treatment

Table 1.11 Effect of cycloheximide on lesion development of protected leaf disks

Treatment	Time of Application[a]	6 Days[b]		8 Days[b]		10 Days[b]	
		LD[c]	LN[d]	LD	LN	LD	LN
H_2O	A	0.59	50	0.92	40	1.43	30
	B	0.64	60	0.77	40	1.28	30
	C	0.88	60	0.75	40	1.28	30
CH 20 ppm	A	4.80	0	9.40	0	14.1	0
	B	3.90	0	10.85	0	14.1	0
	C	3.05	0	8.25	0	13.3	0
CH 10 ppm	A	3.35	0	7.90	0	11.45	0
	B	2.50	20	6.10	0	8.6	0
	C	1.58	40	5.44	10	7.77	10
CH 2 ppm	A	1.71	20	4.94	10	8.44	30
	B	0.50	70	3.13	60	6.80	80
	C	1.50	70	3.33	40	5.14	20

a)
b) See footnotes under Table 1.9
c)
d)

Results means of 10 lesions/5 plants/treatment

Table 1.12 Effect of actinomycin D on lesion development of protected leaf disks

Treatment	Time of Application[a]	6 Days[b]		8 Days[b]		10 Days[b]	
		LD[c]	LN[d]	LD	LN	LD	LN
H_2O	A	0.59	50	0.92	40	1.43	30
	B	0.64	60	0.77	40	1.28	30
	C	0.88	60	0.75	40	1.28	30
AD 20 ppm	A	2.43	30	8.83	30	10.00	30
	B	1.50	80	6.00	80	15.00	80
	C	2.16	40	7.06	20	12.06	20
AD 10 ppm	A	0	100	0	100	0	100
	B	2.33	70	4.83	70	6.50	70
	C	2.00	70	4.25	60	7.50	30
AD 2 ppm	A	3.00	60	5.72	10	8.66	10
	B	2.5	50	6.80	50	10.10	50
	C	2.5	50	8.30	50	10.16	40

a)
b) See footnotes under Table 1.9
c)
d)

Results means of 10 lesions/5 plants/treatment

both protected and unprotected disks. At 20 ppm CH, lesion diameter on unprotected disks was about 400% those of water treated control disks when measured after 10 days. This was reduced to about a 250-300% increase at 2 ppm CH. Lesion diameter on protected leaf disks at all 3 concentrations of CH was not significantly different from unprotected disks.

The 50-60% reduction in the number of lesions developing on water treated protected disks (as measured after 6 days) was completely lost at 20 ppm CH, reduced drastically at 10 ppm but largely unaffected at 2 ppm.

Time of application of CH affected both lesion size and number with application 24 hours prior to challenge inoculation generally giving the greatest effect. CH applied at 2 ppm 24 hours before inoculation of protected disks reduced the number of sites where lesions did not develop whereas when applied at the same time as, or 24 hours after the challenge, had no effect.

Tables 1.11 and 1.12 show the effects of AD to be quite different from those of CH. Although the lesion diameter on unprotected and protected disks were again greatly increased over the water treated controls, the number of sites where lesions developed was greatly reduced. This may be explained by either the inhibition of growth of C. lagenarium or the induction of resistance, by AD, in cucumber to C. lagenarium.

Summary

1. Lesion size and number on water treated protected disks were approximately 50% those of unprotected disks when measured after 6 days.

2. CH at all concentrations increased lesion size of unprotected disks by up to 400%.

3. CH at all concentrations increased lesion size of protected disks so they were not significantly different from unprotected disks.

4. CH at 20 and 10 ppm almost completely negated the reduction in lesion number seen in water treated protected disks.

5. Time of application of CH affected both lesion size and number.

6. AD at all concentrations increased lesion size of protected and unprotected disks.

7. AD at all concentrations reduced the number of lesions developing on both protected and unprotected disks.

II. Following the results of the preceding experiment in which cycloheximide was shown to nullify induced resistance in cucumber, the next experiment was undertaken to assess the effect of CH and AD on the genetic resistance of cucumber to a non-pathogen.

Leaf disks were cut from cucumber leaves and floated on solutions of

CH, AD (both at concentrations between 5-120 ppm) or water for 24, 48 or 72 hours. Disks were then put on moist filter paper in 9 cm petri dishes and inoculated with one 3 $\mu\ell$ drop of spore suspension of C. lindemuthianum (1×10^7 $m\ell^{-1}$).

Disks were monitored for the appearance of lesions. After 14 days, no lesions had appeared in any of the treatments.

Summary

Resistance to the non-pathogen C. lindemuthianum was not affected by CH or AD at the concentrations used.

h. Water status

Infection of a host may have a profound affect on the water status of that plant. Extreme examples are the vascular wilt pathogens, but other pathogens causing less severe symptoms such as necrosis and water soaking must involve some alterations in the water relations of host cells.

To determine whether water status could in some way influence this induction of resistance, the following experiment was carried out.

Cucumber cotyledons were floated on solutions of either mannitol or polyethylene glycol 6000 (PEG) for 3 days prior to inoculation with C. lagenarium (1×10^5 spores $m\ell^{-1}$).

Relative water content (RWC) was determined by weighing cotyledons

after floating them on PEG or mannitol, placing them on distilled water and reweighing until constant weight was obtained. Dry weight was determined after drying in an oven at 60°C.

$$RWC = \frac{\text{(Fresh weight) - (oven dry weight)}}{\text{(Fully turgid weight) - (oven dry weight)}} \times 100$$

Lesion size and number were noted.

FIGURE 1.16 shows that despite a difference in RWC of 40% there was no significant difference in lesion size. Lesion number was not reduced (not shown in FIGURE 1.16).

Tissues taken to a RWC of less than 75% did not survive the duration of the experiment and have therefore not been included.

Summary

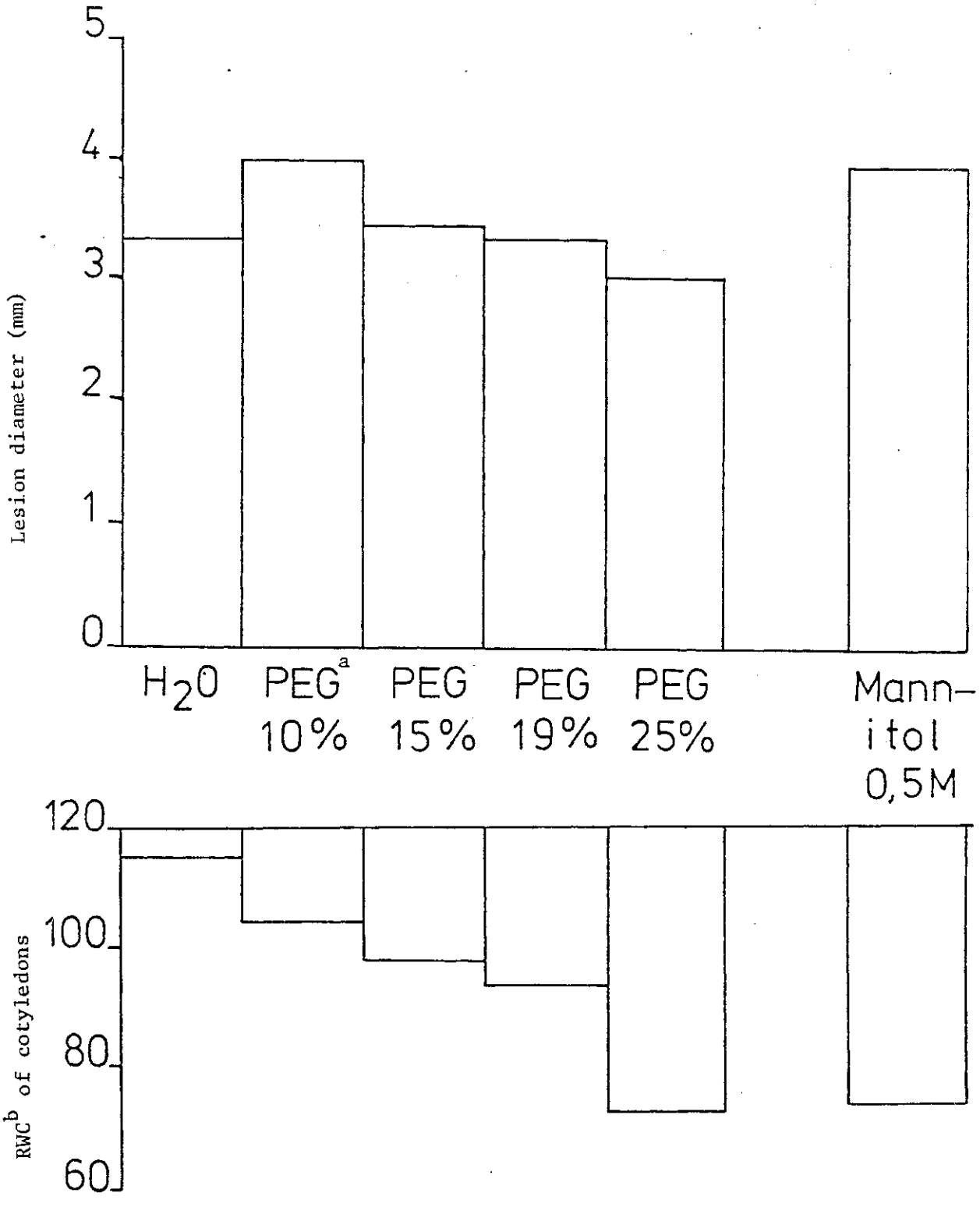
Tissues taken to the survivable limit of dehydration show no significant reduction in either lesion size or number when subsequently challenged with C. lagenarium.

5. The effects of non-multiplying agents as inducers of resistance

a. Fungal cell-wall components.

To determine whether mycelial wall components could induce systemic resistance, fungal cell-wall extracts containing mainly β1-3 glucans were obtained from the pathogen Colletotrichum lagenarium and the

FIGURE 1.16 Effects of water status on lesion development



a PEG = Polyethylene glycol 6000

b RWC = Relative water content

Results means of 24 lesions/6 cotyledons/treatment

non-pathogen Colletotrichum lindemuthianum race δ, as in Materials and Methods. These were solubilised by autoclaving, reduced to required concentrations by rotary evaporation at $40^{o}C$ and injected into cotyledons of 15 day old cucumber plants. Three concentrations at 1000, 500, 250 and 0 $\mu\ell\ m\ell^{-1}$ glucose equivalents were used.

Seven days after injection of the cotyledons, the first leaf was challenge inoculated with 20 3 $\mu\ell$ drops of a suspension of C. lagenarium spores ($1 \times 10^{5}\ m\ell^{-1}$) as in Materials and Methods.

Lesion size and number were noted after a further 7 days (Table 1.13).

Within 72 hours, most cotyledons injected with 1000 and 500 $\mu g\ m\ell^{-1}$ solutions had collapsed and those injected with 250 $\mu g\ m\ell^{-1}$ showed some necrosis.

Table 1.13 Effects of mycelial cell wall material on induction of resistance

Source of mycelial Wall extract	$\mu g\ m\ell^{-1}$ glucose equivalents	Lesion diameter after 7 days	S.E.	% reduction in lesion number
C. lagenarium	0	2.56	0.099	0
	250	2.76	0.115	0
	500	2.49	0.206	0
	1000	2.69	0.104	0
C. lindemuthianum	0	2.48	0.081	0
race δ	250	2.71	0.105	0
	500	2.78	0.097	0
	1000	2.88	0.073	0

Results means of at least 80 lesions/4 plants/treatment

Summary

1. Fungal cell-wall components from C. lagenarium and C. lindemuthianum both cause necrosis when injected at high concentrations into cucumber cotyledons.

2. This necrosis has no effect on lesions which subsequently develop on the first true leaf.

b. I. To determine whether non-multiplying spores of C. lagenarium could replace the induction of systemic resistance by infection with C. lagenarium, spores were heat killed as in Materials and Methods, and injected at 1 x 10^7 spores $m\ell^{-1}$ into the cotyledons of 15 day old cucumber plants.

In addition, other plants were injected with live spores of C. lindemuthianum race δ (1 x 10^7 spores $m\ell^{-1}$).

After 7 days the first true leaf was challenge inoculated with a spore suspension of C. lagenarium (1 x 10^5 $m\ell^{-1}$).

Lesion size and number were noted after a further 7 days (Table 1.14).

Table 1.14 Effect of injection of heat-killed conidia and non-pathogenic conidia on induction of resistance

Cotyledon treatment	Mean lesion diameter after 7 days	S.E.	% reduction in lesion number over control
H_2O	2.56	0.062	-
Heat-killed C. lagenarium spores	2.49	0.104	0
Live C. lindemuthianum spores	2.65	0.095	0

Results means of 80 lesions/4 plants/treatment

II. In a second experiment, the effect of injecting cotyledons with heat-killed conidia of C. lagenarium at 1 x 10^7 spores $m\ell^{-1}$ and non-pathogen Pseudomonas phaseolicola (1 x 10^7 bacteria $m\ell^{-1}$) on lesion development by C. lagenarium in the same cotyledon, when challenged 4 days after injection, was assessed.

Lesion size and number were noted after a further 5 and 8 days (Table 1.15).

Table 1.15 Effect of injection of heat killed conidia and live Pseudomonas phaseolicola on induction of resistance at the same site

Cotyledon treatment	5 Days[a]		8 Days[a]	
	LD[b]	LN[c]	LD	LN
H_2O	2.57	0	3.36	0
Heat killed C. lagenarium	2.52	8.3	3.90	8.3
Live Pseudomonas phaseolicola	2.65	14.5	3.81	14.5

a = days after challenge inoculation

b = mean lesion diameter

c = % reduction in lesion number over control

Results means of 48 lesion sites/8 cotyledons/treatment

Table 1.15 shows that no substantial induction of resistance occurred following injection of cotyledons with an heat-killed pathogen or live non-pathogenic Pseudomonas phaseolicola. The presence of foreign bodies within cucumber cotyledons did not induce resistance to subsequent challenge inoculations.

Summary

1. Injection of cucumber cotyledons with heat-killed conidia of C. lagenarium and live non-pathogenic spores of C. lindemuthianum race 6 had no effect on subsequent development of C. lagenarium on the first leaf.

2. Injection of cotyledons with heat-killed conidia of C. lagenarium or the live non-pathogenic bacterium Pseudomonas phaseolicola had no significant effect on lesion development on the same cotyledons when challenge inoculated after a further 4 days.

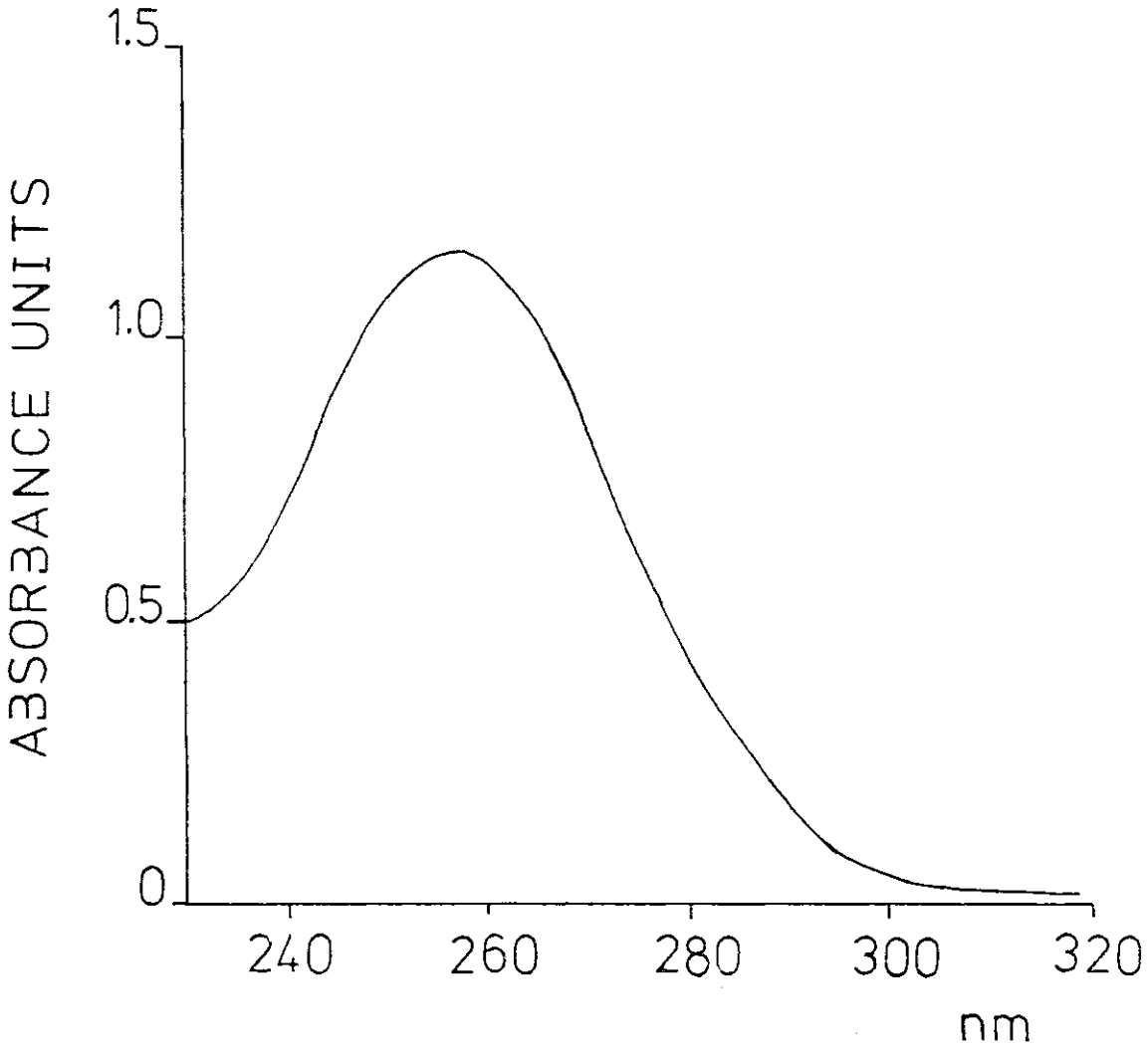
c. Fungal nucleic acid

C. lagenarium was grown in liquid shake culture for 10 days at which point the nucleic acids were extracted, (See Materials and Methods).

Nucleic acid was injected into cotyledons of 15 day old cucumber plants at between 0.25-2500 $\mu g\ m\ell^{-1}$ (assuming an extinction coefficient of 25).

After a further 7 days the first true leaf was challenge inoculated with 3 $\mu\ell$ drops of a suspension of C. lagenarium spores ($1 \times 10^5\ m\ell^{-1}$).

FIGURE 1.17 UV scan of fungal nucleic acid



Fungal nucleic acid extracted from C. lagenarium as described in Materials and Methods.

The final nucleic acid precipitate was dissolved in buffer and scanned in a Beckman 35 spectrophotometer.

Lesion size and number were noted after 7 days (Table 1.16).

Table 1.16 Effect of injecting fungal nucleic acid into cotyledons on lesion development on first leaves

Nucleic acid ($\mu g\ m\ell^{-1}$)	Mean lesion diameter after 7 days (mm)	S.E.	% reduction in lesion number over control
0	2.56	0.062	-
0.25	2.42	0.081	0
2.5	2.38	0.063	0
25	2.41	0.095	0
250	2.25	0.082	0
2500	2.38	0.11	0

Results means of 80 lesions/4 plants/treatment

FIGURE 1.17 shows the ultra violet scan of the fungal nucleic acid between 230 and 320 mm. The 260/280 ratio of 2.55 indicates a relatively pure preparation with very little protein contamination. Nucleic acid concentrations were determined from the absorbance at 260 assuming an extinction coefficient of 25.

Table 1.16 shows that C. lagenarium nucleic acid injected into cucumber cotyledons has only a very slight effect on lesion size on subsequently challenged first leaves. Nucleic acid at 250 $\mu g\ m\ell^{-1}$ has a significant but small (about 13%) effect in reducing lesion size. No reduction in lesion number was noted.

Summary

1. Fungal nucleic acid produced only very small reductions in lesion size and no reduction in lesion number on first leaves following injection of the cotyledons.

d. Tissue diffusates

Water diffusates from infected cucumber tissue, non-infected tissue surrounding lesions, protected tissue and non-protected tissue were prepared as in Materials and Methods. After sterilisation and concentration by rotary evaporation, the diffusates were injected into cotyledons of 15 day old cucumber plants.

Seven days later the first true leaf was challenge inoculated with 3 $\mu\ell$ drops of a spore suspension of C. lagenarium at 1 x 10^5 $m\ell^{-1}$.

Lesion size and number were noted after 5 and 10 days.

Table 1.17 shows that diffusates from all tissues caused necrosis when injected into cotyledons. Diffusates from protected leaf tissue appeared to cause more severe necrosis than those from unprotected tissue. Although unexpected, this was not repeated as lesion size on subsequently challenged first leaves was not significantly different between treatments. No reduction in lesion number was noted.

Summary

1. Necrosis caused by water diffusates of infected, lesion surround or

Table 1.17 Effect of tissue diffusates on lesion development

Tissue	Concentration (μg dry wt $m\ell^{-1}$)	Cotyledon Necrosis	5 Days[a] LS[b]	5 Days[a] LN[c]	10 Days[a] LS	10 Days[a] LN
Infected	10	+++[d]	1.82	0	5.56	0
	2.5	+++	1.51	0	5.82	0
Surrounding Lesion	10	+++	1.82	0	4.82	0
	2.5	+++	1.78	0	-	-
Protected Leaf	10	+++	1.35	0	4.37	0
	2.5	+++	1.63	0	3.97	0
Unprotected Leaf	10	+	1.61	0	4.37	0
	2.5	+	1.87	0	4.24	0

a Number of days after challenge

b Lesion diameter in mm

c % of infected sites not producing lesions

d Degree of necrosis caused by diffusate in cotyledon, +++ severe + mild

\- Not tested

Results means of 80 lesions/4 plants/treatment

protected leaf tissue in cotyledons had no effect on lesion development on challenged first leaves.

e. Seed soaking

To determine whether suspected inducers of resistance, some of which had already been used in various forms in preceding experiments, could induce responses in a germinating plant, seeds were soaked for 48 hours, prior to planting, in the following:-

1. 6-Benzylaminopurine (6-BAP) at 0.1, 1 and 10 $\mu g\ \ell^{-1}$.

2. Abscisic acid (ABA) at 0.1, 1 and 10 $\mu g\ \ell^{-1}$.

3. C. lagenarium cell-wall glucan at 10, 100 and 1000 µg glucose equivalents $m\ell^{-1}$.

4. C. lindemuthianum race δ at 10, 100 and 1000 µg glucose equivalents $m\ell^{-1}$.

5. C. lagenarium nucleic acid at 10 and 1000 $\mu g\ m\ell^{-1}$.

6. C. lagenarium water diffusate from cultures grown on V8 juice.

7. Cell free filtrate from liquid cultures of C. lagenarium.

8. Suspension of heat-killed conidia of C. lagenarium (1×10^7 spores $m\ell^{-1}$).

Seeds were grown under greenhouse conditions, as in Materials and Methods, until the first true leaf was fully expanded. Disks were cut from the leaf and placed on moist filter paper in 9 cm petri dishes. One 3 $\mu\ell$ drop of a spore suspension of C. lagenarium (1×10^5 $m\ell^{-1}$) was placed on each.

Lesion size and number were noted after 6, 8 and 10 days (Table 1.18).

Water soaked seeds were taken as control.

Table 1.18 shows that when measured after 6 days, several of the treatments reduced lesion size and number when subsequently challenged, (6-BAP, ABA and C. lagenarium cell-wall glucan all gave small reductions). These reductions were even smaller when measured after 8 days and, with the exception of 6-BAP at 1.0 and 10 $\mu g\ \ell^{-1}$, all treatments showed an increase in lesion size when measured after 10 days over the water soaked controls.

Summary

1. Seeds soaked in 6-BAP at 1.0 and 10 $\mu g\ \ell^{-1}$ for 48 hours prior to sowing showed a small reduction in lesion size over control treatments.

2. All other treatments, when measured 10 days after challenge inoculation, showed an increase in lesion size.

Table 1.18 Effects of soaking seeds on later development of lesions

Seeds Soaked in	Concentration	6 Days[a]		8 Days[a]		10 Days[a]	
		LS[b]	LN[c]	LS	LN	LS	LN
H_2O	-	3.27	0	4.18	0	5.23	0
6-BAP	0.1 μg ℓ^{-1}	2.65	10	4.56	5	7.30	0
	1.0 μg ℓ^{-1}	2.47	0	3.52	0	4.21	0
	10 μg ℓ^{-1}	2.71	5	3.28	0	4.05	0
ABA	0.1 μg ℓ^{-1}	2.85	0	4.97	0	9.30	0
	1.0 μg ℓ^{-1}	2.30	10	4.13	0	7.30	0
	10 μg ℓ^{-1}	2.45	15	3.97	10	6.91	0
Cell-free culture filtrate	1	3.03	0	4.67	0	7.56	0
	10	3.06	0	4.31	0	6.40	0
	10^2	3.28	0	4.74	0	6.88	0

a = Days after challenge inoculation

b = Lesion diameter

c = % of inoculated sites not producing lesions

Results means of 20 lesions/10 plants/treatment

Table 1.18 Continued

Seeds Soaked in	Concentration	6 Days[a]		8 Days[a]		10 Days[a]	
		LS[b]	LN[c]	LS	LN	LS	LN
H_2O	-	3.27	0	4.18	0	5.23	0
C. lagenarium cell wall glucan	10 μg mℓ$^{-1}$	2.87	5	4.06	0	6.90	0
	100 μg mℓ$^{-1}$	2.70	0	4.69	0	8.15	0
	1000 μg mℓ$^{-1}$	2.36	10	4.63	0	7.63	0
C. lindemuthianum cell wall glucan	10 μg mℓ$^{-1}$	2.91	10	5.69	0	8.52	0
	100 μg mℓ$^{-1}$	4.96	0	7.35	0	10.77	0
	1000 μg mℓ$^{-1}$	3.76	0	5.37	0	7.12	0
C. lagenarium nucleic acid	10 μg mℓ$^{-1}$	3.54	10	5.09	10	7.65	0
	1000 μg mℓ$^{-1}$	3.24	0	4.55	0	6.38	0
C. lagenarium water diffusate	1	3.24	0	5.30	0	7.85	0
C. lagenarium Heat-killed conidia	1 x 10^7 spores mℓ$^{-1}$	2.77	0	5.12	0	7.91	0

a)
b) see footnotes on previous page
c)

6. Induction of necrosis

Induction of extensive necrosis in the first true leaves of 15 day old cucumber plants by:-

a. Exposure to steam (by placing the leaf above a boiling water bath for $\underline{c}$ 30 seconds).

b. Exposure to fire (by placing parts of a leaf through a naked flame so that approximately half of the surface is burnt).

c. Physical damage (caused by manual crushing between thumb and fore-finger so that approximately half the leaf surface becomes necrotic).

d. Injection of chloroform (so that $\underline{c}$ 20 1 cm^2 sites on the leaf become watersoaked).

Following the above treatments the second leaves were challenged 7 days later with a spore suspension of $\underline{C.\ lagenarium}$ (1×10^5 $m\ell^{-1}$).

No significant difference was noted in either lesion size or number between these or control treatments.

Summary

1. Chemical or physical damage leading to necrosis in the first leaf has no effect on subsequent challenge inoculation of the second leaf.

DISCUSSION I

Induction of systemic resistance in cucumber to C. lagenarium has been shown following prior inoculation with the same fungus. This confirms the findings of Hammerschmidt et al. (1976) who were the first to show that cucumber plants could be systemically protected from C. lagenarium by earlier inoculation of lower leaves with either the same fungus or Cladosporium cucumerinum.

Induction of resistance is not limited to C. lagenarium as the inducer organism. Infection of cotyledons or first leaves with tobacco mosaic virus, which causes local lesions, induces resistance in first and second leaves respectively and infection of cotyledons with the bacterium Pseudomonas lachrymans induces resistance in first leaves to challenge inoculation by C. lagenarium. Similar results were reported by Jenns and Kuč (1977) and Caruso and Kuč (1979).

Induction of resistance in cucumber second leaves was apparent 120 hours after infection of first leaves with either C. lagenarium or tobacco necrosis virus (TNV). Maximum effects were normally apparent when the inducer leaf had remained attached for 168-192 hours after inoculation. This compares, with detection of resistance, with removal after 72-96 hours for fungi and 48 hours for TNV reported by Caruso and Kuč (1979) and Kuč and Richmond (1977). The differences between this work and published work may be explained by use of cucumber cultivars exhibiting differing degrees of genetic resistance to C. lagenarium, the use of different races and by the spore concentration used in the challenge inoculations (challenge inocula of 1×10^5 spores $m\ell^{-1}$ were used throughout this work as this was the lowest concentration to produce consistent control infection).

Resistance induced by C. lagenarium was marginally greater than that induced by TNV. This may be a real difference or one dependent on non-optimal concentrations of inoculum or conditions for multiplication of TNV.

Location of the inducer organism seems to play an important part in the degree of protection induced.

Andebrhan (1978) had failed to induce systemic resistance against C. lagenarium in first leaves by prior inoculation of the second leaves with large inocula of C. lagenarium. This work shows that lesion size on first leaves was reduced by 35% and lesion number by 6.8% when second leaves were infected prior to challenge. The degree of induced resistance was much smaller than in the reverse situation.

The apparent 'movement' of resistance within a single leaf reflects movement within the whole plant, in that resistance moves from tip to base much more readily than from base to tip. When a lateral half of a leaf is the induction site, the opposite half leaf is not uniformly protected as areas nearer the petiole show greater resistance to challenge inoculations.

Such movement argues against a cell to cell communication of an induction signal which would be expected to move equally well in all directions. A more likely explanation would be a phloem-borne signal, the main direction of which would be from older mature leaves to the younger growing points.

It is interesting to note that induction of resistance within a single leaf following inoculation of the tip took up to 5 days to appear. This

is the time taken for the induction of resistance in the second leaf following infection of the first. The comparative distances between induction and protection sites were 2-3 cm and 20-30 cm. Simultaneous induction of resistance at 2 and 20 cm from the induction site rules out certain possibilities: 1. Slow signal translocation because a substance taking 5 days to travel 20 cm would have induced resistance at 2 cm within 12 hours. 2. A receptor site requiring 5 days to react to a single signal stimulation because removal of the inducer leaves after 4 days and left for a further day before challenge inoculation gives no resistance.

This leaves two likely alternatives. Either the inducer site needs 5 days to produce a 'signal' or the receptor site requires continuous stimulation for 5 days to activate the mechanism. Jenns and Kuc (1980) reported that systemic induction of resistance to C. lagenarium by inoculation of the lower leaves with TNV took only 24-48 hours. If this is so then the time taken for the receptor site (second leaf in this case) to react is not the limiting factor. This suggests that the production of the signal in the inducer leaf determines the timecourse of induction. It should be pointed out, however, that in the present work, timecourse for induction by TNV was similar to that for induction by C. lagenarium.

Shielding of the inducer leaf from light prevented movement of the 'signal' from that leaf. This suggests a phloem-borne signal but does not rule out the possibility that light is necessary for the production of such a signal.

The number of lesions on the inducer leaf is directly related to the degree of protection induced in the second leaf. This implies that

production of a 'signal' substance takes place at or around the lesions on the inducer leaf. As few as 3 lesions on the first leaf produce a significant reduction in lesion size on the second leaf.

Another important feature is the ability of C. lagenarium to overcome induced resistance when the inoculum concentration is high. C. lagenarium at 1×10^7 spores $m\ell^{-1}$ completely nullifies the effects of induced resistance. This is curious as it implies that a single spore cannot initiate infection on systemically protected cucumber but can do so in the presence of other spores.

One explanation for this may be that symptoms caused by a single spore are masked until, at high spore concentrations, symptoms become apparent.

Richmond, Kuč and Elliston (1979) reported that systemic protection was lost by removal of a portion of the leaf's epidermal tissue and inoculation of the exposed sites.

This was not confirmed because it was found that epidermis removal did not significantly alter the number of lesions developing at those sites. However, those lesions which did develop on epidermis stripped cotyledons were larger than those on cotyledons with the epidermis intact, but there still remained a significant difference between stripped protected and stripped unprotected tissue.

These results do not support the suggestion of Richmond et al. (1979) that induced resistance may be due to changes, induced systemically, in the epidermal cell wall of cucumber plants, but not in underlying cells.

The results of the experiment in which cycloheximide (CH) and actinomycin D (AD) were applied to cucumber leaf disks must be interpreted with care. An important point is whether the metabolic inhibitors affect the host or the fungus. CH has been widely reported to be highly active against many fungi and to be systemically distributed within plants, (Ford, Klomparens and Hammer, 1958). Fawcett and Spencer (1970), using leaf disks cut from cucumber leaves floated on solutions of CH, showed the inhibitor to have no deleterious effect on reduction of growth of C. lagenarium applied as a drop to the leaf disk. This was confirmed because CH did not inhibit C. lagenarium but caused an increase in lesion size on healthy unprotected cucumber leaf disks. This suggests an active defense mechanism operating in normal susceptible cucumber plants, restricting fungal growth to a limited extent.

The reduction in lesion number in water treated protected leaf disks compared with water treated unprotected leaf disks was up to 60%. This reduction was lost completely when disks were floated on the higher concentrations of CH. The time of application of CH was also important. The greatest effect was generally seen when CH was applied 24 hours prior to inoculation, the effect diminishing when application was 24 hours after inoculation. All these results suggest that the inhibitor affects the host rather than the fungus and that inhibition of 80s ribosomes suppresses resistance, indicating that induced resistance requires metabolic activity.

The effects of AD appear to be quite different from those of CH. In healthy unprotected cucumber disks, the number of inoculated sites producing lesions was decreased drastically (one treatment combination caused 100% reduction in lesion number) indicating that AD was probably

fungistatic. Heath (1979), using CH and AD to induce haustoria formation of cowpea rust on non-host pea, found that CH gave a three fold increase whereas AD reduced the number to less than the control plants.

The effect in this work at high AD concentrations seems to be largely on the fungus rather than the host. AD at low concentrations increases lesion size in both unprotected and protected leaf disks despite a low level of inhibition of lesion development. As AD inhibits DNA dependent RNA synthesis and mRNA synthesis this could imply the involvement of de novo protein synthesis in both genetic and induced resistance in cucumber.

In conclusion it seems that both the normal susceptible response and induced resistance to C. lagenarium in cucumber involve some active metabolic process which is adversly affected by CH, and to some extent by AD.

The use of both metabolic inhibitors in an attempt to induce infection by the non-pathogen C. lindemuthianum race δ was not successful even at very high inhibitor concentrations. It is concluded therefore that the resistance mechanisms operating against C. lagenarium are not the same as those protecting cucumber from other non-pathogens.

That shortwave ultra-violet (UV) (254 nm) irradiation is able to inactivate mRNA directly has been shown by Swenson and Setlow (1964). Levy et al. (1974) in their work with cucumber mosaic virus in a resistant cucumber cultivar suggested that UV irradiation, in a similar way to AD (Nachman et al. 1971) inhibits the formation of or operation of a mechanism that suppresses virus multiplication.

It was found that 20 minutes exposure to UV irradiation increases the susceptibility of cucumber leaves to C. lagenarium. Leaf disks that had been cut from systemically protected leaves lost their protection after the same exposure time. The ability of UV light to inhibit mRNA may be significant in both these instances although UV light undoubtedly affects many other processes and structures within plant tissues.

Heat-shock of plant tissues has been used to prevent or delay disease resistance responses. Bean leaves immersed in water at 40-55°C for various periods of time up to six minutes increase their susceptibility to several viruses and fungi (Yarwood, 1956).

This work shows that temperatures from 5-35°C have no effect on lesion size or number when applied for periods up to 300 seconds. Unprotected leaf disks immersed at 45°C for 300 seconds and 55°C for only 5 seconds showed a marked increase in lesion size. Similarly, protected disks immersed for the same periods showed increases in both lesion size and number indicating that heat-shock has a significant effect on the mechanism(s) that restrict lesion expansion in healthy susceptible cucumber as well as the mechanism(s) for induced resistance.

In contrast, Lindner et al. (1959) found that susceptibility of cucumber to infection with tobacco mosaic virus was considerably reduced when cotyledons were dipped into water at 45°C for 1 to 2 minutes and Hicks and Frost (1979) found that susceptibility of cucumber to Prunus necrotic ringspot was increased by a period at 14°C.

There are many effects that have been ascribed to heat-shock. Depression of respiration, photosynthesis and RNA and protein synthesis are a few (see literature cited by Aist and Israel, 1977).

Comparison of the effects of predisposition by heat-treatment is difficult as treatments have been applied in many different ways for differing time intervals by various workers.

It has been suggested that plant water status may influence the susceptibility of plants to infection (Yarwood, 1959). Susceptibility of French bean to tobacco necrosis virus was greatly reduced when plants were grown in culture solutions at high suction tensions, (Kimmins and Litz, 1967). Increase in osmotic values due to carbohydrate build-up was the suggested mechanism of resistance of vines to Uncinula necator, (Goheen and Schnathorst, 1961).

Changes induced in the water status of cucumber cotyledons prior to inoculation with C. lagenarium had no significant effect on either lesion size or number in this work. It is unlikely, therefore, that changes in water status affect the induction of resistance in cucumber.

Section 2. Chemical induction of resistance

1. To assess the potential of various chemicals as inducers of resistance, the following were dissolved in phosphate buffer (0.01 M pH 6.8) and injected into 15 day old cucumber cotyledons from a hypodermic syringe:-

1. acetylsalicylic acid
2. 4-aminobenzoic acid
3. 4-aminosalicylic acid
4. gentisic acid
5. 4-hydroxybenzoic acid
6. polyacrylic acid (molecular weight $\underline{c}$ 1500-2000)
7. protocatechuic acid
8. resorcinol
9. salicylic acid

Following the injection of test substances, (in which cotyledons were completely watersoaked) cotyledons were washed with distilled water and the plants were put into high humidity chambers for 2 days. Cotyledons were challenge inoculated with six 3 $\mu\ell$ drops of a suspension of C. lagenarium spores (1×10^5 $m\ell^{-1}$) 24 or 96 hours after injection.

Lesion size and number were noted after 5, 7 and 9 days.

None of the test substances used in Table 2.1 had a significant effect on either lesion size or number when challenge inoculation followed injection by 24 hours.

Table 2.1: Effects on lesion development of test substances applied 96 hours before challenge inoculation

Treatment of cotyledons	Concentration	5 Days[a]		7 Days[a]		9 Days[a]	
		LD[b]	LN[c]	LD	LN	LD	LN
Control (PO_4^- buffer)	0.01 M	3.76	0	6.32	0	8.00	0
4-hydroxybenzoic acid	0.02%	2.42	10	4.18	6	6.01	6
Polyacrylic acid	0.2%	2.25	98	3.5	96	4.5	93
Protocatechuic acid	0.02%	2.28	54	2.46	35	2.38	31
Resorcinol	0.02%	2.54	88	2.32	27	2.71	27
Salicylic acid	0.02%	0	100	2.01	73	1.97	48

a Days after challenge inoculation

b Mean lesion diameter of developed lesions

c % of inoculated sites not producing lesions

Results means of at least 48 lesions/8 cotyledons/treatment

Table 2.1 continued

Treatment of cotyledons	Concentration	5 Days[a]		7 Days[a]		9 Days[a]	
		LD[b]	LN[c]	LD	LN	LD	LN
Control (PO_4^- buffer)	0.01 M	3.76	0	6.32	0	8.00	0
Acetylsalicylic acid	0.02%	1.00	98	2.25	85	2.87	79
4-aminobenzoic acid	0.02%	2.68	38	4.81	27	4.14	21
4-aminosalicylic acid	0.02%	1.93	40	3.45	32	4.95	23
Gentisic acid	0.02%	3.00	98	2.00	47	2.00	33

a)
)
b) See footnotes under preceding page
)
c)

All treatments gave lesion diameter significantly different from control (PO_4^-) by at least 0.01% probability

When challenge inoculated after 96 hours, most test substances showed some reduction in lesion size and number (Table 2.1).

In this experiment polyacrylic acid, acetylsalicylic acid, gentisic acid and salicylic acid all induced an almost 100% reduction in lesion number, compared with phosphate buffer injected control cotyledons, when measured 5 days after challenge inoculation. Measurement of the same cotyledons showed, after 10 days, induced resistance remaining high in polyacrylic acid and acetylsalicylic acid treated cotyledons, but dropping below 50% for salicylic acid and gentisic acid.

Results varied when the experiment was repeated. Polyacrylic acid (at 0.2%), acetylsalicylic acid, salicylic acid, p-hydroxybenzoic acid and gentisic acid (all at 0.02%) gave 100% reduction in lesion number on some occasions but much lower levels (even 0%) on others (data not presented). Polyacrylic acid, acetylsalicylic acid and salicylic acid gave the most consistent results with reduction in lesion number normally between 60-85% compared with buffer injected control plants.

These three substances were chosen for further experiments.

2. Method of application

To determine whether chemical inducers would be active when applied to cucumber plants using methods other than cotyledon injection, the following experiments were done:-

(a) Absorption through cut petiole

Cucumber plants were grown as in materials and methods until the first true leaf was fully expanded. The leaf was then removed and the petiole placed in a vial containing either phosphate buffer (0.01 M pH 6.8) or phosphate buffer containing polyacrylic acid (0.2 and 0.02%), acetylsalicylic acid, gentisic acid (0.02 and 0.002%) or salicylic acid (0.002%).

Leaves were challenge inoculated with a suspension of C. lagenarium spores (1×10^5 $m\ell^{-1}$) 4 days later.

Lesion size and number were noted after 5 days (Table 2.2).

Table 2.2: Effect of absorption through petiole on lesion development

Treatment	Concentration	5 Days after inoculation		
		LD[a]	SD[b]	LN[c]
Phosphate buffer	0.01 M	3.21	0.17	0
Polyacrylic acid	0.02%	3.11	0.11	0
	0.2%	2.89	0.19	0
Acetylsalicylic acid	0.002%	3.30	0.15	0
	0.02%	3.44	0.20	0
Salicylic acid	0.002%	3.54	0.16	0
Gentisic acid	0.002%	3.13	0.19	0
	0.02%	3.29	0.17	0

a mean lesion diameter

b 95% confidence limits

c % of inoculated sites not producing lesions

Results means of 24 lesion/6 leaves/treatment

Table 2.2 shows that absorption of test substances through cut petioles for 4 days has no significant effect on lesion size or number.

(b) Watered on to plants in soil

Polyacrylic acid (0.04%), acetylsalicylic acid (0.004%) both in phosphate buffer (0.01 M pH 6.8) or phosphate buffer alone were watered on to young cucumber plants with fully expanded cotyledons, grown in John Innes No. 1 compost in 3 inch pots, for 5 days prior to challenge inoculation of the cotyledons with a suspension of C. lagenarium spores (1×10^5 mℓ^{-1}).

Lesion size and number were noted after 5, 7 and 9 days.

Table 2.3 shows that polyacrylic acid causes no reduction in lesion size or number but acetylsalicylic acid gives a small but significant reduction in both. Concentrations of polyacrylic acid and acetylsalicylic acid used in this experiment were less than those used for injection of cotyledons as higher concentrations caused wilting of test plants.

(c) Watered on to plants grown in vermiculite

Cucumber seeds were sown in seed trays containing vermiculite. Plants were watered with Long Ashton nutrients solution (LANS) (see materials and methods) containing polyacrylic acid, acetylsalicylic acid, salicylic acid or with no additive as control. Concentrations used were 0.04%, 0.002 and 0.002% respectively. Plants were grown in growth cabinets at 24-25°C using Philips fluorescent tubes. They were

Table 2.3: Effects of test substances watered on to cucumber plants in soil on lesion development

Treatment	Concentration	5 Days[a]		7 Days[a]		9 Days[a]	
		LD[b]	LN[c]	LD	LN	LD	LN
Phosphate buffer	0.01 M	2.92	0	3.63	0	4.49	0
Polyacrylic acid	0.04%	3.18	0	4.00	0	4.75	0
Acetylsalicylic acid	0.004%	2.40[d]	5	3.07[d]	5	3.80[d]	0

a Days after challenge inoculation

b Mean lesion diameter

c % of inoculated sites not producing lesions

d Significantly different at 0.02% level to PO_4^- treatment

Results means of 18 lesions/3 plants/treatment

inoculated when cotyledons were fully expanded and the first leaf just appearing. Six 3 $\mu\ell$ drops of a suspension of C. lagenarium spores (1×10^5 $m\ell^{-1}$) were placed on each cotyledon.

Lesion size and number were noted after 6, 8 and 10 days.

Plants grown on vermiculite in LANS were shorter than plants grown ordinarily on compost. Plants grown in LANS with addition of polyacrylic acid, acetylsalicylic acid or salicylic acid were smaller than those in LANS without additives. These plants also showed some signs of phytotoxicity, leaves being faintly chlorotic at the margins.

Table 2.4 shows the effect of these substances on lesion size and number.

Control plants grown in LANS without additives showed a 31% reduction in the number of lesions developing compared to plants grown normally in compost and water (results of other experiments).

Addition of polyacrylic acid, acetylsalicylic acid and salicylic acid caused a further reduction of 17%, 50% and 38% in lesion number.

Lesion size was reduced in acetylsalicylic acid treated plants and slightly increased in polyacrylic acid and salicylic acid treated plants.

(d) Other methods

I. Test substances (polyacrylic acid, acetylsalicylic acid and salicylic acid) were sprayed once daily on to fully expanded first leaves of cucumber plants for 3 successive days at various concentrations. Plants

Table 2.4: Effects of test substances watered on to cucumber plants in vermiculite on lesion development

Treatment	Concentration	6 Days[a]		8 Days[a]		10 Days[a]	
		LD[b]	LN[c]	LD	LN	LD	LN
LANS	-	2.39	35	2.89	31	3.69	31
Polyacrylic acid	0.04%	2.53	48	3.32	48	4.15	48
Acetylsalicylic acid	0.004%	1.75[d]	81	2.15[d]	81	2.84[d]	81
Salicylic acid	0.004%	2.38	69	3.33	69	4.21	69

a Days after challenge inoculation

b Mean lesion diameter

c % of inoculated sites not producing lesions

d Significantly different at 0.01% from LANS control

Results means of 48 lesions/8 cotyledons/treatment

were left for 5 days, the leaves washed and then challenge inoculated with a suspension of C. lagenarium spores (1×10^5 mℓ^{-1}).

II. Prior to sowing, seeds were soaked for 48 hours in various concentrations of polyacrylic acid, acetylsalicylic acid and salicylic acid. Seeds were then sown in John Innes No. 1 compost and grown in the greenhouse as in Materials and Methods until cotyledons were fully expanded. Six 3 $\mu\ell$ drops of a suspension of C. lagenarium spores (1×10^5 mℓ^{-1}) were placed on each cotyledon.

Subsequent lesion development showed that neither method produced a significant reduction in either lesion size or number.

SUMMARY

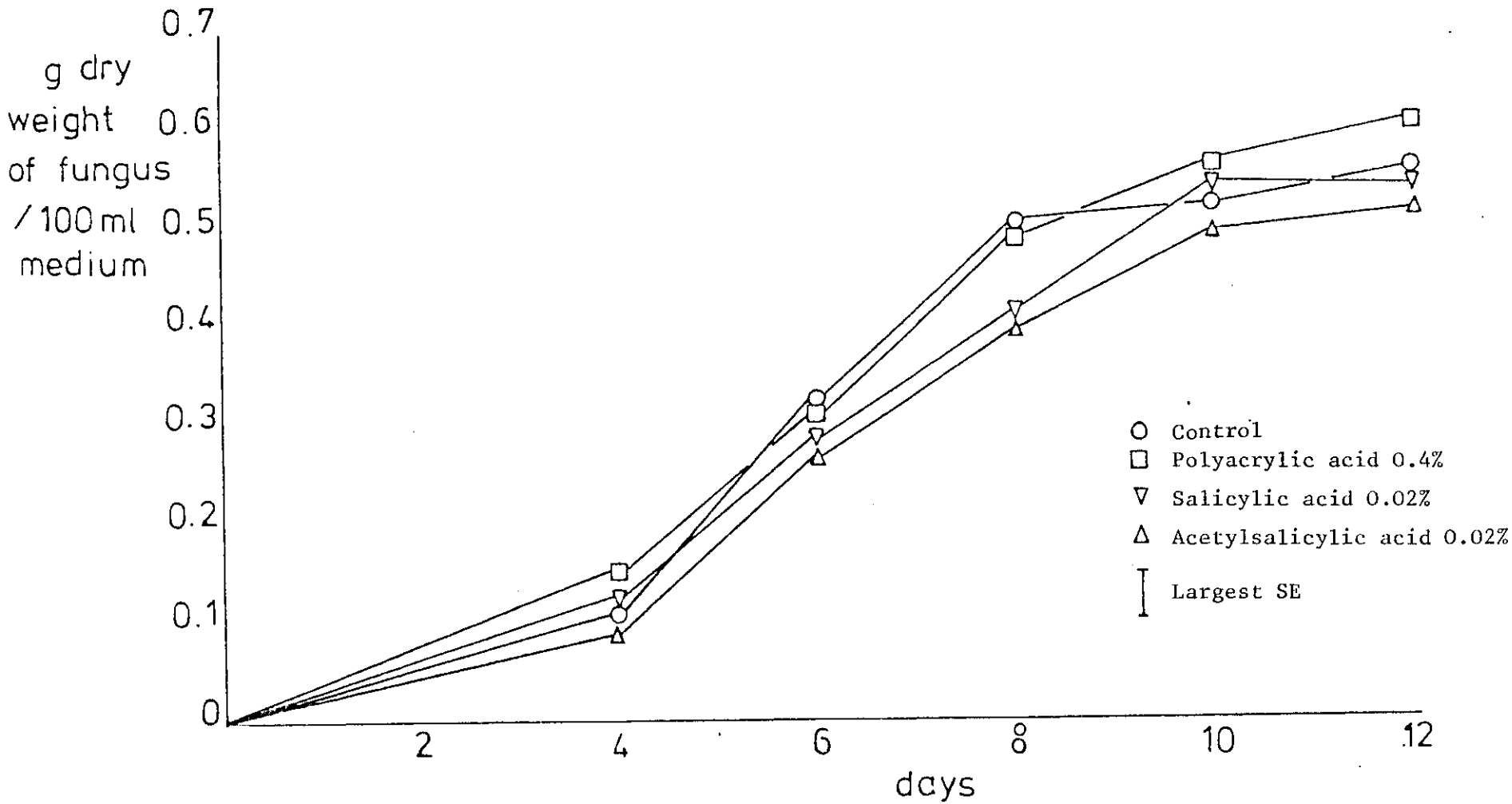
1. Injection of cotyledons with polyacrylic acid, acetylsalicylic acid and salicylic acid gave reduction in lesion number (up to 100%) and lesion size when challenge inoculated with C. lagenarium 96 hours later.

2. Challenge inoculations of cotyledons 24 hours after injection of the above chemicals had no effect on lesion size or number.

3. Uptake through roots for the life of the plant (grown in vermiculite) induced slightly higher levels of resistance but was phytotoxic causing stunting and marginal chlorosis.

4. Test substances sprayed on to leaves, watered on to plants in soil, used to soak germinating seeds or absorped through cut petioles were ineffective methods of inducing resistance to C. lagenarium.

FIGURE 2.1 Effects of test substances on growth of C. lagenarium in liquid medium



Results means of 3 replicates/treatment

5. Injection was the most efficient method of application.

3. Effects of test substances on C. lagenarium in vitro

To determine whether these substances were themselves acting in a fungitoxic manner, C. lagenarium was grown in liquid culture (100 mℓ in 250 mℓ Erlenmeyer flasks) containing 1% carbon source, incubated at 24-25°C on an orbitol shaker at 100 rpm with addition of test substance at concentrations used for cotyledon injection in earlier experiments.

Culture media (3 replicates per treatment) were seeded with a suspension of C. lagenarium spores at c 1 x 10^6 $m\ell^{-1}$. Fungus was harvested at intervals and dry weight measured after drying in an oven at 60°C until constant weight.

FIGURE 2.1 shows that, at the concentration used, polyacrylic acid (0.2%) had no effect on the growth of C. lagenarium. Acetylsalicylic acid and salicylic acid (both at 0.02%) caused a very slight reduction.

This suggests that none of the three test substances acts directly on C. lagenarium when injected in to cucumber cotyledons.

4. Systemic induction of resistance

To determine whether the induction of resistance was systemic, phosphate buffer or test substances in buffer were injected into only half the cotyledon of 15 day old cucumber plants. Plants were kept in humidity chambers for 2 days and 4 days after injection, the injected and non-injected halves were challenge inoculated with 3 μℓ drops of a suspension of C. lagenarium spores (1 x 10^5 $m\ell^{-1}$).

Lesion size and number were noted after 6 and 8 days (Table 2.5).

In addition, cucumber plants with fully expanded cotyledons and first leaves were injected with phosphate buffer or test substances in phosphate buffer. Plants were kept in humidity chambers for 2 days. Disks were cut from the first and second leaves seven days after injection and placed in 9 cm petri dishes on moist filter paper. One 3 $\mu\ell$ drop of C. lagenarium was then placed on each disk.

Lesion size and number were noted after 5, 7 and 9 days (Table 2.6).

Table 2.5 shows that injecting cotyledons with phosphate buffer (0.01 M) increases the susceptibility of cucumber tissue to subsequent inoculation by C. lagenarium.

Injecting half cotyledons with polyacrylic acid, acetylsalicylic acid and salicylic acid gave a reduction in lesion size between 41-59% and lesion number between 44-50% as measured after 6 days. Reduction, in adjacent non-injected tissue, in lesion size varied between 30-52% and in lesion number between 21-56%.

When measured after 8 days, more lesions had developed on non-injected halves on all 3 treatments, but reduction compared with buffer injected controls for polyacrylic acid, acetylsalicylic acid and salicylic acid was 44%, 21% and 4% respectively.

Table 2.6 shows the effects of injection of cotyledons on lesion development on first and second leaves. Polyacrylic acid and salicylic acid both caused slight, but significant, reduction in lesion size in

Table 2.5: Effect of injection of half cotyledons on lesion development on the same and adjacent half

Treatment	Concentration	Cotyledon half	6 Days[a]		8 Days[a]	
			LD[b]	LN[c]	LD	LN
Control (Phosphate buffer)	0.01 M	injected	3.49	0	4.54	0
		non-injected	2.57	0	3.36	0
Polyacrylic acid	0.4%	injected	2.07	44	2.98	22
		non-injected	1.81	56	1.97	44
Acetylsalicylic acid	0.02%	injected	1.50	48	2.53	50
		non-injected	1.23	38	1.88	21
Salicylic acid	0.02%	injected	1.40	50	2.16	42
		non-injected	1.46	21	2.44	4

a Days after challenge inoculation

b Mean lesion diameter

c % of inoculated sites not producing lesions

Results means of 24 lesions/4 plants/ treatment

All treatment lesion diameters are significantly different to PO_4^- control by at least 0.01% probability

Table 2.6: Effect of cotyledon injection on lesion development on first and second leaves

Treatment	Concentration	Effect on leaf number:-	5 Days[a] LD[b]	5 Days[a] LN[c]	7 Days[a] LD	7 Days[a] LN
Control (Phosphate buffer)	0.01 M	1	3.03	0	3.78	0
		2	3.19	0	3.94	0
Polyacrylic acid	0.4%	1	3.39	0	4.12	0
		2	2.21[d]	13	2.96[d]	0
Acetylsalicylic acid	0.02%	1	2.89	0	3.86	0
		2	2.87	12.5	3.40	0
Salicylic acid	0.02%	1	2.91	14	-	-
		2	2.23[d]	0	2.76[d]	0

a) b) c) See footnotes under previous table

d Significantly different from control at 0.05% level

Results means of 24 lesions/6 plants/treatment

second leaves but not in first leaves. Polyacrylic acid and acetylsalicylic acid caused a small reduction in the number of lesions developing on the second leaf and salicylic acid caused a similar drop on the first leaf.

5. Gel electrophoresis of soluble proteins

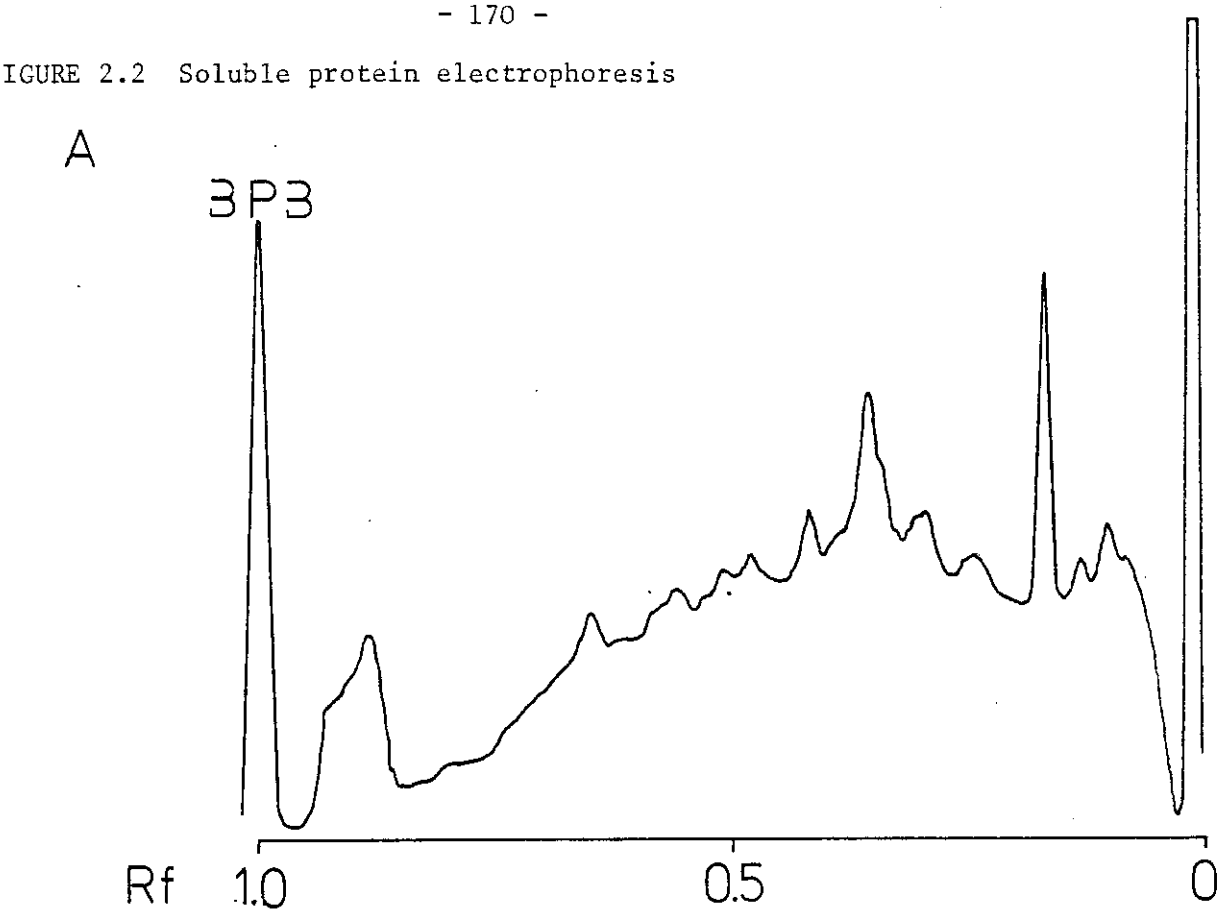
To assess the effects of injection of cotyledons with test substances on the soluble protein fraction in cucumber, proteins were extracted and electrophoresed as follows:-

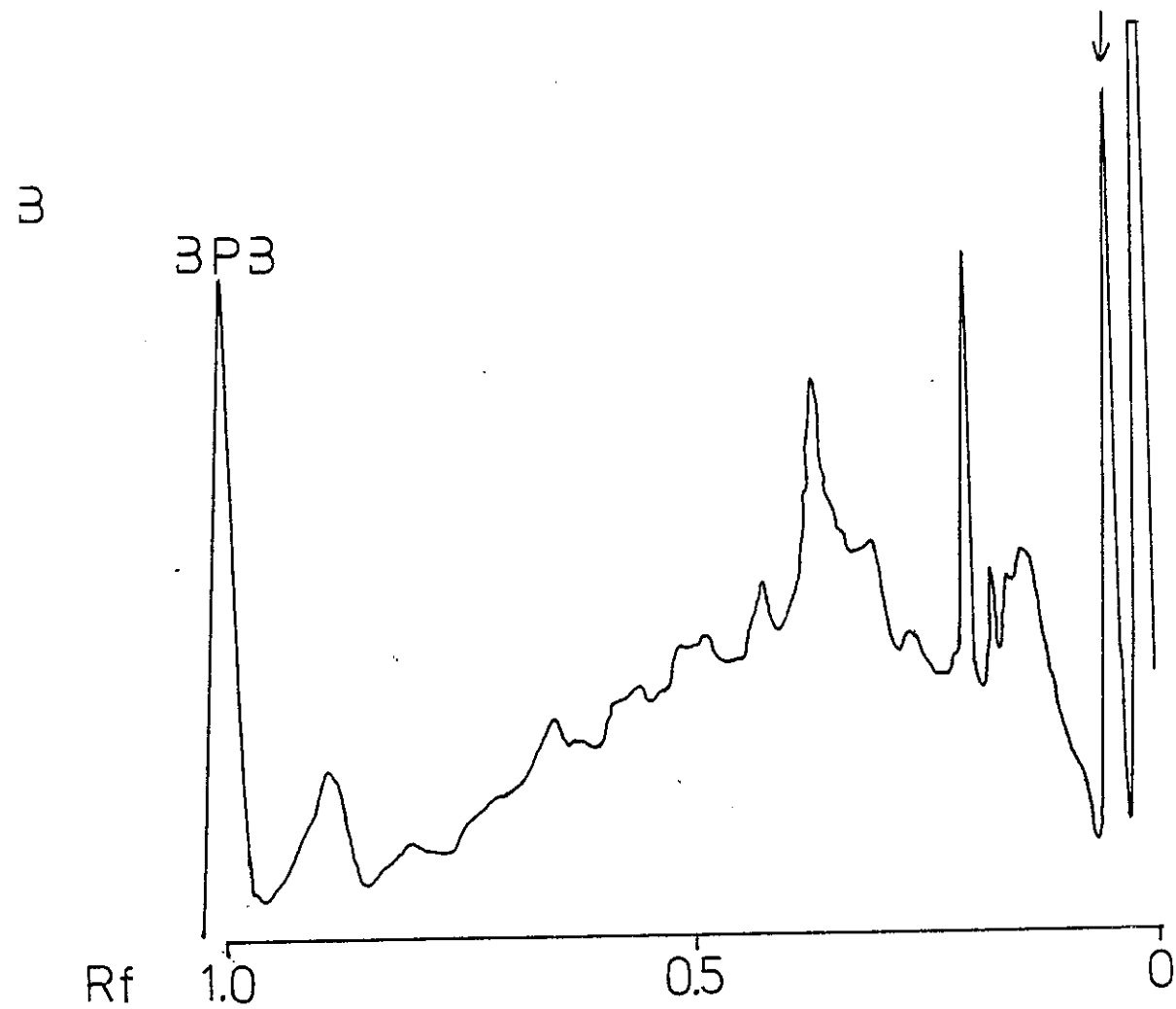
Cucumber cotyledons were injected with phosphate buffer (0.01 M pH 6.8) or phosphate buffer containing polyacrylic acid (0.4%), acetylsalicylic acid (0.02%) or salicylic acid (0.02%). Plants were kept in a humidity chamber for 2 days then in a growth cabinet at 25°C. Cotyledons were sampled after 24 and 96 hours by grinding the tissue in a mortar at 4°C in 0.1 M phosphate/citrate buffer pH 2.8 containing 0.5 M sucrose and 0.3% (V/V) mercaptoethanol. Extracts were centrifuged at 45,000 g for 30 minutes and the supernatant fluid used immediately for disc-electrophoresis (see Materials and Methods) in cylindrical 10% acrylamide gels with bromophenol blue as a marker.

Gels were stained with Coumassie blue for 24 hours and detained in 10% acetic acid. The gels were then scanned in a spectrophotometer at 280 nm.

FIGURES 2.2-2.7 show that the soluble protein pattern was similar for control treated cotyledons and test substance treated cotyledons after 24 hours and, more importantly, after 96 hours. This suggests that induced resistance, which is apparent 96 hours after injection, is not due to the formation of novel protein.

FIGURE 2.2 Soluble protein electrophoresis





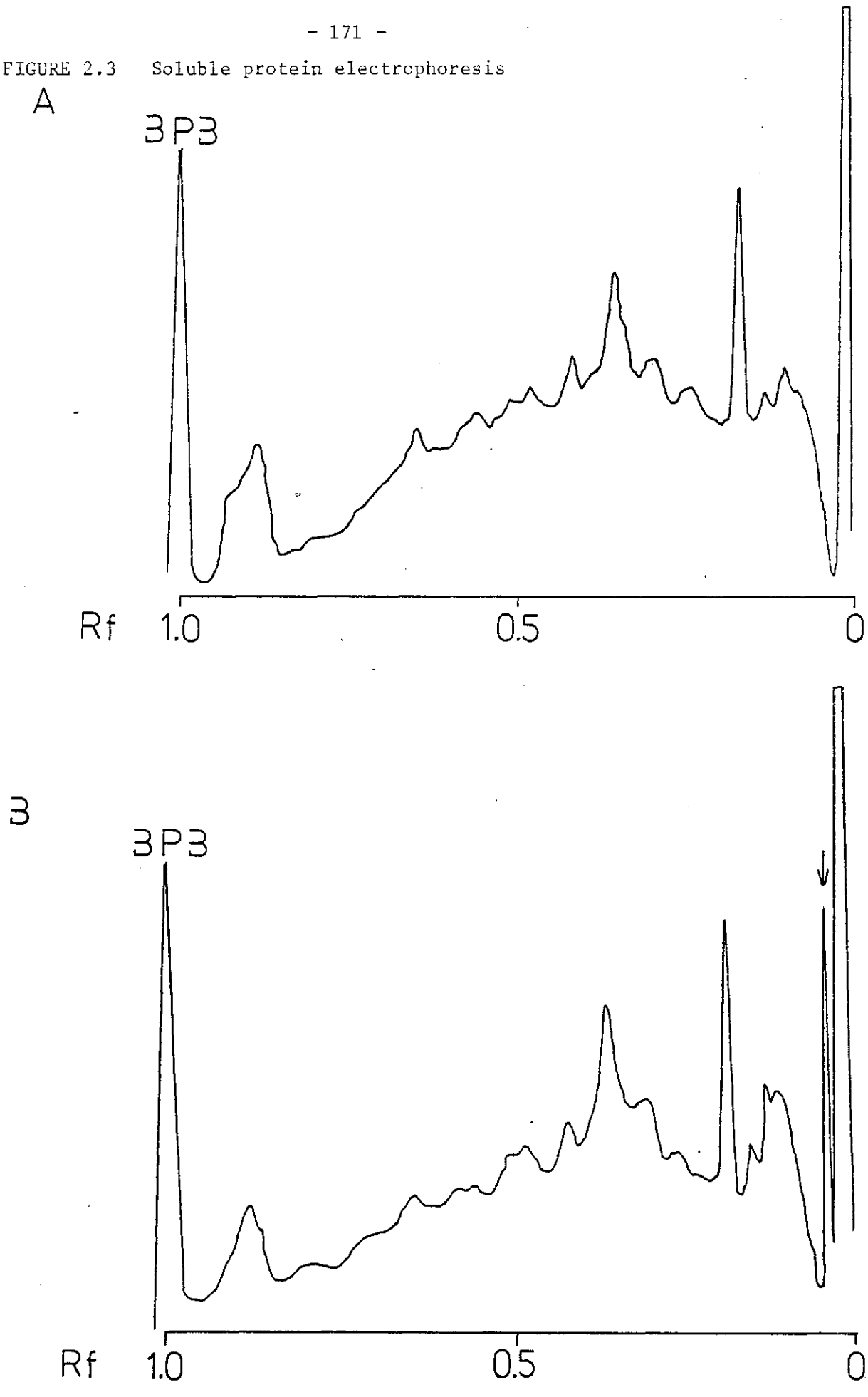
A Proteins from PO_4^- injected cotyledons after 1 day

B Proteins from acetylsalicylic acid injected cotyledons after 1 day

BPB = Bromophenol blue marker

↓ = New protein

FIGURE 2.3 Soluble protein electrophoresis



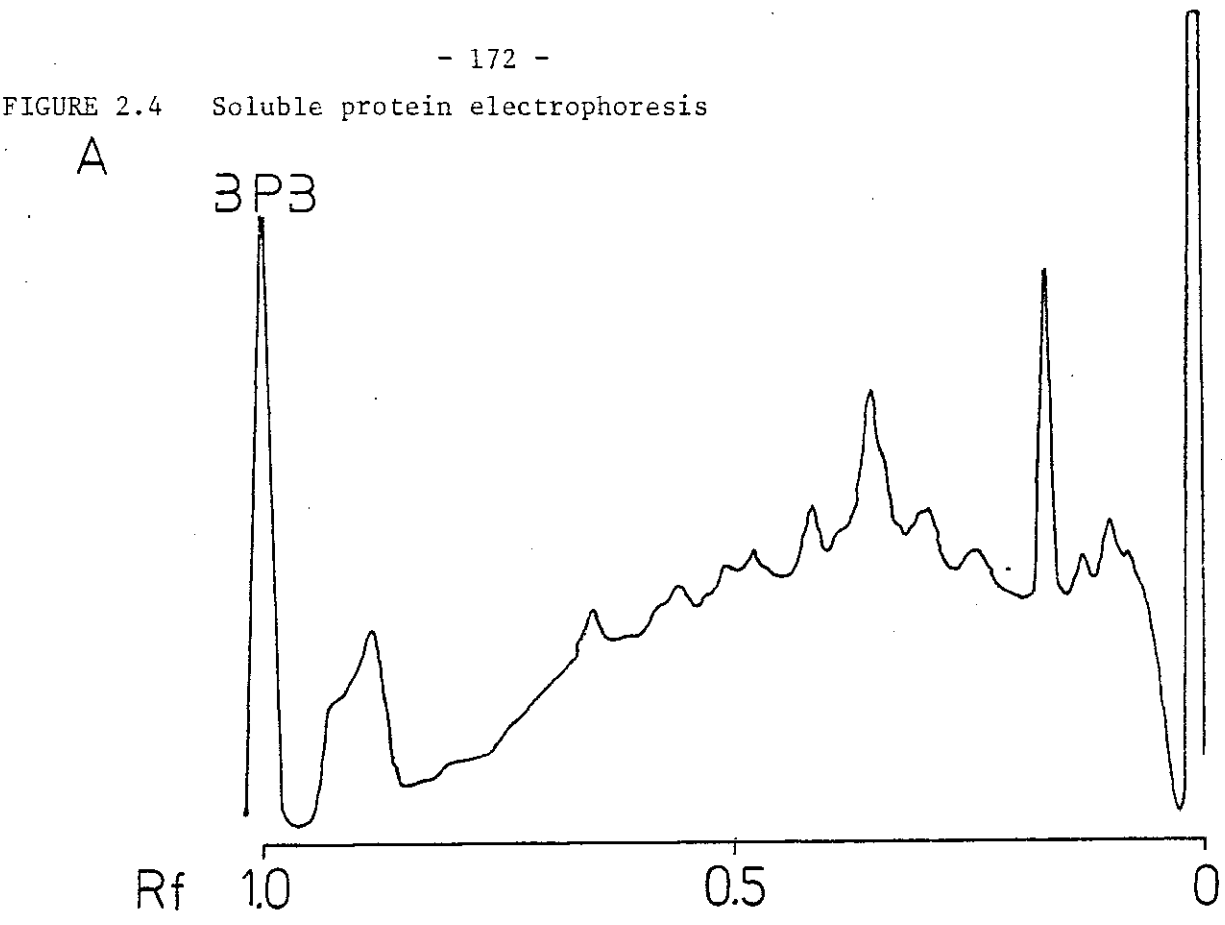
A Proteins from PO_4^- injected cotyledons after 1 day

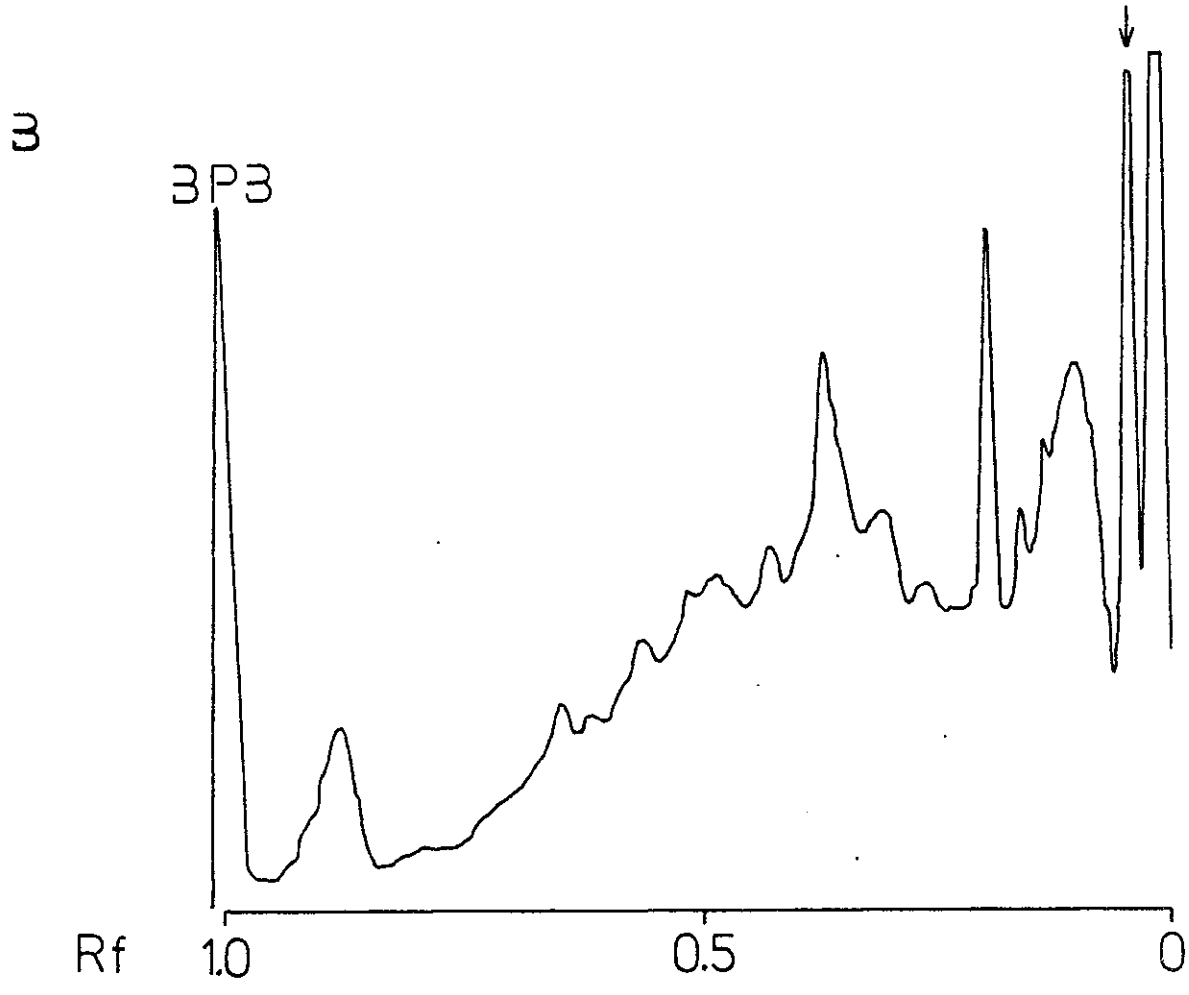
B Proteins from polyacrylic acid injected cotyledons after 1 day

BPB = Bromophenol blue marker

↓ = New protein

FIGURE 2.4 Soluble protein electrophoresis





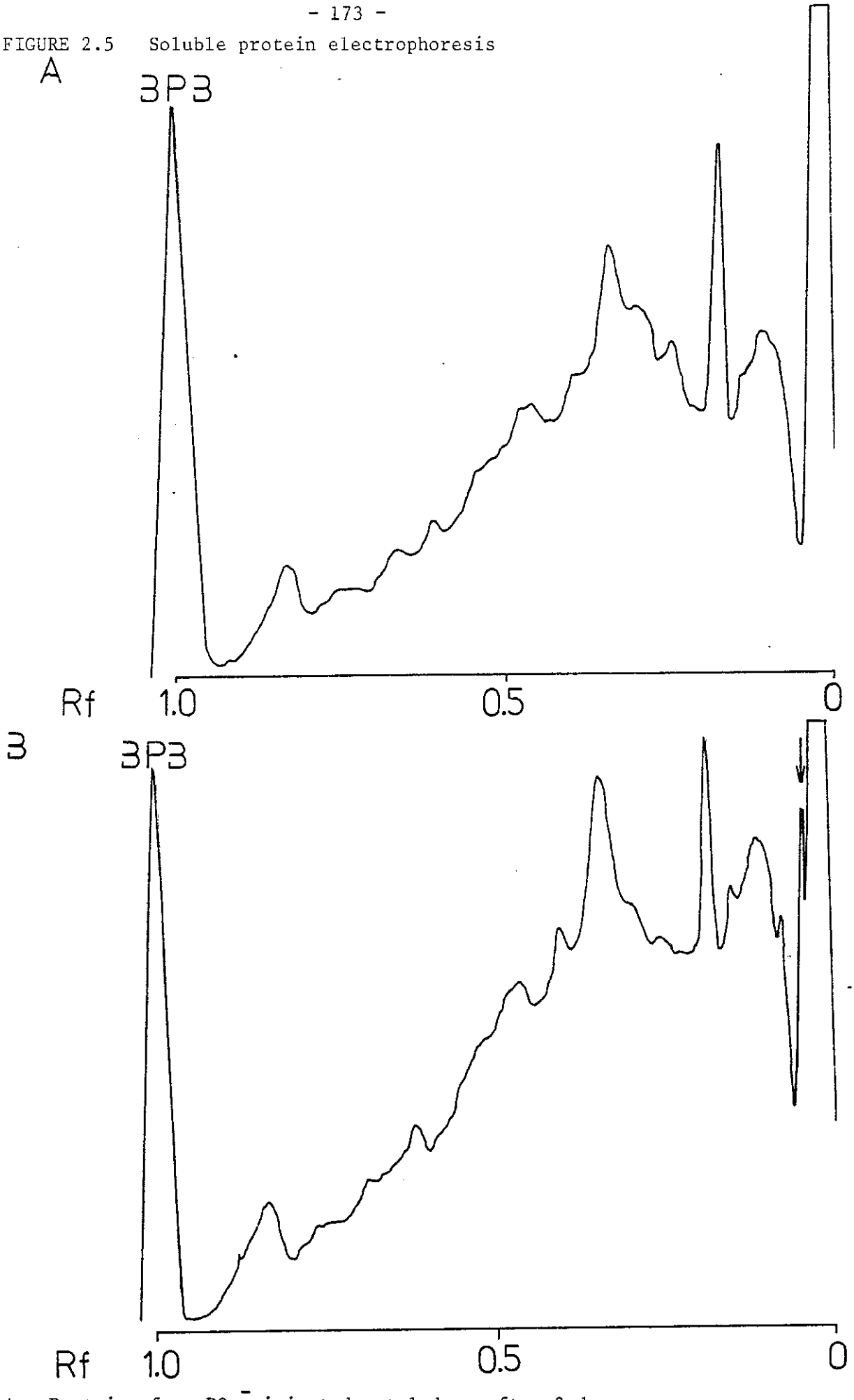
A Proteins from PO_4^- injected cotyledons after 1 day

B Proteins from salicylic acid injected cotyledons after 1 day

BPB = Bromophenol blue marker

↓ = New protein

FIGURE 2.5 Soluble protein electrophoresis



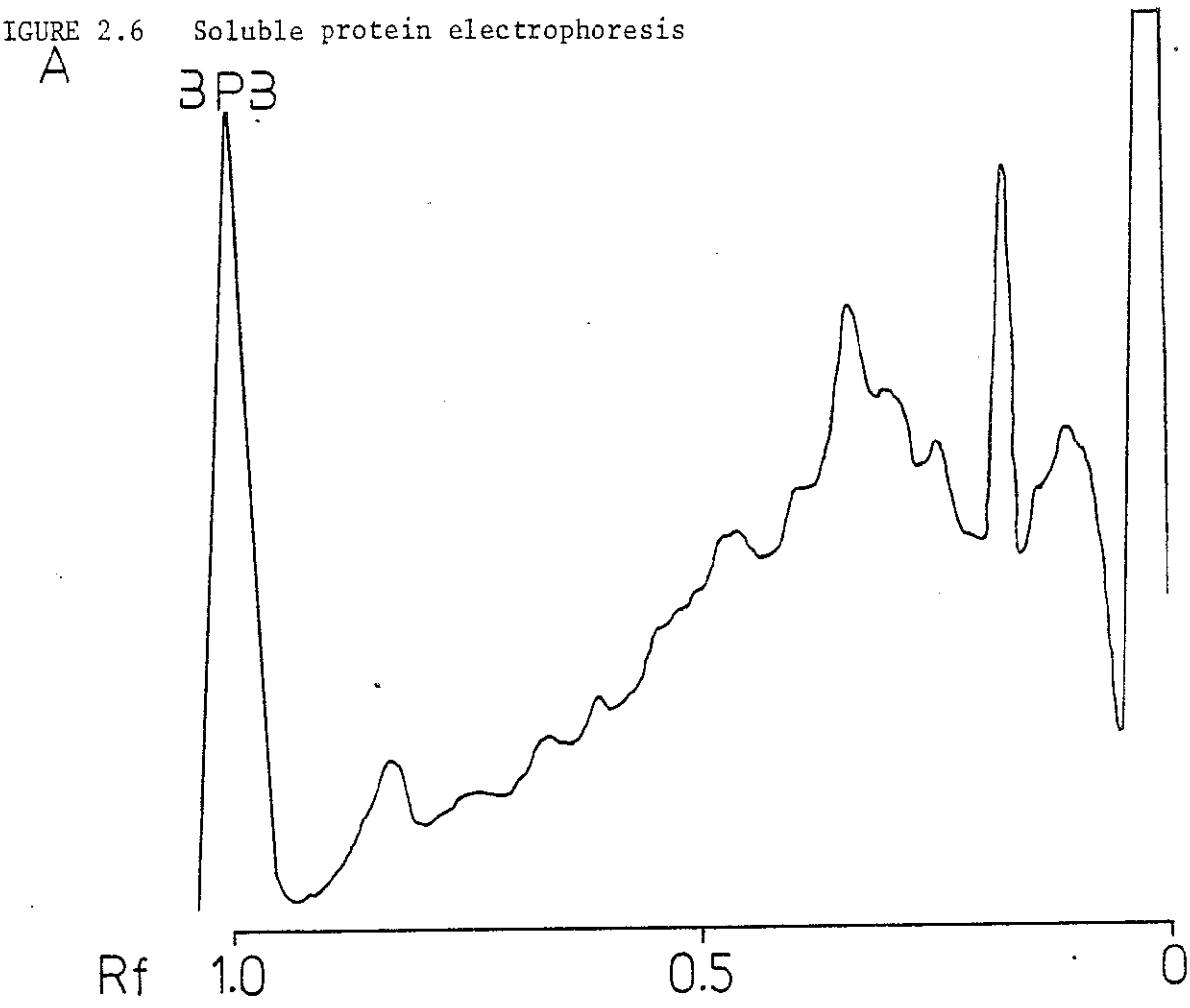
A Proteins from PO_4^- injected cotyledons after 3 days

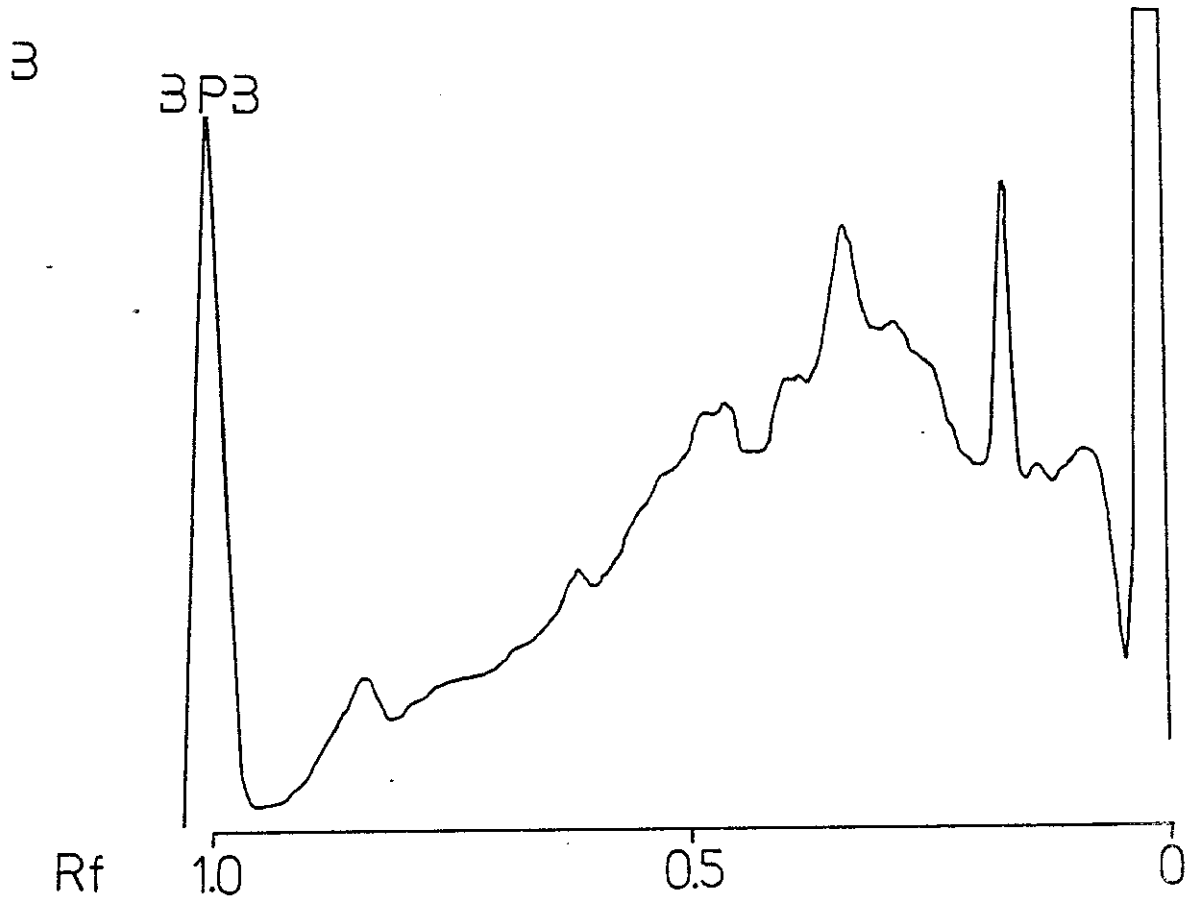
B Proteins from acetylsalicylic acid injected cotyledons after 3 days

BPB = Bromophenol blue marker

↓ = New protein

FIGURE 2.6 Soluble protein electrophoresis



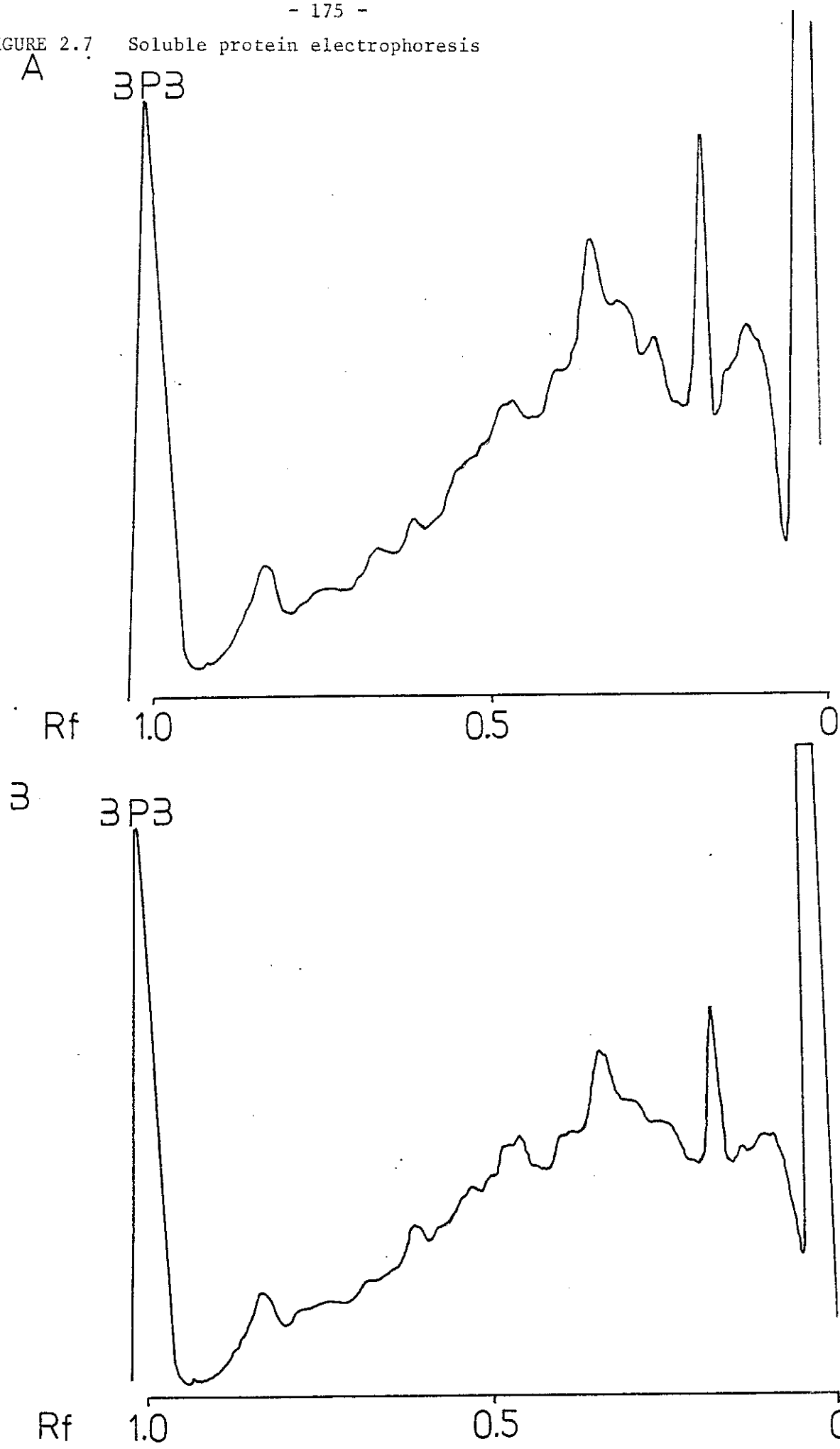


A Proteins from PO_4^- injected cotyledons after 3 days

B Proteins from polyacrylic acid injected cotyledons after 3 days

BPB = Bromophenol blue marker

FIGURE 2.7 Soluble protein electrophoresis



A Proteins from PO_4^- injected cotyledons after 3 days

B Proteins from salicylic acid injected cotyledons after 3 days

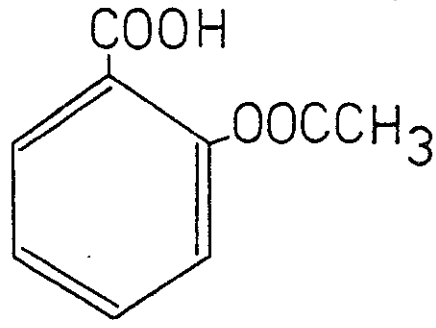
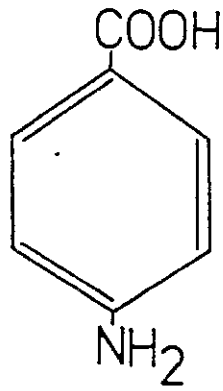
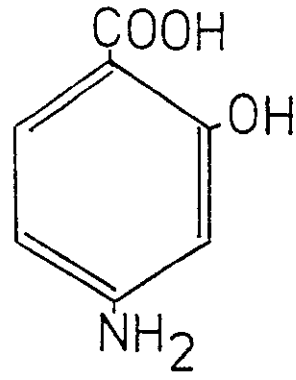
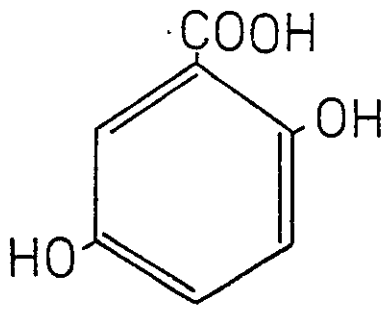
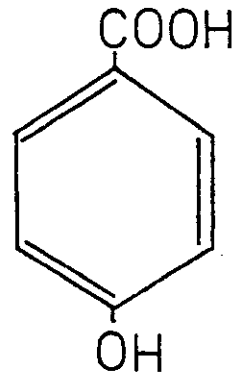
BPB = Bromophenol blue marker

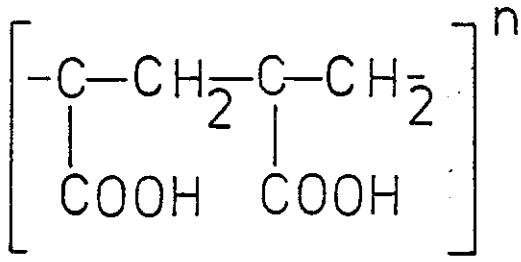
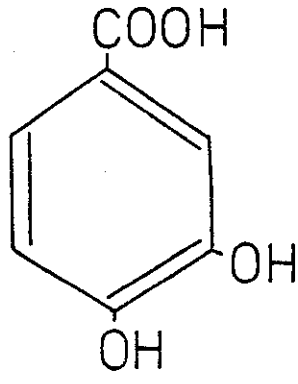
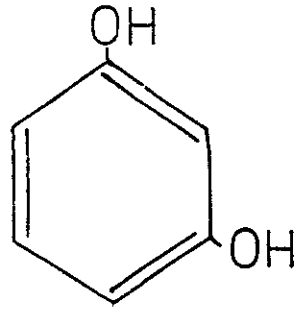
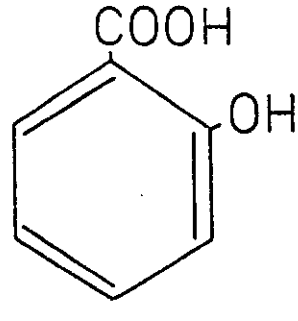
6. Induction of accumulation fungitoxic compounds

To determine whether test substances were either inducing fungitoxic compounds, or were themselves being converted into such compounds, ethanol extracts from injected cotyledons were prepared as in Materials and Methods. Extracts were spotted on to t.l.c. plates and run in chloroform: methanol (25:1), as described by Andebrhan (1978).

The chromatograms were assayed for fungitoxic compounds with a heavy suspension of spores of C. lagenarium applied in the same medium as used for liquid culture (see Materials and Methods).

Phosphate buffer injected controls showed two main inhibition zones at Rf 0.23 and 0.86. Three fainter zones were noted between these. Cotyledons injected with polyacrylic acid, acetylsalicylic acid and salicylic acid all showed similar chromatographic patterns. No extra zone or zone amplification was noted.

	% reduction in lesion number[a]
 acetylsalicylic acid	79
 4-aminobenzoic acid	21
 4-aminosalicylic acid	23
 gentisic acid	33
 4-hydroxybenzoic acid	6

		% reduction in lesion number[a]
	polyacrylic acid	93
	protocatechuic acid	31
	resorcinol	27
	salicylic acid	48

a Reduction in lesion number on cucumber cotyledons, injected 96 hours before challenge inoculation, as measured 9 days after inoculation. Figure taken from Table 2.1.

DISCUSSION II

Polyacrylic acid (PA), acetylsalicylic acid and salicylic acid are not inhibitors of C. lagenarium when present in liquid shake culture at the concentrations used in these experiments. The substances have no effect on lesion size or number when injected 24 hours prior to inoculation with C. lagenarium, but do so when injected 96 hours before inoculation. Inhibition of lesion formation, in some experiments, was 100% compared to phosphate buffer injected controls and was routinely found to be between 60-80%.

This compares well with the findings of Gianinazzi and Kassanis (1974) who found that PA, injected into tobacco cv Xanthi-nc, induced complete resistance to tobacco mosaic virus when applied 2-3 days before virus inoculation but had no effect when injected some hours before or after inoculation. The same workers reported considerable variation in the ability of certain high molecular weight polymers of PA to induce resistance, apparently due to age differences of the host.

White (1979) reported that injection of tobacco cultivar Xanthi-nc with solutions of acetylsalicylic acid, salicylic acid and benzoic acid, 2-3 days prior to inoculation with tobacco mosaic virus, significantly increased the resistance to that virus.

Application of test substances by placing cut petioles in solutions, watering on to pots or spraying leaves had very little effect on subsequent lesion development. This contrasts with the work of Kassanis and White (1975) using PA, tobacco and tobacco mosaic virus in which they reported up to 97% fewer lesions produced in PA sprayed

or watered plants after only one application. However, the concentrations of PA used were over 300 times more than the toxic limit for cucumber.

Induced resistance was obtained when test substances were watered on to roots of plants grown in LANS in vermiculite for the duration of the plant's life (c 4 weeks). Lesion number was reduced by 48, 81 and 69% for PA, acetylsalicylic acid and salicylic acid respectively, but test plants showed some signs of phytotoxicity, especially to PA, by slight leaf distortion and chlorotic leaf margins.

Injection of one half of a cucumber cotyledon with test substances induced resistance in the non-injected half. Lesion size and number were generally less in the injected half than in the non-injected half. This may be due to movement of the test substance after injection or to stimulation of an unknown factor thought to be involved in the systemic induction of resistance in cucumber second leaves following infection of the first. Gianinazzi and Kassanis (1974) injected half a tobacco leaf with PA but used the opposite half (water injected) as their control so it is not possible to determine whether the effect is mobile in tobacco.

In all cases where resistance has been induced by PA, acetylsalicylic acid or salicylic acid to viruses, resistance has been associated with the formation of new proteins. Gianinazzi and Kassanis (1974) showed that three additional soluble proteins appeared in PA injected leaves, but only in conditions in which resistance to infection was induced. White (1979) reported that PA does not induce resistance to tobacco mosaic virus or the formation of the so-called 'b'-proteins in the

tobacco cv Samsun NN or White Burley, but acetylsalicylic acid, salicylic acid and benzoic acid induce both resistance and the formation of new proteins in these cultivars.

Similar de novo synthesis of a protein (Rf 0.70) has been reported in cucumber following infection with viruses (Tas and Peters, 1977; Ziemiecki and Wood, 1975), and fungi (Andebrhan et al. 1980). Resistance induced as a result of injection of test substances was not accompanied by the formation of such a protein although all three test substances induced a high molecular weight protein 24 hours after injection (not coinciding with resistance). This protein had completely disappeared 3 days after injection of PA and salicylic acid, and only a trace was detectable in acetylsalicylic acid treated cotyledons. It is therefore thought unlikely that these test substances act on C. lagenarium by stimulating de novo synthesis of host proteins.

It is possible that these non-toxic test solutions could be converted into fungitoxic substances during the 3 day period between injection and inoculation. Ethanol extracts of injected cotyledons run on t.l.c. plates and assayed for fungitoxic compounds with a heavy suspension of spores of C. lagenarium showed no differences between test substance and phosphate buffer injected treatments.

A preliminary experiment on the effects of test substances on host enzymes has suggested that phenylalanine ammonia-lyase (PAL) activity was up to 100% higher in plants grown on vermiculite and watered with LANS containing either PA, acetylsalicylic acid or salicylic acid. These increases, if confirmed, are similar to those found in systemically protected second leaves following infection of first leaves with C. lagenarium, (see section D).

The effects of PA on virus infection of plants has been likened to the interferon mechanism in animals, in which a substance (the interferon) can induce an antiviral state which results in the slowing of virus replication, possibly by affecting the rate of synthesis of virus-coded proteins. New proteins produced in response to PA, acetylsalicylic acid and salicylic acid have been suggested to act as interferons to plant viruses (Gianinazzi and Kassanis, 1974; White, 1979). No such effect was found by Cassells et al. (1978) for tobacco mosaic virus replication in protoplasts from PA-treated plants showing a 60% reduction in lesion number. Spraying leaves with an anti-transpirant abolished PA-induced resistance and it was suggested that PA-induced changes in susceptibility are related to its effects on cell water relations, a less turgid cell being less susceptible to viral infection. In contrast, acetylsalicylic acid has itself been shown to have antitranspirant effects, closing stomata in Phaseolus vulgaris and Commelina communis (Larque-Saavedra, 1979) and would therefore appear to have the opposite effect to PA as suggested by the work of Cassells et al. (1978), yet both induce resistance in tobacco.

None of the above explanations can account for PA, acetylsalicylic acid or salicylic acid induction of resistance to C. lagenarium. The water status of cucumber leaves has no effect on infection by C. lagenarium (see section A) and no new proteins were detected.

In summary, it is unlikely that PA, acetylsalicylic acid or salicylic acid induced resistance of cucumber to C. lagenarium can be attributed to either the direct action of the test substances on the parasite or the production of fungitoxic derivatives within the plant. There is evidence that the test substances sensitise the host over a 3 day period

so that subsequent inoculation results in substantially less infection. Action of chemicals in this way is not common, although 2, 2-dichloro-3, 3-dimethyl cyclopropane carboxylic acid may exert its systemic fungicidal activity against the rice blast disease caused by Piricularia oryzae in this way (Cartright et al. 1977).

Section 3. Induction of resistance with 6-benzylaminopurine

Preliminary experiments by Andebrhan (1978) indicated that kinetin may have an effect on development of C. lagenarium lesions on cucumber.

To determine whether changes induced in cucumber leaves by cytokinins would be similar to those induced in systemically protected leaves, the following was done.

First true leaves of 15 day old cucumber plants were sprayed daily to run-off for 11, 6 or 3 days with 10, 1, 0.1 or 0 mg ℓ^{-1} of 6-benzyl-aminopurine (6-BAP), a substance with cytokinin-like activity.

Leaf disks were taken from the sprayed first leaves and unsprayed second leaves, placed on moist filter paper in 9 cm petri dishes and inoculated with one 3 $\mu\ell$ drop of a suspension of C. lagenarium spores (1×10^5 $m\ell^{-1}$) as in Materials and Methods.

As a control treatment, first leaves of plants of a similar age were inoculated with c. 100 3 $\mu\ell$ drops of a suspension of C. lagenarium spores (1×10^6 $m\ell^{-1}$) on all but a central 12 cm^2 circle of each leaf.

Leaf disks were cut from this central healthy 12 cm^2 and from second leaves of the same plants 9 days later and placed in petri dishes and inoculated as above.

Lesion size and number were noted after 10 days.

FIGURE 3.1 shows that prior inoculation of the first leaf reduces lesion

size by 60% on leaf disks taken from the central uninoculated area. Spraying leaves with 6-BAP at 10 mg ℓ^{-1} for 11 days also gave a 60% reduction in lesion size. 6-BAP sprayed at 10 mg ℓ^{-1} for 6 and 3 days and at 1.0 mg ℓ^{-1} for 6 and 3 days gave smaller reductions. 6-BAP at 0.1 mg ℓ^{-1} increased lesion size.

Reduction in lesion number only occurred in C. lagenarium treated first leaves. No reduction was noted for any 6-BAP treated leaves.

FIGURE 3.2 shows reductions in lesion size and number in second leaves for plants whose first leaves were inoculated with C. lagenarium or sprayed with 6-BAP. Reductions in both size and number occurred in plants where the first leaf was infected, but only small reductions in size occurred in second leaves in plants where the first leaves were sprayed with 6-BAP, and there was no reduction in lesion number.

To assess whether important changes induced in cucumber leaves by spraying with 6-BAP would be induced in systemically protected tissue, chlorophyll, protein, amino-acid and water soluble carbohydrate (WSC) contents were estimated.

6-BAP sprayed leaves, the healthy central 12 cm^2 of infected first leaves (as described earlier) and the second leaves of both treatments were used to estimate the following:-

a. Chlorophyll

Three 4 mm diameter leaf disks were taken from treated tissue and left overnight in 5 mℓ of methanol. Chlorophyll content was measured

directly on a Beckman model 35 spectrophotometer at 665 nm.

FIGURE 3.3 shows the chlorophyll content of disks taken from 6-BAP sprayed first leaves, disks from the central area of infected first leaves and disks from second leaves of plants with first leaves either sprayed or infected. The histograms show that 6-BAP sprayed on to cucumber leaves increases the chlorophyll content. The greatest increases were in leaves sprayed with the highest concentration of 6-BAP for the longest period (11 days). Disks taken from the healthy centre of infected leaves showed no increase.

Chlorophyll content in second leaves of first leaf 6-BAP treated plants also increased but mainly in plants where the first leaf had been sprayed with the highest concentration for the longest period. A small decrease was observed in several 6-BAP treatments as well as in second leaves of plants with infected first leaves.

b. Protein

Three 4 mm leaf disks were taken from treated plants and left overnight in 5 mℓ of methanol. The bleached leaf disks were then left overnight in 3 mℓ NaOH(IN) to solubilise the protein. Following a low speed spin the protein content was determined as the ratio of absorbances at 260 nm and 280 nm.

FIGURE 3.4 shows that spraying cucumber leaves with high concentrations of 6-BAP for the longer periods increases the protein content of disks subsequently taken from those leaves. Disks taken from the healthy centre of infected leaves showed a slight decrease in protein content.

Protein content in second leaves did not show similar trends.

c. Amino-acids

Three 4 mm diameter leaf disks were taken from leaves following the treatments outlined above and left overnight in 5 mℓ of methanol. 1 mℓ of methanol extract was used to estimate amino-acid content by the method of Yemm and Cocking (1954) (Table 3.1).

Table 3.1: Amino-acid content of 6-BAP sprayed or infected cucumber leaves

Treatment	Amino-acid content (units[b])	
	First leaf	2nd leaf
Control[a]	1	1
1st leaf sprayed with 6-BAP, 10 mg ℓ^{-1}, 11 days	1.00 ± 0.01	0.95 ± 0.06
1st leaf infected with *C. lagenarium*	0.98 ± 0.01	0.99 ± 0.1

a. Control plants sprayed with water.

b. 1 unit of amino-acid = amount of amino-acid in control plants.

Table 3.1 shows that amino-acid content does not vary significantly in plants sprayed with 6-BAP or infected with *C. lagenarium* compared with water treated control plants.

d. Water soluble carbohydrate (WSC)

Five 12 mm diameter disks taken from treated plants were homogenised in 15 mℓ water. The homogenate was centrifuged at 12,000 g for 10 min and the supernatant used for carbohydrate determination by the anthrone method (see Materials and Methods).

FIGURE 3.5 shows that WSC content increases in all 6-BAP sprayed leaves and the healthy centre of infected leaves compared with water sprayed control leaves. Largest increases in WSC were found in leaves sprayed with the highest concentration of 6-BAP for the longest period (11 days).

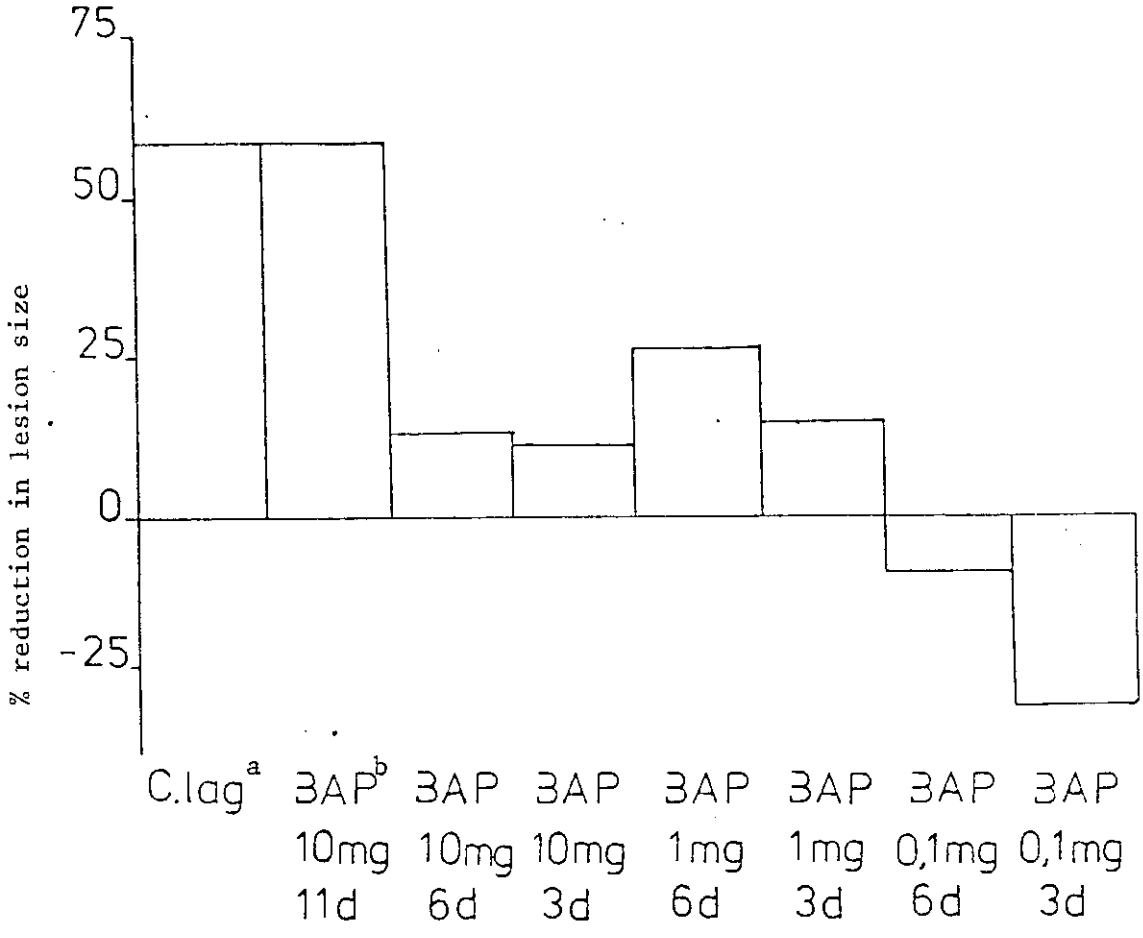
Increases were also noted in the second leaves of first leaf treated plants. These did not, however, relate well to concentration or duration of 6-BAP treatment of the first leaves.

To determine whether 6-BAP would inhibit the growth of C. lagenarium, the fungus was grown in 100 mℓ liquid medium (Sucrose 1.0 g; KH_2PO_4 0.1 g; $MgSO_4.7H_2O$ 0.05 g; Casein hydrolysate 0.46 g; mineral salt solution 0.1 mℓ) in 250 mℓ Erlenmeyer flasks containing 6-BAP at concentrations of 0-10 mg ℓ^{-1}.

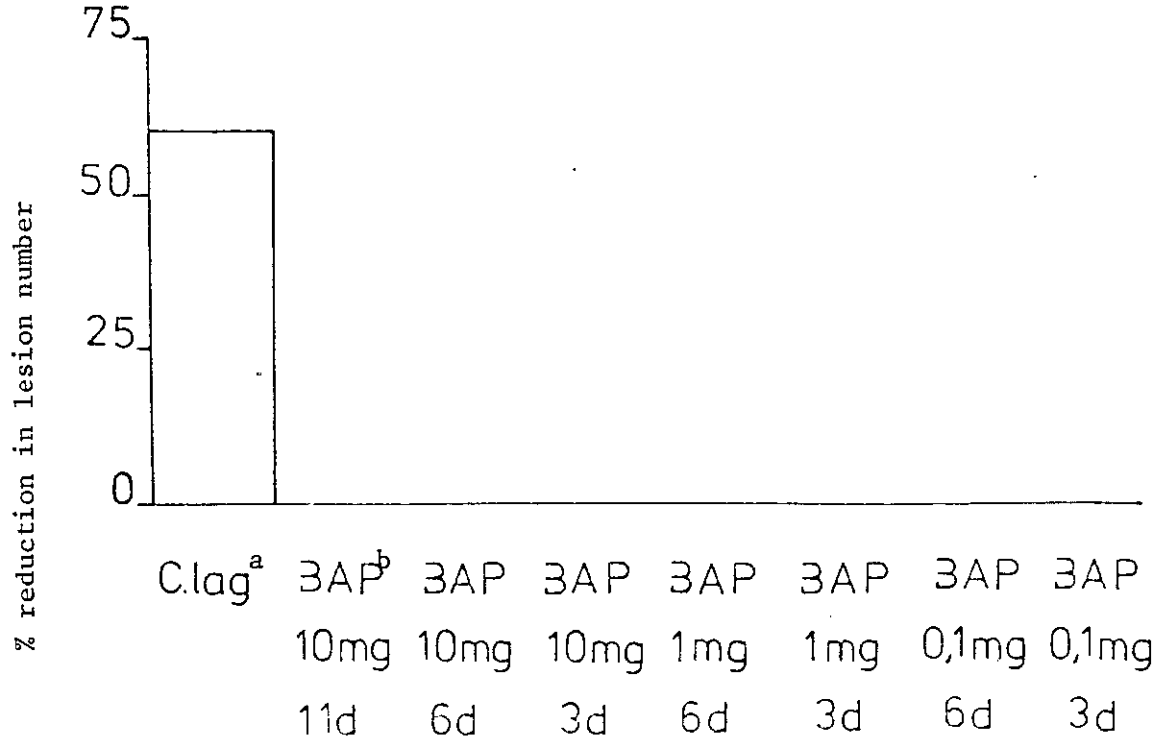
Table 3.2 shows the mean dry weight of 3 replicates for each concentration.

FIGURE 3.1: Effect of 6-BAP on lesion development on first leaves

a. Reduction in lesion size on disks taken from first leaves



b. Reduction in lesion number on disks taken from first leaves



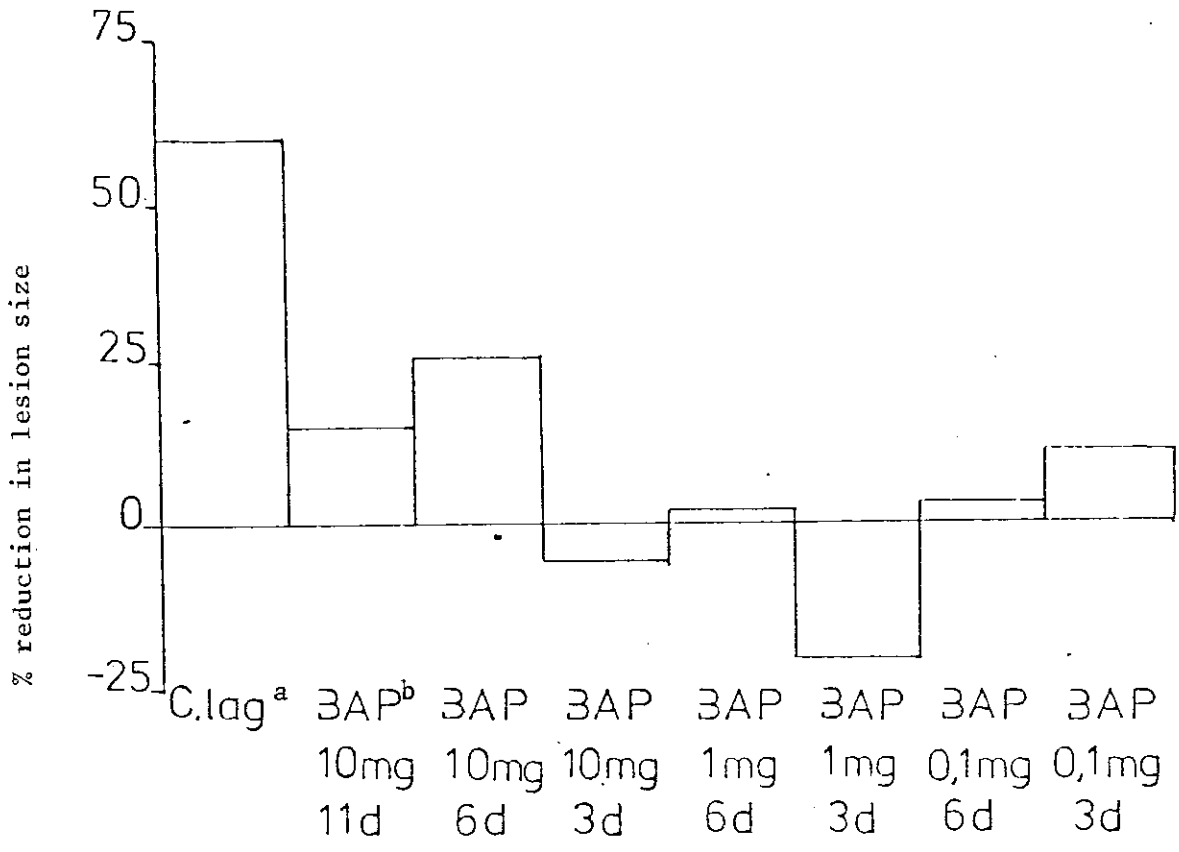
a. Disks taken from central healthy area of first leaves infected with C. lagenarium.

b. First leaves sprayed with 6-BAP at 10 mg ℓ^{-1} daily for 11 days

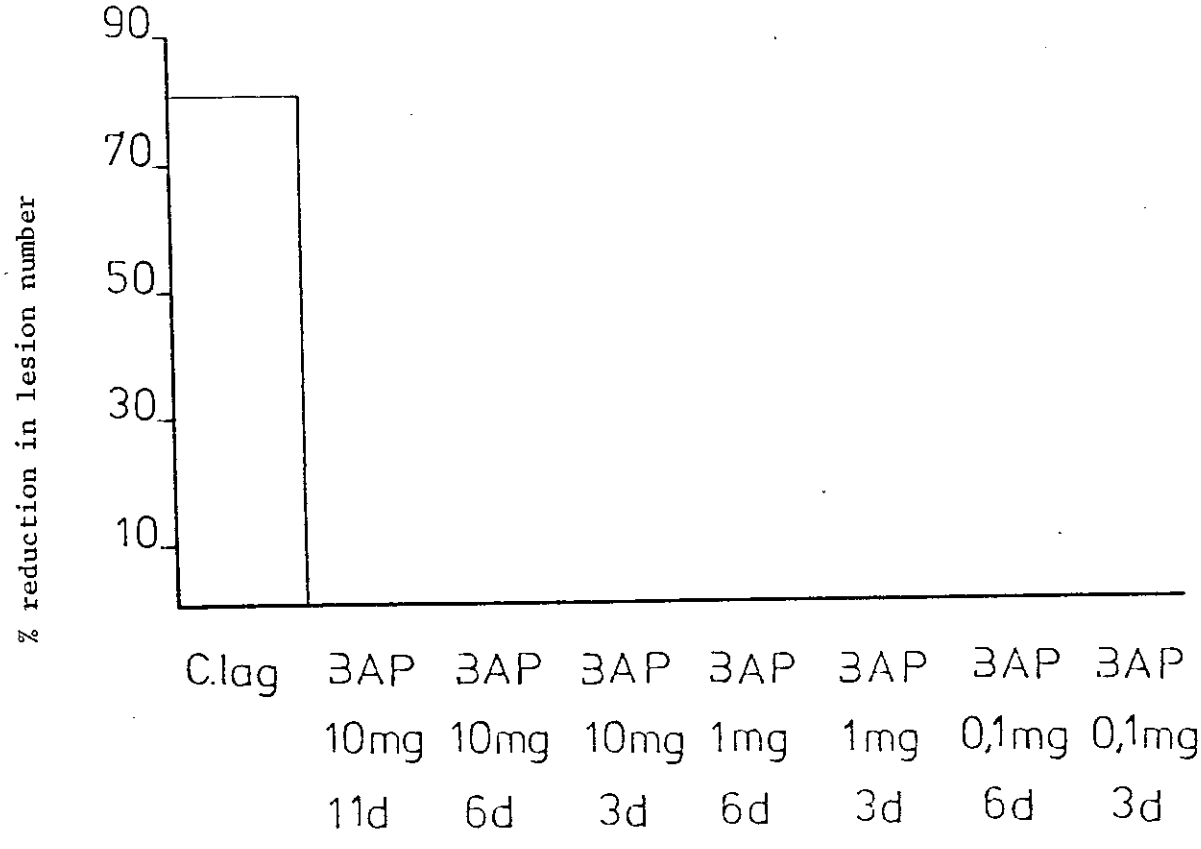
Results means of 20 disks/5 plants/treatment

FIGURE 3.2: Effect of 6-BAP treatment of first leaves on lesion development on second leaves

a. Reduction in lesion size on disks from second leaves



b. Reduction in lesion number on disks taken from second leaves



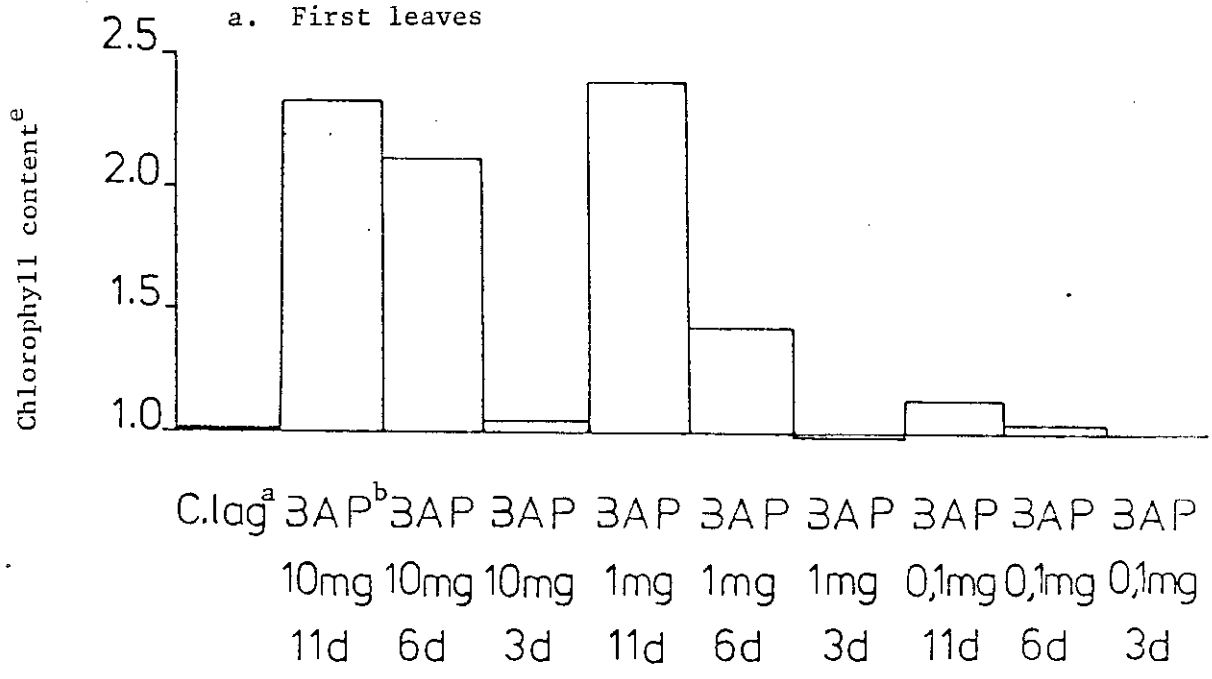
a. First leaves infected with C. lagenarium.

b. First leaves sprayed with 6-BAP, 10 mg ℓ^{-1} daily for 11 days.

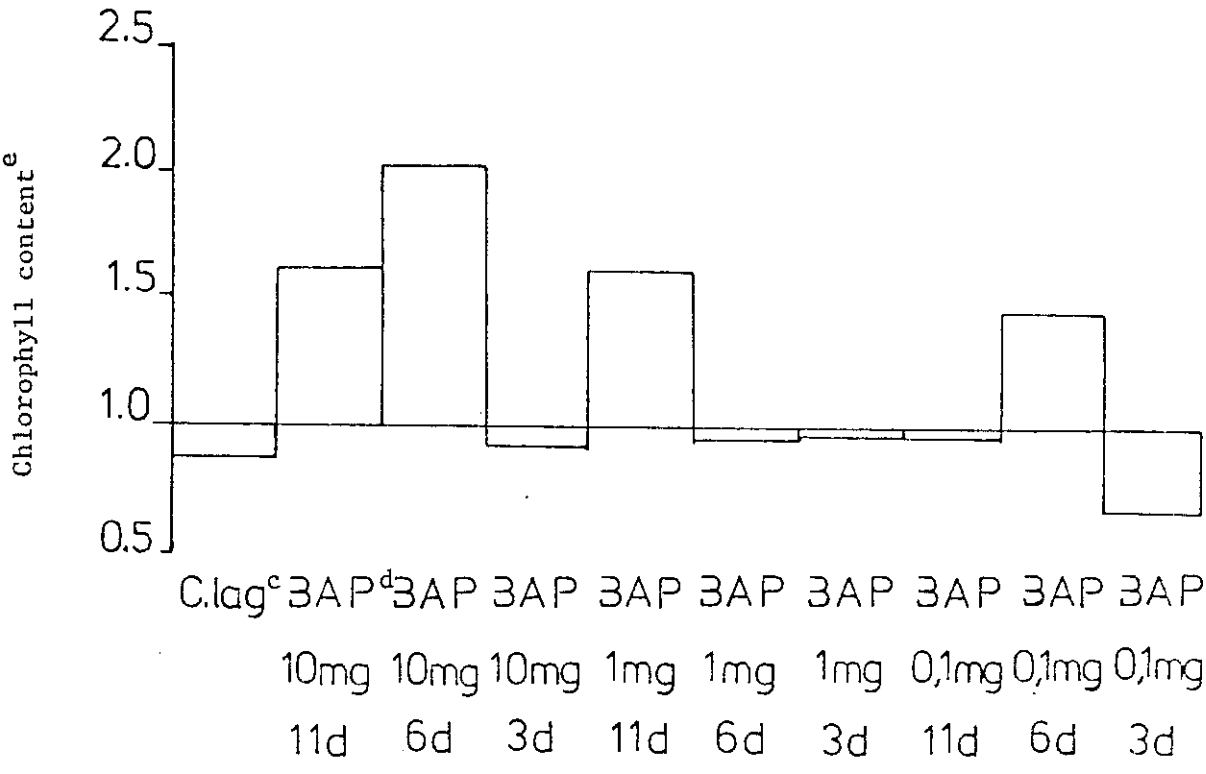
Results means of 20 disks/5 plants/treatment

FIGURE 3.3: Effect of spraying first leaves with 6-BAP on chlorophyll content of first and second leaves

a. First leaves



b. Second leaves



a. Three 4 mm disks taken from the central healthy area of a C. lagenarium infected leaf.

b. Three 4 mm disks taken from leaves sprayed with 6-BAP, 10 mg ℓ^{-1} daily for 11 days.

c. Three 4 mm disks taken from second leaves of plants where the first leaf was infected with C. lagenarium.

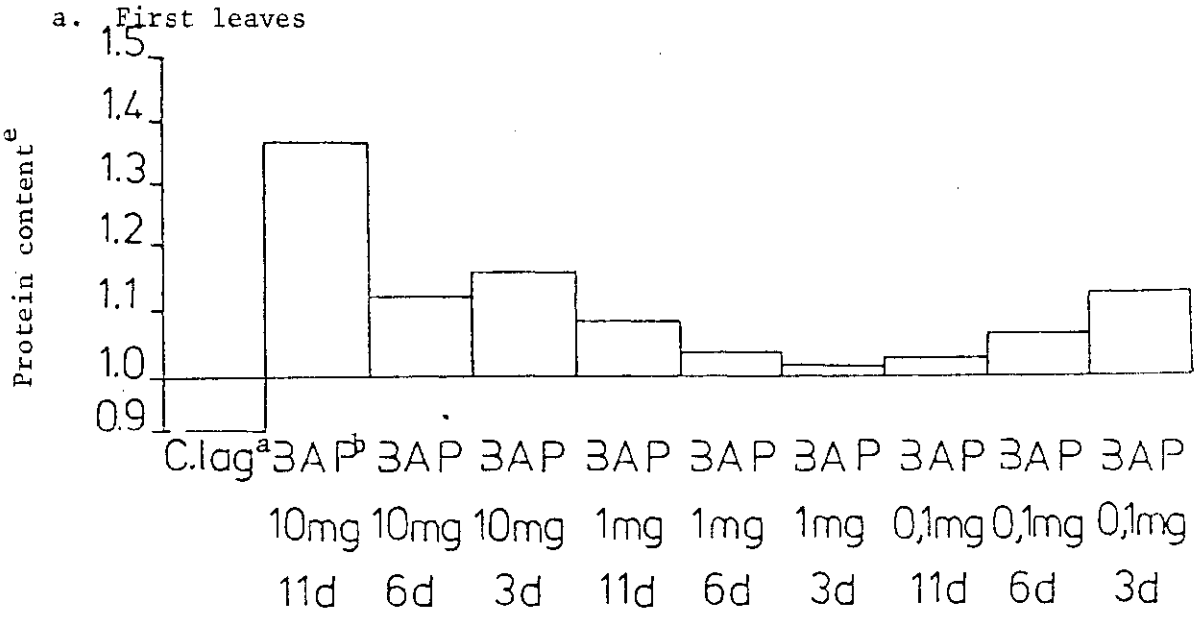
d. Three 4 mm disks taken from second leaves of plants where the first leaf was sprayed with 6-BAP, 10 mg ℓ^{-1} daily for 11 days.

e. Chlorophyll content assessed spectrophotometrically and expressed in comparison to water treated control leaves (control = 1).

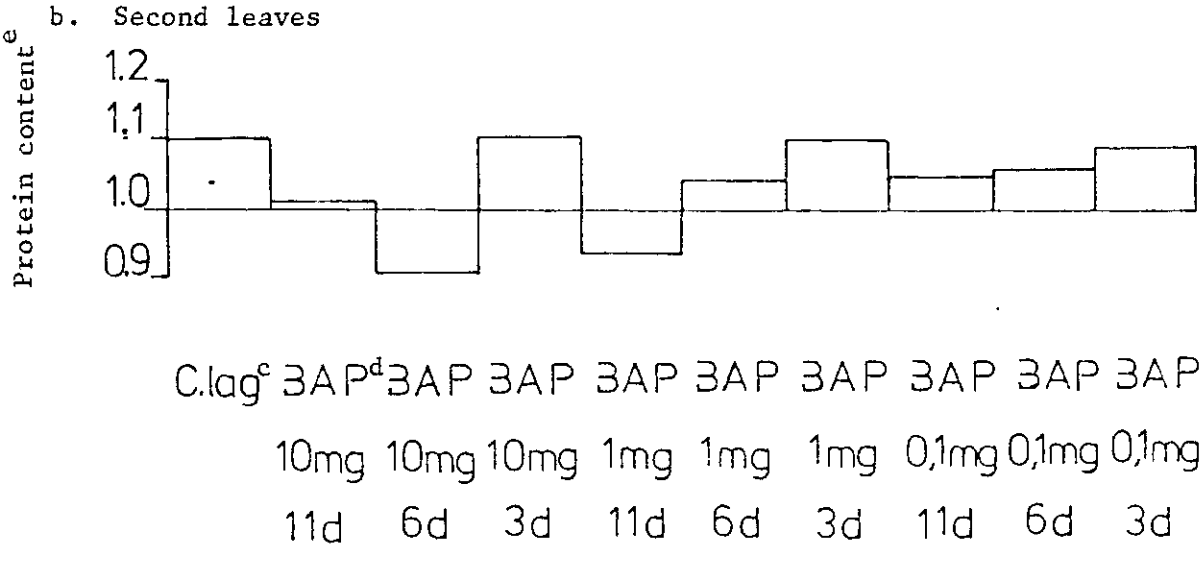
Results means of 3 replicates/treatment

FIGURE 3.4: Effect of spraying first leaves with 6-BAP on protein content of first and second leaves.

a. First leaves



b. Second leaves



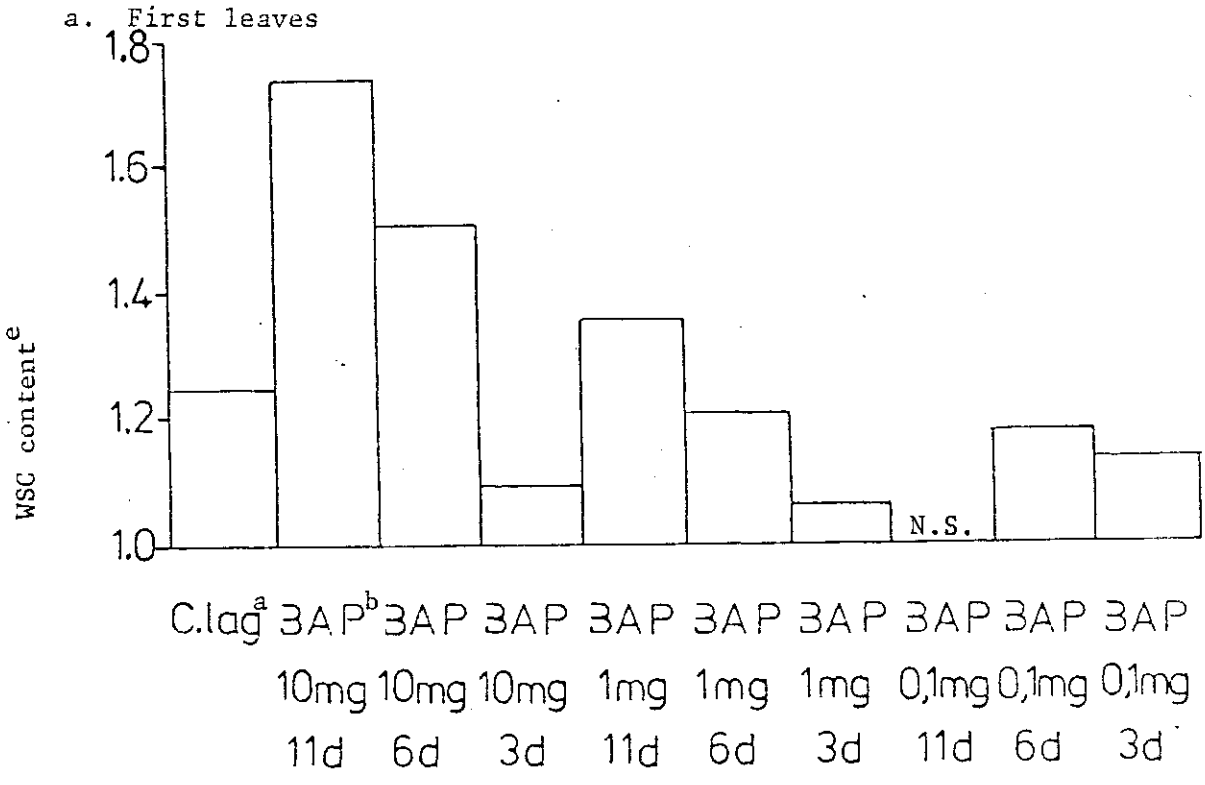
a.)
b.) See footnotes under figure 3.3
c.)
d.)

e. NaOH solubilised protein content estimated by the 260 nm and 280 nm absorbance and expressed in comparison to water treated control leaves (control = 1)

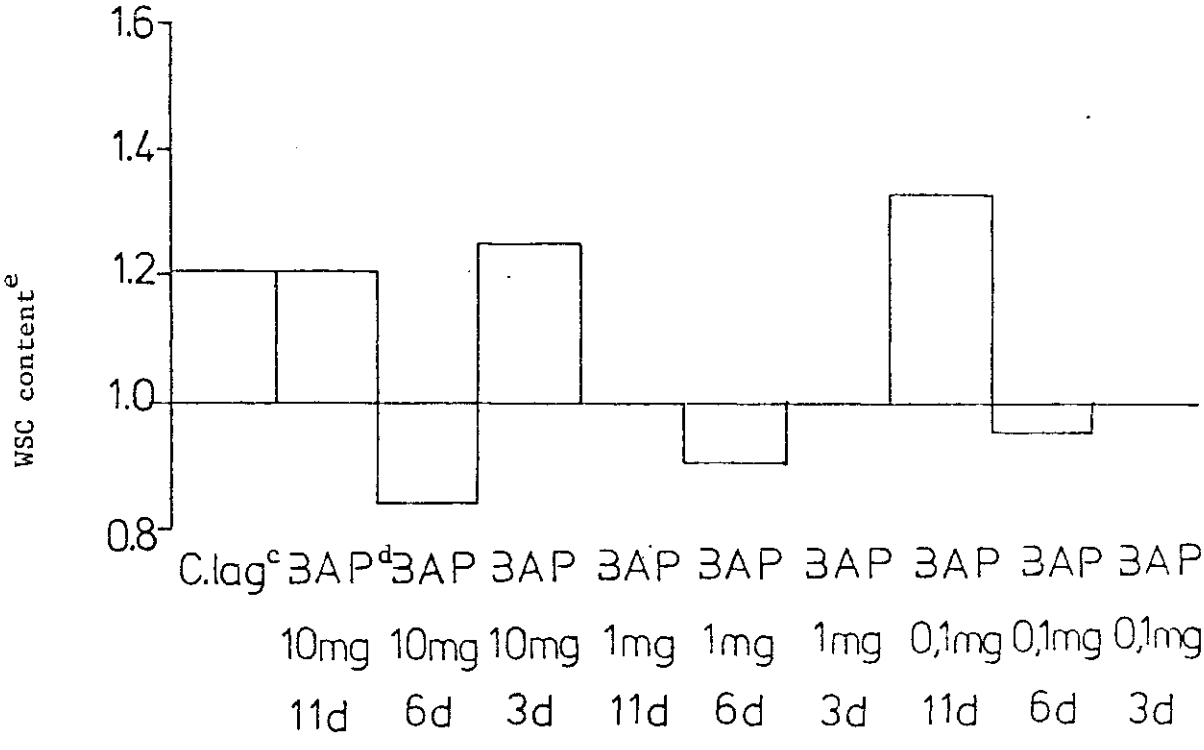
Results means of 3 replicates/treatment

FIGURE 3.5: Effect of spraying first leaves with 6-BAP on water soluble carbohydrate content (WSC) of first and second leaves.

a. First leaves



b. Second leaves



a.)
b.) See footnotes under FIGURE 3.3
c.) N.B. Five 12 mm disks used.
d.)

e. Water soluble carbohydrate content assessed using the anthrone assay and expressed in comparison to water treated control leaves (control = 1)
Results means of 3 replicates/treatment
N.S. = No sample taken

Table 3.2: Effect of 6-BAP on growth of C. lagenarium in liquid shake culture

Concentration of 6-BAP (mg ℓ^{-1})	Dry wt of fungus (mg)	S.E.
0	0.2544	0.057
0.1	0.3356	0.066
1.0	0.4077	0.055
10.0	0.3955	0.034

Figures means of 3 flasks per treatment

Table 3.2 shows that 6-BAP is not toxic to C. lagenarium in liquid shake culture at the concentrations used.

Summary

1. Number and size of lesions on challenged disks taken from healthy uninoculated central areas of inoculated first leaves decreased by c. 60%.

2. 6-BAP, 10 mg ℓ^{-1}, sprayed on first leaves for 11 days, decreased lesion size on first leaves by c. 60% but had no effect on lesion number.

3. Number and size of lesions on disks taken from second leaves of first leaf infected plants was decreased by c. 60% and c. 80% respectively.

4. Number and size of lesions on second leaves of first leaf 6-BAP sprayed plants were not decreased.

5. 6-BAP, 10 mg ℓ^{-1}, did not decrease growth of C. lagenarium in shake culture.

6. 6-BAP, 10 mg ℓ^{-1}, sprayed on first leaves for 11 days, significantly increased content of chlorophyll, protein and water-soluble carbohydrate in first leaves.

7. 6-BAP at all concentrations used, sprayed on first leaves, had no consistent effect on lesion size or metabolic products in second leaves.

DISCUSSION III

There are a considerable number of physiological responses attributed to application of cytokinins. Adding kinetin to dormant Spirodela oligorrhiza caused increased DNA, RNA and protein synthesis (McCombs and Ralph, 1972) and stimulation of the incorporation of amino-acids into plant proteins (Osborne, 1962; Anderson and Rowan, 1966). In some cases increases in protein synthesis appear to be preceded or accompanied by increased RNA synthesis (Letham, 1967). Local application of kinetin to detached leaves not only delays senescence at the treated sites but also promotes the movement of many metabolites to those sites (Kende, 1971; Müller and Braütigam, 1973). Pretreatment of etiolated cucumber cotyledons with benzyladenine increases the amount of chlorophyll produced in the light (Fletcher and McCullagh, 1971) caused by inducing production of proteins including δ-amino-levulinic acid synthetase, the rate limiting enzyme in chlorophyll synthesis.

Cytokinins have also been linked with changes in host pathogen interactions. Spraying intact tobacco leaves daily with kinetin (0.1 mg ℓ^{-1}) for 9 days increased the infectivity of tobacco ringspot virus, whereas at 1.0 and 10.0 mg ℓ^{-1} this treatment reduced symptoms (Tavantzis et al, 1978). The authors suggested that continuous "occupation" or modification by kinetin of a large number of virus replication sites such as ribosomes may hinder virus replication or infectivity to a considerable extent.

In contrast, cytokinin content increased in Xanthi tobacco leaves systemically resistant to challenge inoculation by tobacco mosaic virus (TMV) (Balázs, 1977). Here it was suggested that the reduction in number

of visible local lesions in challenge resistant upper leaves was a result of suppression of necrosis, as virus production was not reduced.

In the current work 6-benzylaminopurine (6-BAP) reduced lesion size on sprayed leaves by up to 60%. This was the same reduction as that induced by prior inoculation with C. lagenarium. However, there was no reduction in lesion number on 6-BAP sprayed leaves whereas in systemically protected leaves there was a 60% reduction.

6-BAP sprayed leaves also showed large increases in chlorophyll, protein and water soluble carbohydrates (WSC), whereas in systemically protected leaves there was only a small increase in WSC and no increase in chlorophyll or protein. There was no detectable change in amino-acid content in either sets of leaves.

In the second leaves of plants, the first leaf of which had been sprayed with 6-BAP, lesion size was slightly reduced when the highest concentration of 10 mg ℓ^{-1} was sprayed for 11 and 6 days. There were also increases in chlorophyll content for some treatment combinations. This indicates that 6-BAP may have been translocated from sprayed leaves.

Reduction in lesion size in 6-BAP sprayed leaves was probably not due to fungitoxicity as in vitro tests with 6-BAP included in the growth medium of C. lagenarium in shake culture had no deleterious effects.

Some concentrations of 6-BAP caused substantial increases in certain metabolites and decreases in size of lesions caused by C. lagenarium. In systemically protected leaves showing similar decreases in lesion

size, corresponding increases in metabolites were not detectable.

This fact, together with the inability to detect endogenous changes in cytokinin levels in protected cucumber leaves (data not presented) make it unlikely that systemic protection in cucumber, caused by prior inoculation with a pathogen, is mediated *via* the action of 6-benzylamino-purine.

The ability of 6-BAP to retard senescence and prevent loss of chlorophyll (Jacoby and Dagan, 1970; Fletcher and McCullagh, 1971) may mean that the apparent reduction in lesion size depends on the suppression of chlorosis caused by *C. lagenarium*.

A second explanation may be that the high soluble sugar concentration found in 6-BAP sprayed leaves represses extracellular enzyme synthesis by *C. lagenarium*. Byrde (1963) suggested that this catabolite repression of enzyme synthesis could explain the "high and low sugar" disease hypothesis of Horsfall and Dimond (1957).

Section 4. Changes in host enzymes

To assess whether infection of cucumber plants by C. lagenarium induced changes in host enzymes, activities of ribonuclease, phenylalanine-ammonia lyase, peroxidase, chitinase and β-(1-3) glucanase were determined.

a. Ribonuclease

Ten grams (fresh weight) of tissue from the second leaf of a healthy or systemically protected plant (assumed to be protected from earlier experiments) was ground in a pestle and mortar in 100 mℓ of cold 50 mM potassium phosphate buffer pH 6.8 containing 10 g polyvinylpolypyrrolidone. The slurry was passed through 'Miracloth' to remove larger debris and the filtrate centrifuged at 10000 g for 30 minutes. The pellets were discarded and the pH of the supernatant adjusted to 5.0 with 1 N HCl. Three replicates per treatment were used.

After standing overnight at 0^{o}C the precipitate formed was collected by centrifugation as above. The supernatant was regarded as 'soluble RNase'. The pellet was redissolved in 50 mM phosphate buffer pH 6.8 and clarified by centrifugation and regarded as 'pH 5 insoluble RNase'.

RNase activity was assayed with high molecular weight yeast RNA.

The reaction mixture contained, in 1 mℓ final volume, 40 mM sodium acetate buffer pH 5.8, 0.6 mg RNA and the enzyme fraction (0.05 mℓ for 'soluble' and 0.2 mℓ for 'insoluble' fractions).

Protein was estimated by the Lowry method as described in Materials and Methods.

FIGURE 4.1 shows the activity of both 'soluble' and 'insoluble' RNase from healthy and systemically protected tissue of second leaves at various periods after inoculation of the first leaves with either C. lagenarium or water.

The trend is for RNase activity to increase with the age of the plant from 4-13 days after inoculation, but no significant quantitative differences were observed between healthy and protected tissue.

b. Phenylalanine-ammonia lyase (PAL)

One gram of healthy or systemically protected leaf material was extracted in 5 mℓ of 0.025 M borate (pH 8.8) and centrifuged at 14000 g for 15 minutes.

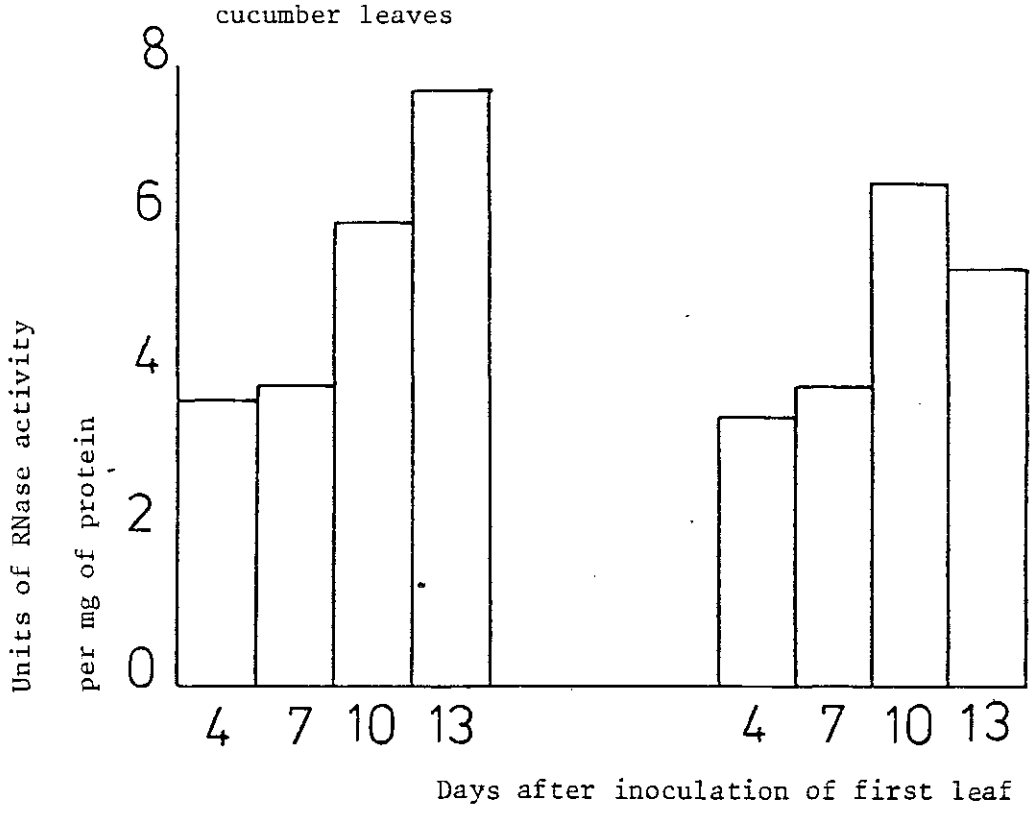
The supernatant was used as the enzyme extract.

At least 3 replicates per treatment were used. The reaction mixture containing 1.5 mℓ of 0.05 M borate buffer (pH 8.8), 1 mℓ 0.06 M phenylalanine solution and 0.5 mℓ enzyme extract was incubated for one hour at 37°C.

The change in OD at 290 nm was read as a measure of enzyme activity (FIGURE 4.2).

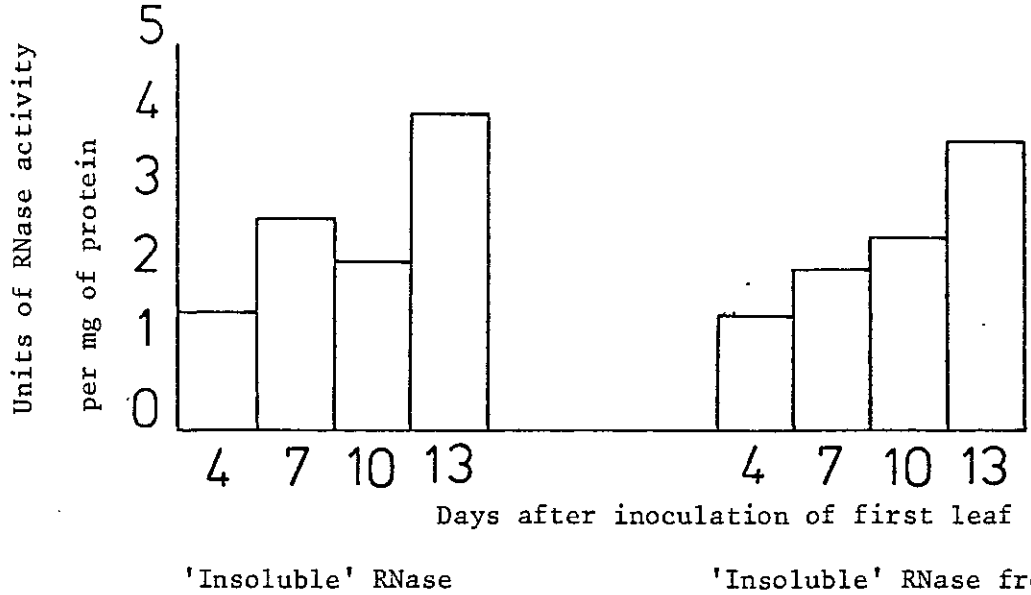
FIGURE 4.2 shows the activity of PAL in the protected second leaves of

FIGURE 4.1: Ribonuclease activity from protected and unprotected cucumber leaves



'Soluble' RNase from healthy tissue

'Soluble' RNase from protected tissue



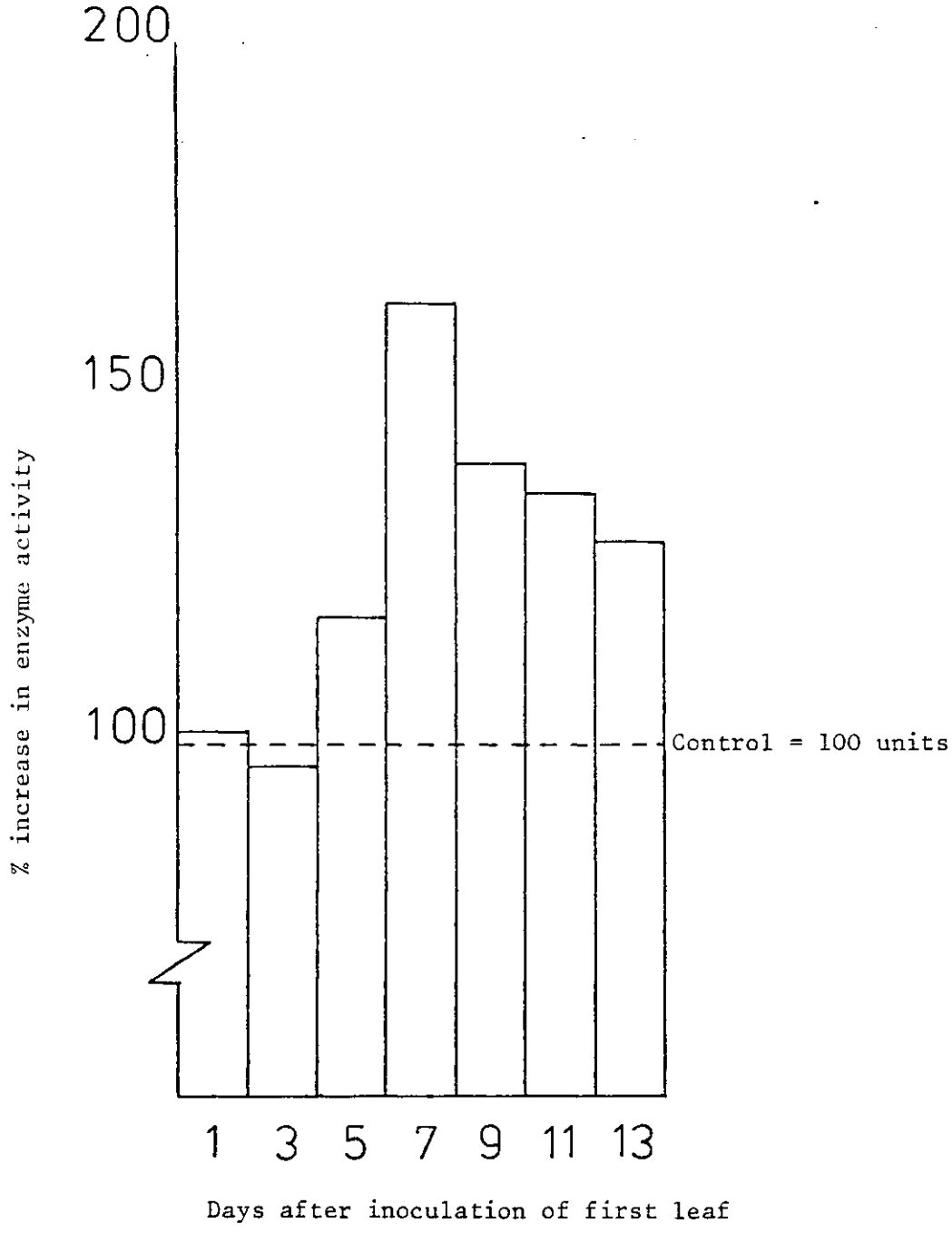
'Insoluble' RNase from healthy tissue

'Insoluble' RNase from protected tissue

One unit of RNase activity is defined as the amount of enzyme catalysing an increase in the absorbance at 260 nm of 1.0 under standard assay conditions.

Results means of at least 3 replicates per treatment

FIGURE 4.2: PAL activity in protected cucumber second leaves



Activity of protected leaves is expressed as a % of control leaves which was approximately 33 nM of cinnamic acid released h^{-1} g^{-1} fresh tissue for a reaction mixture containing 20 mM L-phenylalanine $m\ell^{-1}$.

Results means of at least 3 replicates per treatment

cucumber at various times after the infection of the first leaves. Activity (expressed in comparison to PAL levels of second leaves of plants where the first leaf had been water inoculated) increased by over 60% 7 days after infection of the first leaf and remained significantly higher than control treatments for the duration of the experiment.

c. Peroxidase

Peroxidase activity was assayed according to the method of Loebenstein and Lindsey (1963).

Tissue from healthy unprotected, systemically protected and infected cucumber leaves (infected leaves having $\underline{c}$ 40 5 mm lesions per leaf) was ground in a pestle and mortar with 0.01 M sodium phosphate buffer pH 6.0. This was centrifuged at 10000 g for 15 minutes and the supernatant used as the enzyme preparation. Enzyme (0.5 mℓ) was added to 2.5 mℓ of pyrogallol reagent in a spectrophotometer tube. The instrument was zeroed against a blank at 404 nm and 0.5 mℓ of peroxidase added. The tube was inverted once and immediately re-inserted into the spectrophotometer. The time taken for an increase in optical density of 0.2 units was recorded. As there was no appreciable autodecomposition of the substrate during the period of assay, enzyme activity can be assumed to be inversely proportional to the time taken for a required change in OD.

Absolute peroxidase values are therefore expressed as the reciprocal value sec. mg. fresh wt.$^{-1}$. (See Table 4.1).

Table 4.1: Peroxidase activity in cucumber leaves

Tissue	[a]Time for OD change/secs	S.E.	[b]Enzyme activity
Healthy 1st leaf	5.01	± 0.118	1.97
Infected 1st leaf	1.36	± 0.103	7.33
Healthy 2nd leaf	5.06	± 0.016	1.97
Protected 2nd leaf	4.80	± 0.125	2.08

a = time taken for a change in OD of 2.0 units per mg of fresh tissue

b = absolute peroxidase values expressed as the reciprocal value sec. mg fresh wt^{-1}.

Results means of 6 replicates per treatment

Table 4.1 shows that enzyme activity in infected leaves was over three times higher than that in control leaves. Activity in healthy and protected second leaves was not significantly different.

d. Chitinase and β-(1-3) glucanase

Healthy unprotected and systemically protected cucumber tissue were ground in a pestle and mortar with extraction buffer (see Materials and Methods). The homogenate was centrifuged, the pellet resuspended, centrifuged again, supernatants combined and dialysed overnight. This was used as the enzyme preparation. Substrates were prepared as in Materials and Methods.

β-(1-3) glucanase activity was determined in cell free extracts in a reaction mixture consisting of 8 mg of soluble laminarin dissolved in citrate buffer pH 4.7, plus 0.2 mℓ enzyme preparation. Reaction time was 2 hours at 25°C after which the enzyme product (free glucose) was determined using the Nelson's (1944) modification of the Somogyi method. Control mixtures were the same, reaction stopped at zero time.

Chitinase activity was determined using a reaction mixture of 20 mg purified chitin dispersed in 0.8 mℓ of sodium citrate buffer (pH 5.8, 0.1 M) plus 0.2 mℓ enzyme preparation (see Materials and Methods). The reaction mixture was incubated for 2 hours at 25°C. Control mixtures were the same, reaction stopped at zero time. Enzyme product was determined by the Nelson/Somogyi method.

Table 4.2: Activity of chitinase and β-(1-3) glucanase in unprotected and protected cucumber leaves

Tissue	Activity (µg glucose equivalents, mg protein^{-1} hr^{-1})			
	β-(1-3) glucanase	S.D.	Chitinase	S.D.
Healthy unprotected	16.21	± 2.0	22.72	± 3.31
protected	24.69	± 3.52	25.63	± 3.52

Results means of 6 replicates per treatment

Table 4.2 shows the activity of β-(1-3) glucanase and chitinase from cucumber leaves. There was no significant difference between chitinase activity from healthy unprotected and systemically protected leaves.

There was, however, a 50% increase in β-(1-3) glucanase activity in protected tissue compared to unprotected control leaves.

DISCUSSION IV

Systemic induction of resistance in cucumber almost certainly involves changes in host metabolism. These changes would require the synthesis of new messenger RNA (mRNA) molecules and enzymes. Such changes have been reported following infection of higher plants with obligate parasites such as rusts and powdery mildews (Chakravorty and Shaw, 1977). Pure et al. (1979) reported the presence of a number of different size classes of mRNA in the polysomes from rust infected wheat leaves which are either absent or present in much lower quantities in the polysomes from healthy leaves. This confirmed earlier work by Tani et al. (1973) who reported changes in the transcription patterns of oat leaves infected by Puccinia coronata. These results show that infection can alter the host's primary metabolism.

It was found that there are no quantitative changes in RNase activity in cucumber tissue which is systemically protected against C. lagenarium compared to unprotected tissue. Activity increases as tissue ages in both unprotected and protected leaves. Qualitative changes, that is changes in substrate preference of RNase to different polynucleotides, would not have been detected in this investigation. Such changes may influence the response of protected tissue to challenge inoculation by C. lagenarium.

There are several reports showing evidence of increased peroxidase activity following infection (see Literature Review). These have led to the hypothesis that changes in peroxidase activity may be involved in the defence mechanisms of plants. The action of peroxidase in connection with disease resistance has been linked, amongst other things,

to its ability to oxidise certain substrates such as phenolic compounds. Increases in peroxidase activity have been shown to coincide with induction of systemic resistance to tobacco mosaic virus in tobacco (Simon and Ross, 1971).

In C. lagenarium infected cucumber leaves, peroxidase activity increased by over 300%. It was not possible to find significant increases in enzyme activity in upper, systemically protected leaves.

In contrast, Hammerschmidt and Kuć (1980) reported increases in peroxidase activity in second leaves of cucumber with as few as one lesion on the first leaf. More recently, Kuć (1982) expanded this work to show that total peroxidase activity in challenged leaves of unprotected plants may, in time, be up to three times that in protected leaves and that injury to a leaf with dry ice or carborundum also enhances peroxidase activity in that leaf but does not induce resistance. Similarly, senescence or treatment with ethylene increase peroxidase activity but neither induce resistance. Kuć went on to argue that peroxidase may be one component of a mechanism for containment of a pathogen in a protected plant. In order that the enzyme should be effective a non-limiting supply of substrate should be available. He suggests that senescence or ethylene treatment increase peroxidase but do not induce resistance due to a possible lack of substrate.

Failure to protect plants by inducing changes in peroxidase activity in leaves prior to inoculation has led other workers to believe that peroxidase is not involved directly in defence mechanisms. Saprophytic bacteria such as Bacillus subtilis do not induce resistance in tobacco to Pseudomonas solanacearum but do increase peroxidase levels (Lozano

and Sequiera, 1970). Induction of high peroxidase activity in tobacco following infiltration with heat-killed bacteria then shading the leaves with aluminium foil did not induce resistance to P. solanacearum (Nadolny and Sequiera, 1980). Rathmell and Sequiera (1975) were unable to obtain protection of tobacco to P. solanacearum after infiltrating leaves with horseradish peroxidase. Van Loon (1976) concluded that peroxidase increases are not directly involved in disease resistance but may reflect the physiological state of the plant.

The role of phenylalanine-ammonia lyase (PAL) in infected tissue is well known but not well understood. PAL is the first enzyme of the phenylpropanoid pathway and is therefore considered to be the key enzyme in the regulation of synthesis of phenylpropanoid compounds and their derivatives (Camm and Towers, 1973). Many workers have reported the involvement of PAL in phytoalexin synthesis following infection. Increases in PAL in protected second leaves of cucumber occurred five days after the infection of the first leaf, reaching a maximum of about 160% of control values after 7 days. This was followed by a slow decline up to 13 days after first leaf infection (last measurement taken after 13 days) at which time PAL levels were still well above control values. These increases were seen in tissue which was not infected and was at least 10 cm away from tissue that was. These increases in activity were small in comparison with increases found in infected tissue which often reached 200-300%.

The failure of Deverall (1977), Anderbrhan (1978) and this author to detect any post-infectional increase in substances by methods which readily reveal phytoalexins in the Leguminoseae suggests that PAL probably has roles other than formation of antifungal compounds. The

formation of lignin, in which PAL converts phenylalanine to trans-cinnamic acid, is one possible function (see Section 6).

It has been shown that β-(1-3) glucanase is present in higher plant tissue (Abeles et al. 1971; Wargo, 1975). Wargo (1975) suggested that lytic enzymes may form a defence mechanism in resistant tissue to invasion by Armillaria mellea. Netzer et al. (1978) concluded that the increase in activity of muskmelon β-(1-3) glucanase in a resistant cultivar provided a potential defence mechanism against Fusarium oxysporum f. sp. melonis, although Rabenantoandro et al. (1976), despite increase in β-(1-3) glucanase activity in muskmelon following infection by C. lagenarium, concluded that the increases were mainly of pathogen origin and not the result of a host defence mechanism.

In systemically protected, but not infected cucumber tissue (to eliminate the possibility of any enzyme activity being of pathogen origin), a significant but small increase in β-(1-3) glucanase activity was noted. The level of increased activity was less than one twenty fifth of the activity found by Netzer et al. (1978) in muskmelon and is therefore thought not to be sufficiently high to be a component of the induced defence mechanism.

Chitinase activity has also been reported for higher plants. Powning and Irzykiewicz (1965) described chitinase activity in seeds from the families of woody and herbaceous dicotyledons and in the Gramineae. Abeles et al. (1970) partially purified a chitinase system from bean leaves. Pegg and Vessey (1973) reported that healthy and Verticillium albo-atrum infected tomato plants possessed an enzyme capable of hydrolysing chitin. Infection resulted in a significant increase in

chitinase activity which was greater in susceptible reactions.

Healthy cucumber tissue also possesses chitinase activity. Systemically protected cucumber tissue showed a similar level of activity so it is concluded that chitinase is not important in the induced defence mechanism against C. lagenarium.

Section 5. Extracellular enzymes from C. lagenarium

Many pathogenic fungi have the ability to produce extracellular enzymes capable of degrading the complex polysaccharides of plant cell walls. These cell wall constituents, in turn, may be important as inducers of such enzymes.

This section attempts to determine the possibilities of:-

a. Changes in the enzymes induced in cultures of C. lagenarium when grown on cell wall material from protected rather than healthy unprotected plants.

b. Alteration in the ability of extracellular enzymes to degrade protected cell wall material.

1. Enzyme production

C. lagenarium was grown in 500 mℓ of liquid shake culture in 2 litre conical flasks containing 0.5% dried cell wall material from healthy cucumber plants and 0.1% sucrose. Additional nutrients were as described in Materials and Methods. The medium was adjusted to pH 6 prior to seeding with 10 mℓ of a spore suspension at 1×10^7 spores $m\ell^{-1}$. Flasks were kept at 24-25°C on an orbital shaker (100 rpm).

Protein solutions were prepared as described in Materials and Methods. Following dialysis, culture filtrates were stored at -20°C.

2. Pectic enzymes

Enzymes were assayed against sodium polypectate (NaPP) or pectin for:-

a. reduction in substrate viscosity;

b. saturated and unsaturated galacturonides with thiobarbituric acid (TBA);

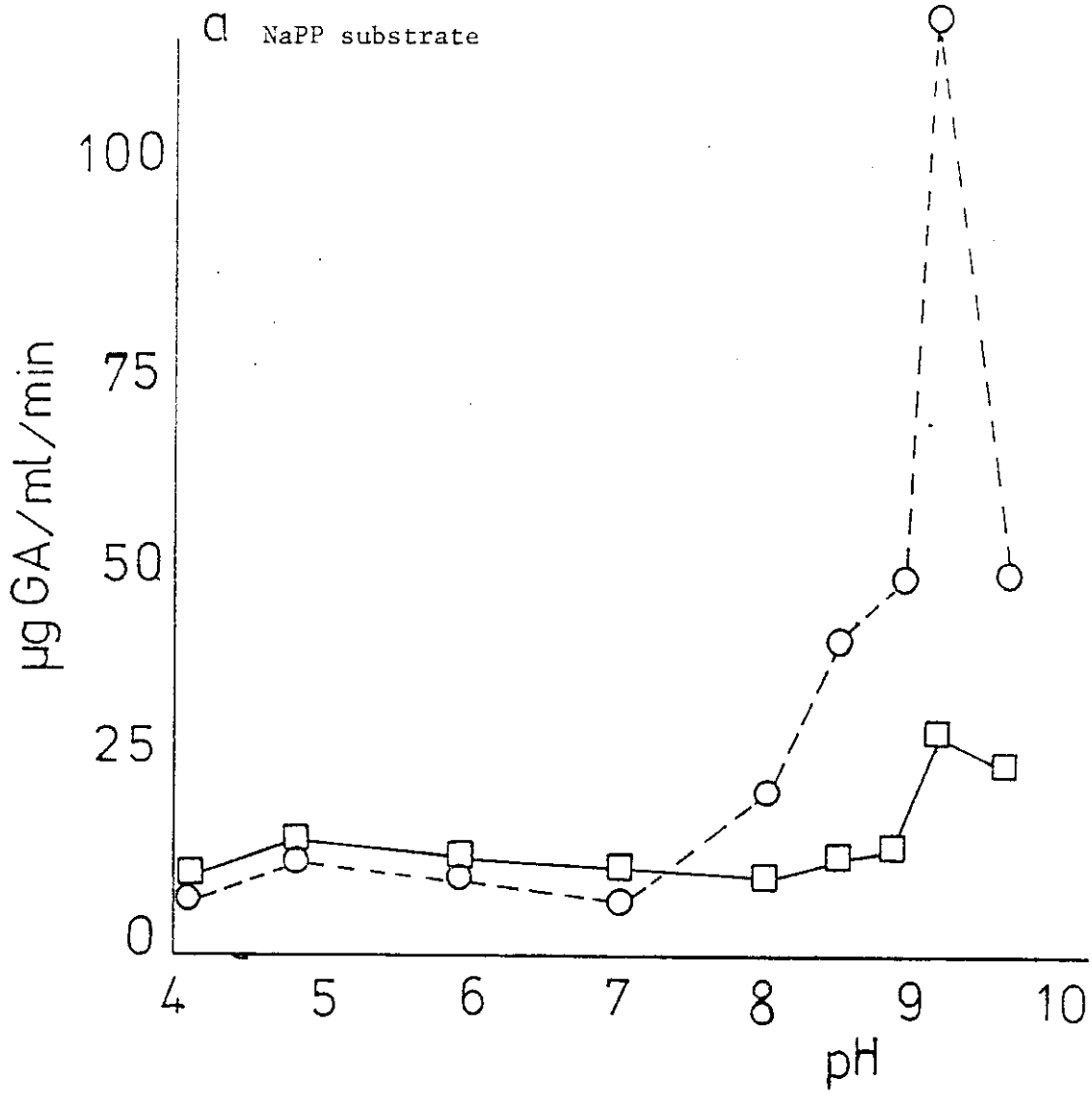
c. soluble reducing sugars using Nelson's modification of the Somogyi method;

d. appearance of unsaturated bonds which absorb at 232 nm.

Filtrates from cultures of C. lagenarium containing pectic enzymes induced by cucumber cell walls exhibited two peaks of activity (FIGURE 5.1). One major peak around pH 9 and a minor peak at pH 5. FIGURE 5.1 shows enzyme activity of culture filtrate measured by the release of galacturonic acid per mℓ of enzyme per minute, with and without the addition of $CaCl_2$ at various pHs on NaPP (FIGURE 5.1a) and on pectin (FIGURE 5.1b).

The two peaks of absorbance represented distinct enzyme types as shown by reaction products at 550 nm, representing action of a trans-eliminase enzyme, and 515 nm representing hydrolysis by a polygalacturonase (PG) following reaction with TBA, (FIGURE 5.2). This test also confirmed the paucity of PG production compared to trans-eliminase.

FIG. 5.1 pH optima of pectic enzymes



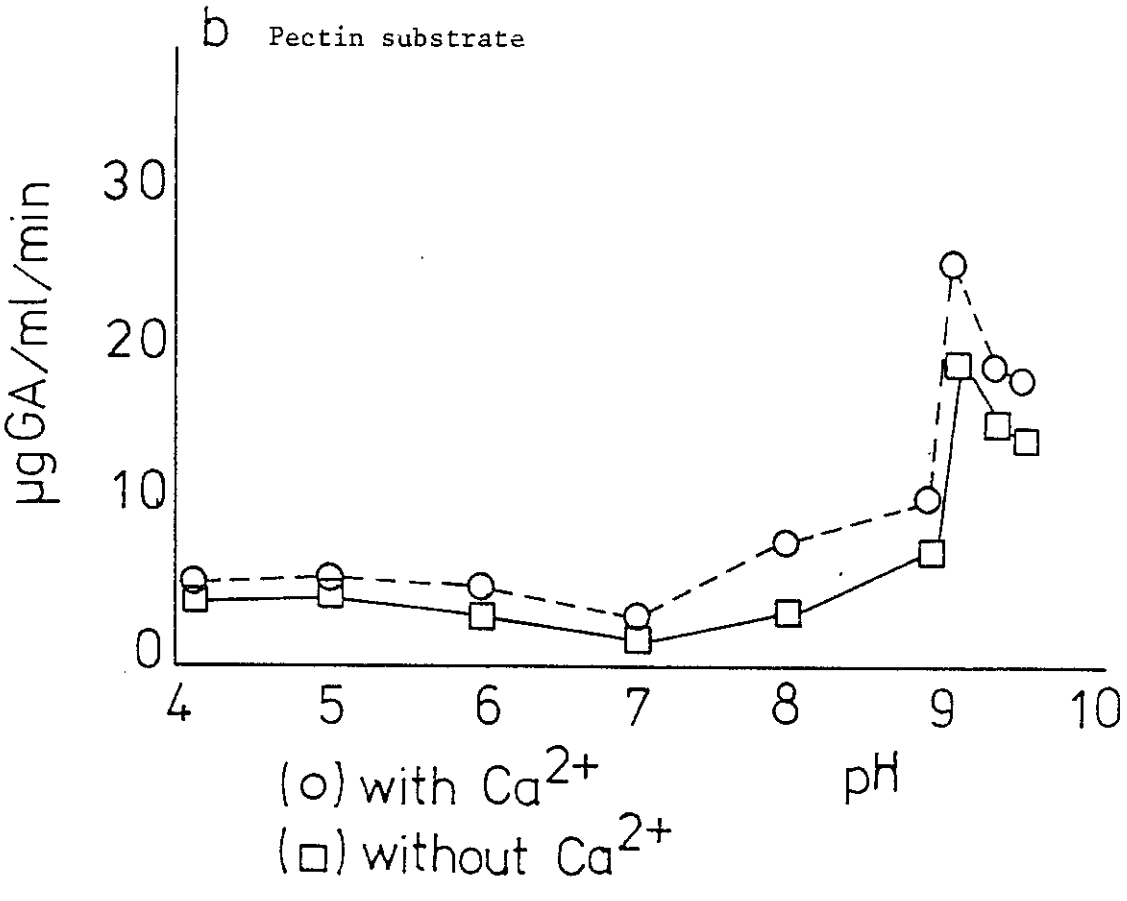
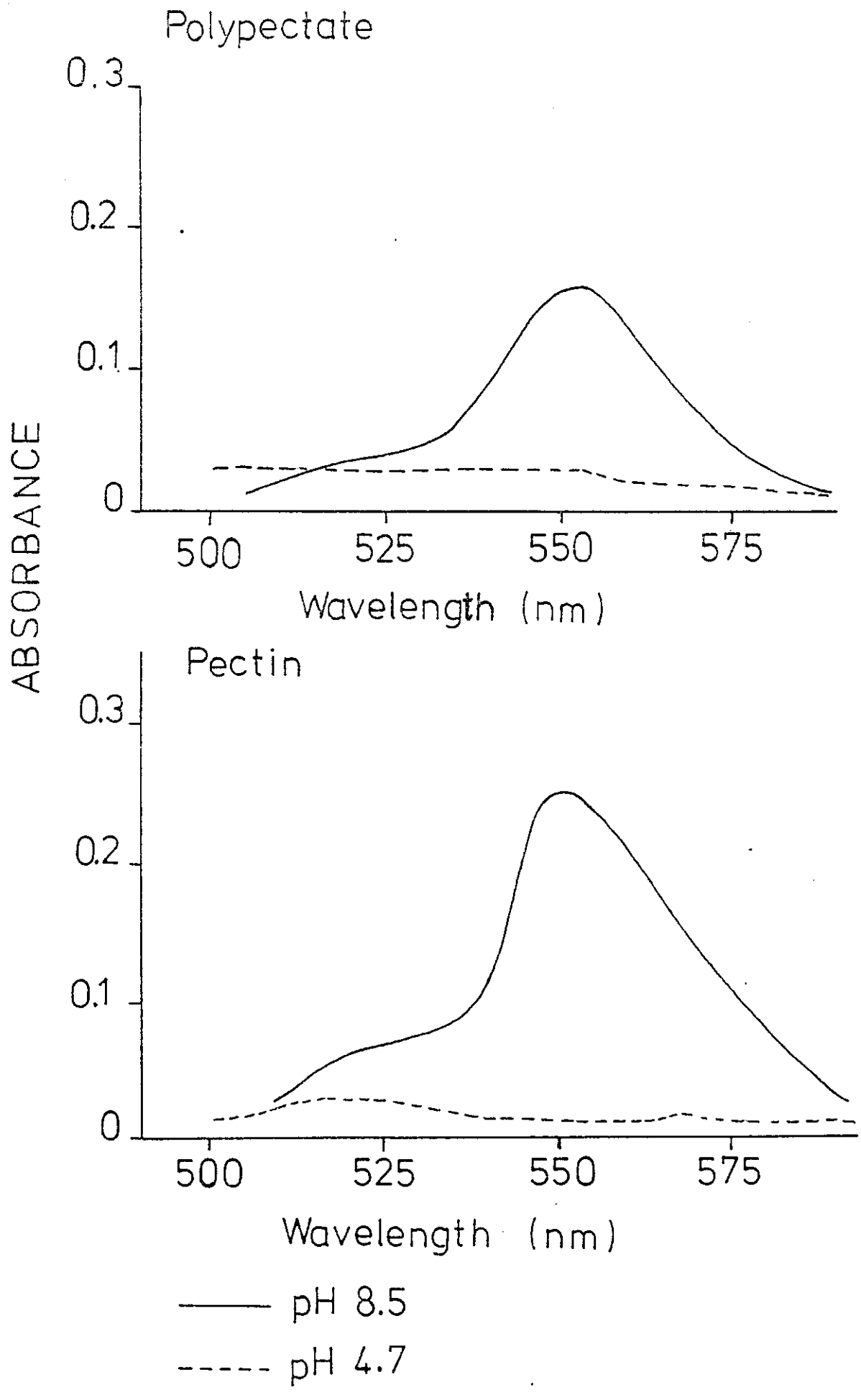


FIG 5.2 TBA test for reaction products



3. Effect of calcium ions

Calcium chloride was added to a reaction mixture of 1.9 mℓ 0.6% NaPP or pectin buffered in 0.1 M glycine/NaOH pH 9.2 and 1 mℓ enzyme to give a final concentration between 0.001 M and 0.2 M and a final volume of 3 mℓ.

FIGURE 5.3 shows enzyme activity, measured by the increase of absorbance at 232 nm, compared to the concentration of $CaCl_2$.

Pectate trans-eliminase had a strong requirement for Ca^{2+}. Activity increased 16 fold to an optimum at 0.02 M above which activity declined, possibly due to soft gel formation. When assayed against pectin, the optimum was again 0.02 M but the requirement for Ca^{2+} was less than that on pectate.

4. Relationship between viscosity reduction and substrate degradation

To determine whether cleavage of pectic substances by enzymes of C. lagenarium is random ("endo-") or terminal ("exo-") the relationship between reduction of substrate viscosity and substrate breakdown was examined.

Enzymes were assayed against NaPP or pectin at 0.6 and 1.2% viscometrically and in identical reaction mixtures for release of reducing groups by the Nelson/Somogyi method.

Time taken for 50% reduction in relative viscosity of substrate (t_{50}) were related to the µg quantities of galacturonic acid released (Table 5.1).

FIGURE 5.3 Effect of Ca^{2+} on enzyme activity

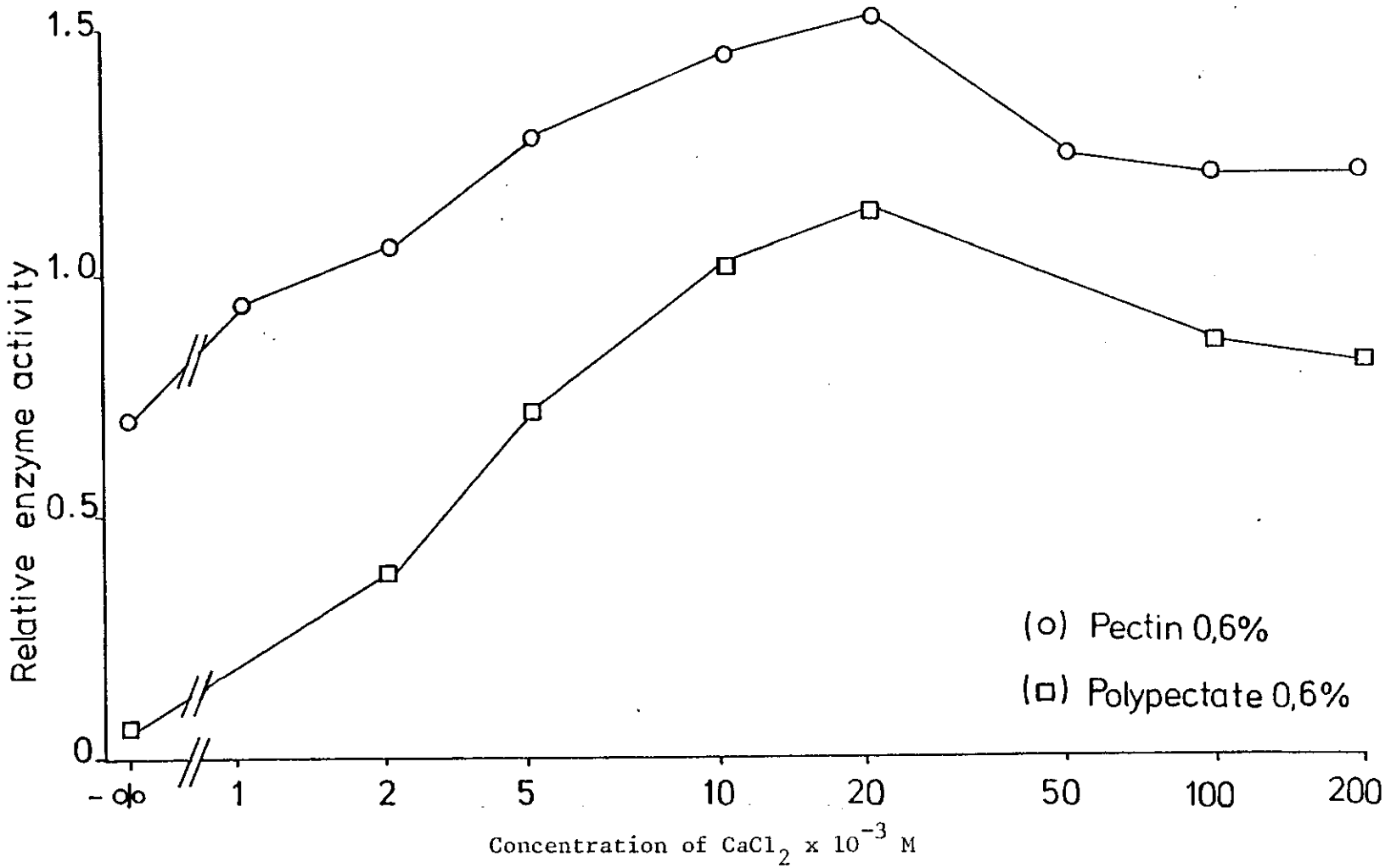


Table 5.1: Relationship between viscosity reduction and substrate degradation.

Substrate	Substrate conc ($g\ell^{-1}$)	t_{50} (mins)[a]	Galacturonic[b] acid released (mg $m\ell^{-1}$)	%[c] substrate hydrolysis
NaPP	12	51	0.035	0.37
	6	11.7	0.025	0.53
Pectin	12	28	0.095	1.01
	6	21.7	0.073	1.56

a. Viscometric assay: 8.9 $m\ell$ 0.6% or 1.2% NaPP/Pectin
1 $m\ell$ 0.005 M $CaCl_2$, 0.1 $m\ell$ enzyme

b. Concentration of GA released in reaction mixtures identical to those in viscometric assay after times corresponding to the t_{50} values (Nelson/Somogyi method).

c. Substrate hydrolysis after times corresponding to the t_{50} values calculated on the basis of 78% (W/W) galacturonide content of substrates.

Results in Table 5.1 show that a 50% reduction in the relative viscosity of NaPP and pectin were accompanied by hydrolysis of less than 2% of the substrate. The figures imply a random action of degradation.

5. Isoelectric focusing

Culture filtrates of C. lagenarium were applied to an LKB 8101 column and resolved in a pH 3 to 10 gradient. Trans-eliminase activity was found in 2 main zones at pI 5 and between pI 7.5-8.5 (FIGURE 5.4).

No PG activity was detected using the cup plate assay.

6. Determination of Michaelis constant for trans-eliminase

The Michaelis constant (Km) is the substrate concentration which gives half the maximum velocity of a chemical reaction.

The affinity of an enzyme for a particular substrate is given by the dissociation or substrate constant (K_s).

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E + P \qquad (1)$$

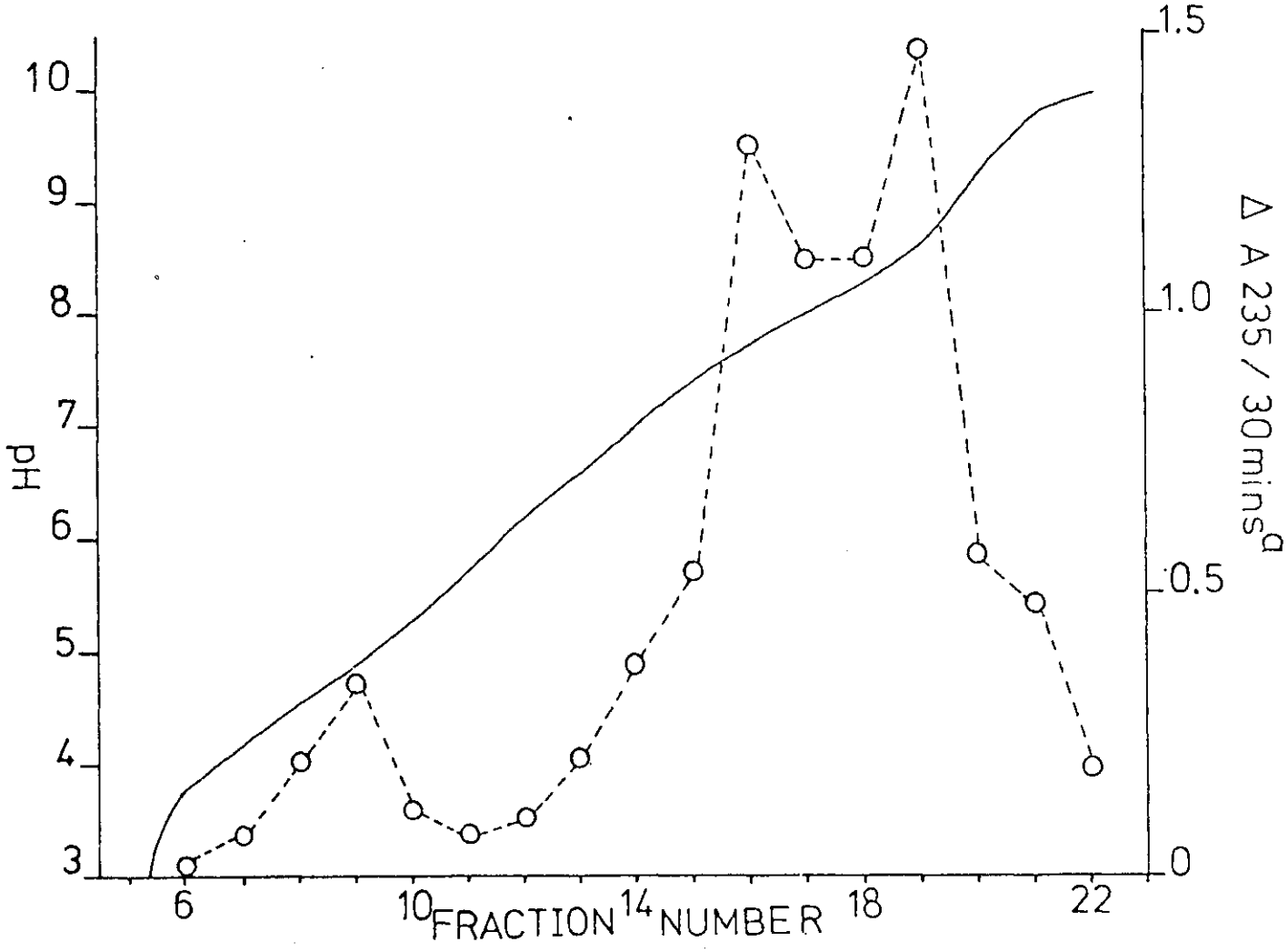
E = Enzyme, S = substrate, ES = enzyme-substrate complex and P = product.

$$K_s = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_{+1}} \qquad (2)$$

$$K_m = \frac{[E][S] + [E][P]}{[ES]} = \frac{k_{-1} + k_{+2}}{k_{+1}} \qquad (3)$$

Although ES can also be obtained from E and P by reversal of the second

FIGURE 5.4 Isoelectric focusing of trans-eliminase



a Enzyme activity assessed by change of absorbance of reaction mixture at 235 nm.

part of equation 1 above, the rate of this back reaction may be neglected as it is the first part of the reaction which is being considered when $[S]$ is very high and $[P]$ is zero or close to zero.

Equations 2 and 3 show that K_m, which can be determined experimentally, approximates to the dissociation constant (K_s) when k_{-1} is very large compared with k_{+2}.

$$K_s = \frac{k_{-1}}{k_{+1}} \approx K_m$$

The Michaelis constant for <u>trans</u>-eliminase was determined by incubation of the enzyme with various concentrations of NaPP or pectin.

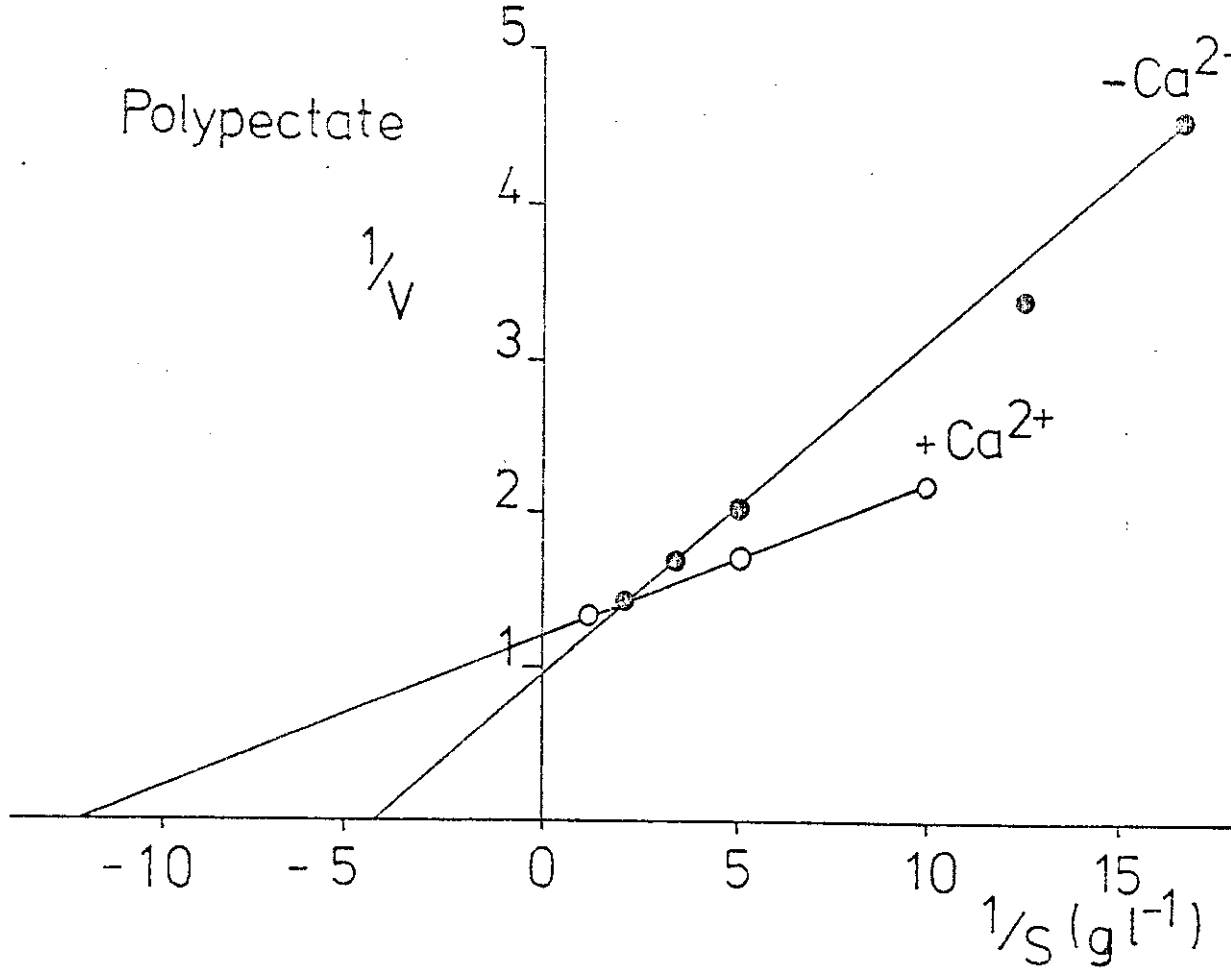
FIGURE 5.5 shows the double-reciprocal (Lineweaver-Burk) plot of enzyme activity (measured by the increase in absorbance at 232 nm) against substrate concentration (in $g\ell^{-1}$).

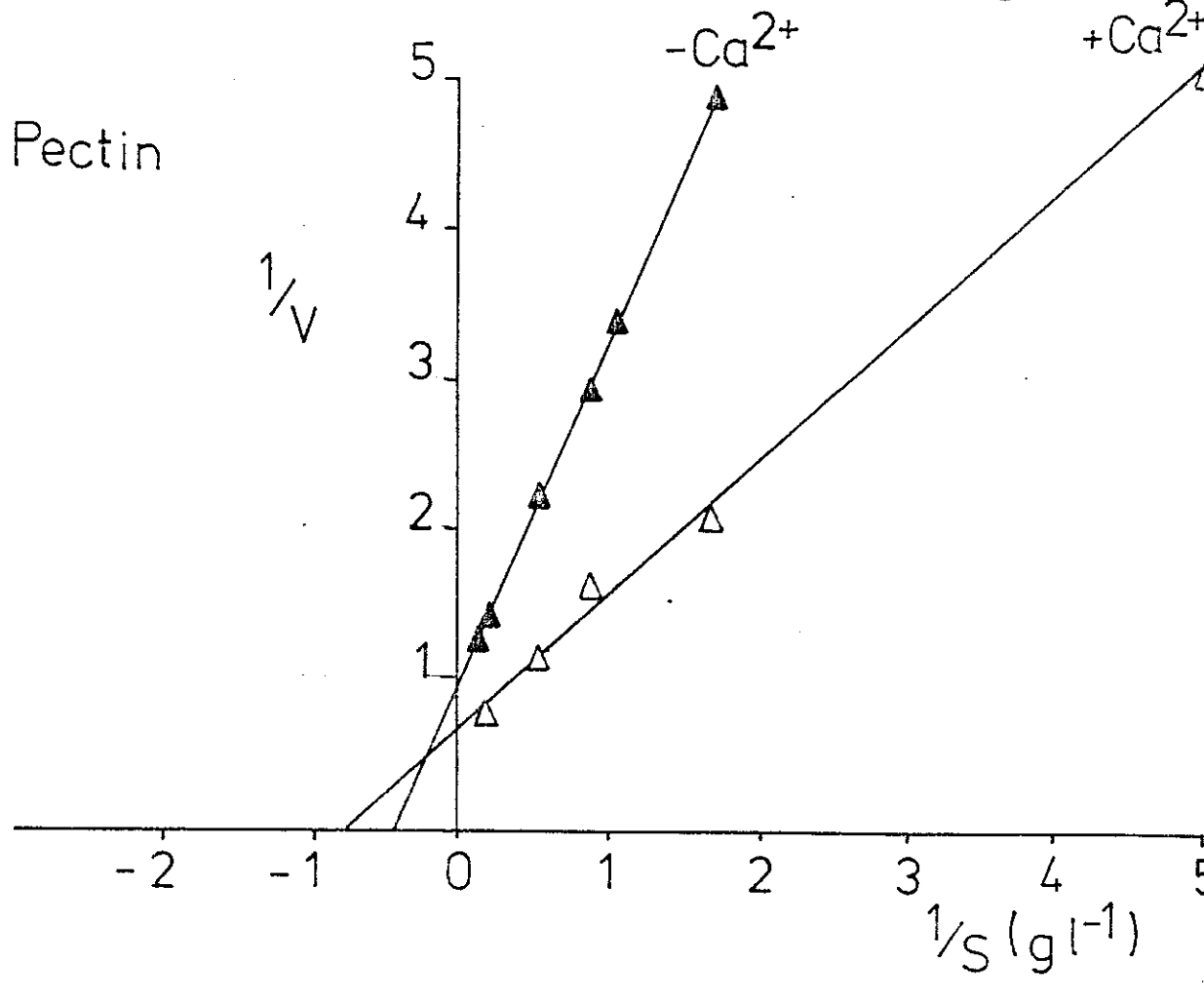
K_m values estimated are the means of 3 replicates per substrate concentration. Values are normally expressed in units of concentration (mole $litre^{-1}$).

Due to the large variability of the molecular weights of pectic substances the units of concentration used are grams $litre^{-1}$.

K_m values for NaPP and pectin were 0.053 $g\ell^{-1}$ and 0.792 $g\ell^{-1}$ (corrected values of substrate concentration in reaction mixtures in cuvette). The smaller K_m value on NaPP suggests that the enzyme had a greater affinity for this substrate than for pectin, although the enzyme is capable of breaking down pectin albeit at a slower rate. This may be due to the greater methylation of pectin.

FIG. 5.5 Lineweaver-Burk plot of trans-eliminase





7. Pectin methylesterase (PME)

To determine whether the above enzyme could be affecting assays using pectin as substrate, activity was assessed as described in Materials and Methods. Reaction mixtures were titrated against 0.0001 M NaOH. Table 5.2 shows the μ equivalents of alkali consumed per hour.

Table 5.2: PME activity

Assay time/hr	μ equivalents of alkali[a]
1	0.15
2	0.14
3	0.11

a = μ equivalents of alkali added to return the reaction mixture to pH 5.

Table 5.2 shows that PME activity was relatively low and it was therefore considered not to be important as a substrate modifier.

8. Hemicellulases

Culture filtrates of C. lagenarium grown on healthy cell wall material produced enzymes capable of degrading xylan (ex larch) and araban (ex larch).

Xylanase had a pH optimum of 5.7 (FIGURE 5.6) and arabanase, a broad optimum between pH 4-6 (FIGURE 5.7).

FIG. 5.6 pH optimum of xylanase

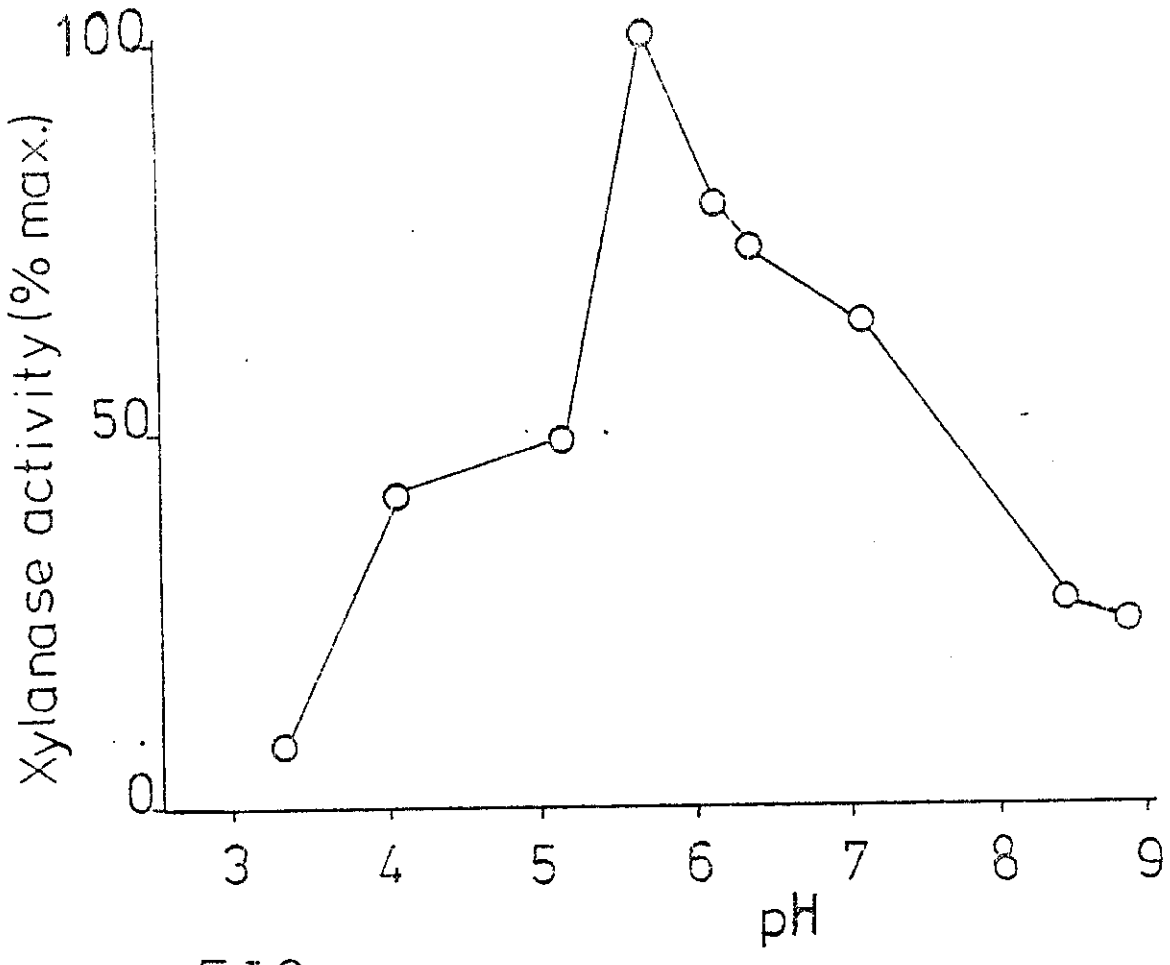
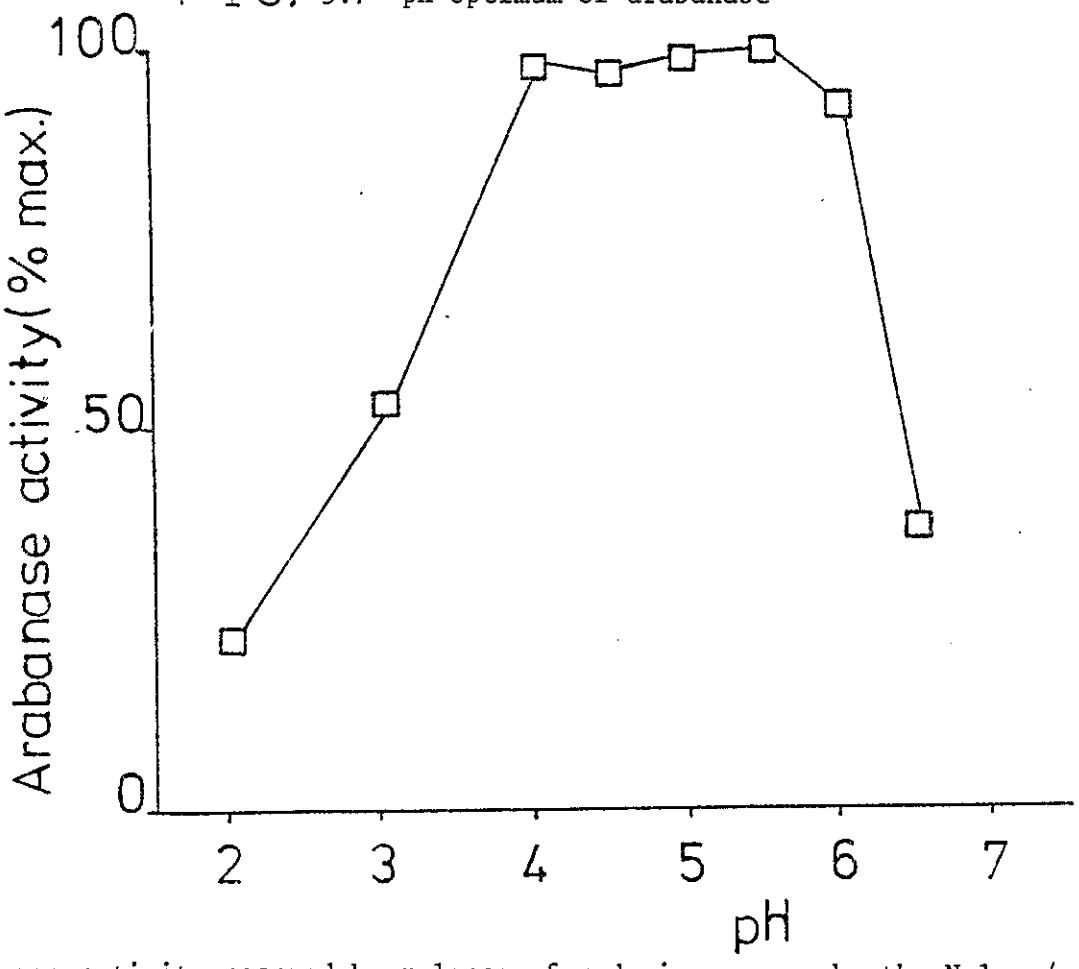


FIG. 5.7 pH optimum of arabanase



Enzyme activity assayed by release of reducing groups by the Nelson/ Somogyi method.

Both enzymes were assayed by the release of reducing groups by the Nelson/Somogyi method.

FIGURE 5.6 shows that xylanase activity was detected over the whole pH range tested. A sharp increase in activity between pH 5 and 5.7 was observed.

FIGURE 5.7 shows that the pH optimum for arabanase was considerably broader than that for xylanase. Maximum activity was detected between pH 4-6.

9. Cellulase

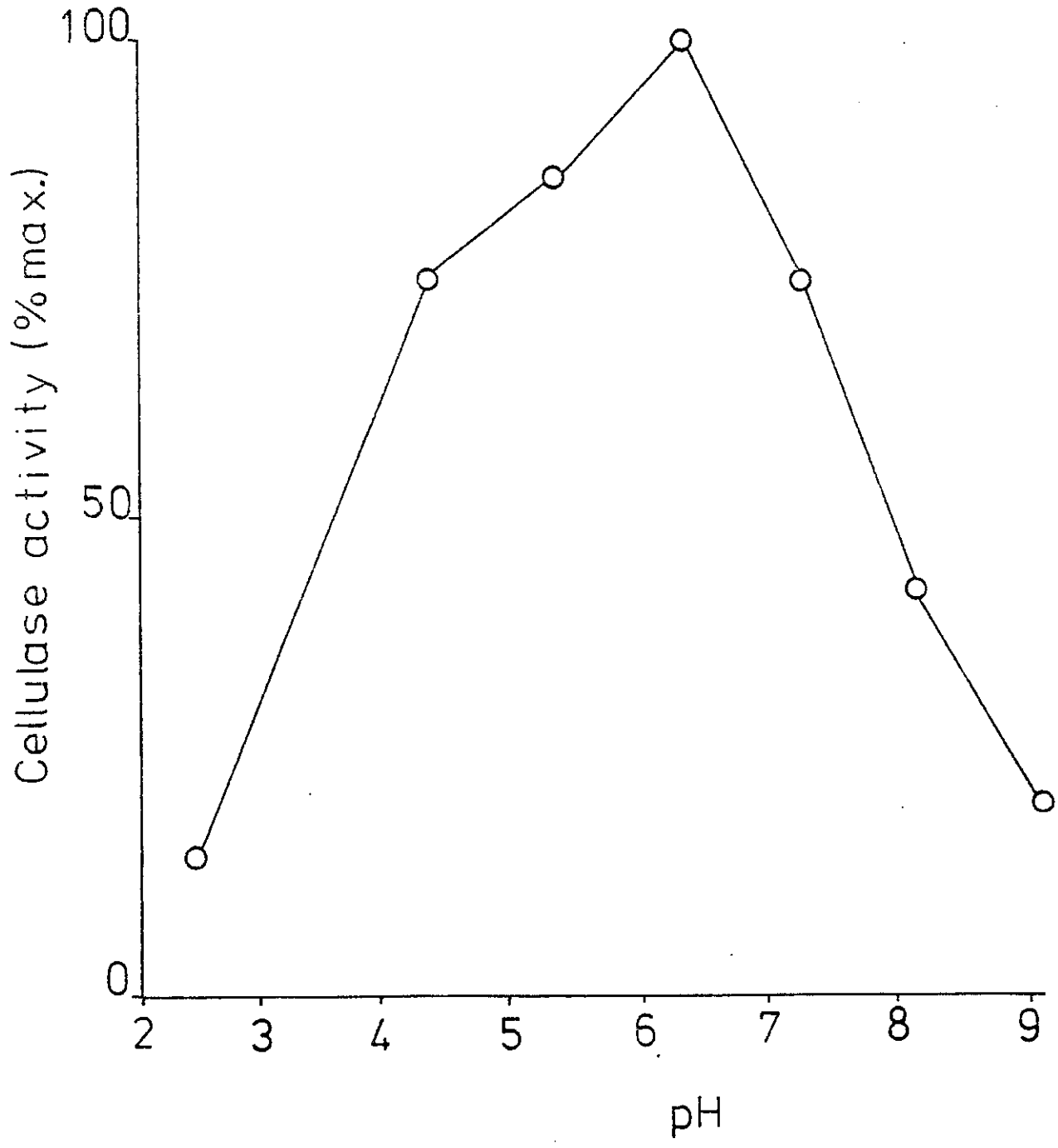
Cellulase of C. lagenarium, induced in shake culture by healthy cell wall material, decreased the viscosity of solutions of carboxymethyl-cellulose over the entire pH range assayed of pH 2.4-9.1. FIGURE 5.8 shows that the optimum pH was 6.3.

10. Sequential formation of enzymes

C. lagenarium was grown in 25 mℓ in 100 mℓ Erlenmeyer flasks containing 1% dried cell wall material from either healthy or systemically protected cucumber plants as the sole carbon source. Additional nutrients were as described in Materials and Methods.

Flasks were harvested at 2 day intervals and assayed for polygalacturonase (PG), trans-eliminase (TE) and xylanase activity at their respective pH optima.

FIG. 5.8 pH optimum of cellulase



Enzyme activity assayed by viscometry on carboxymethylcellulose.

FIG. 5.9 Activity of trans-eliminase with time

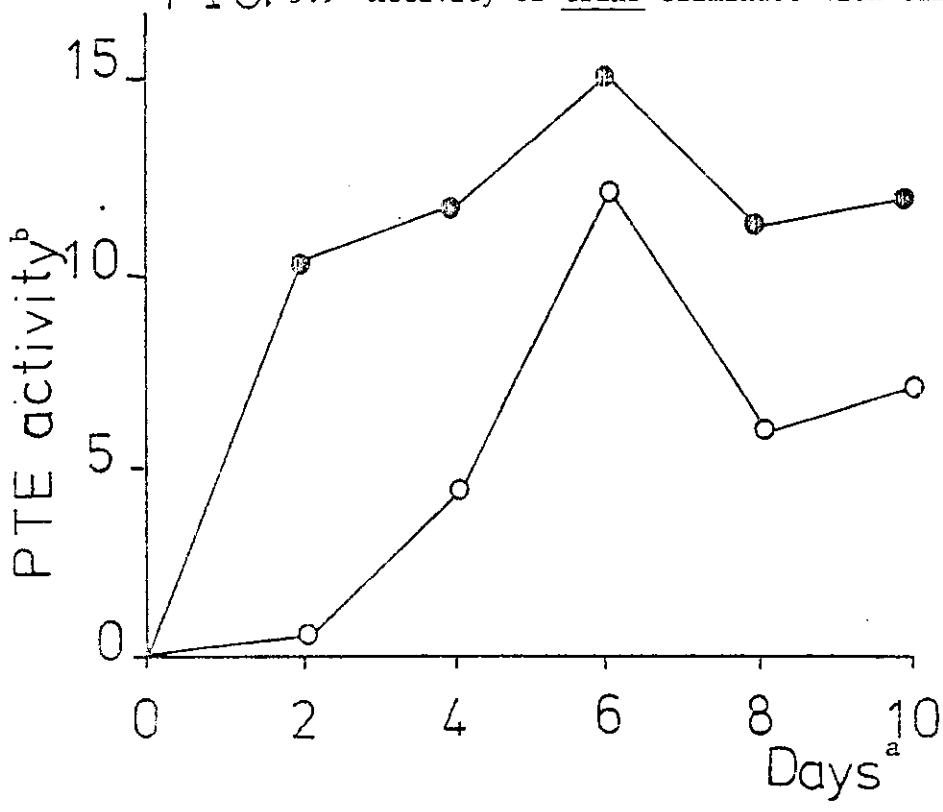
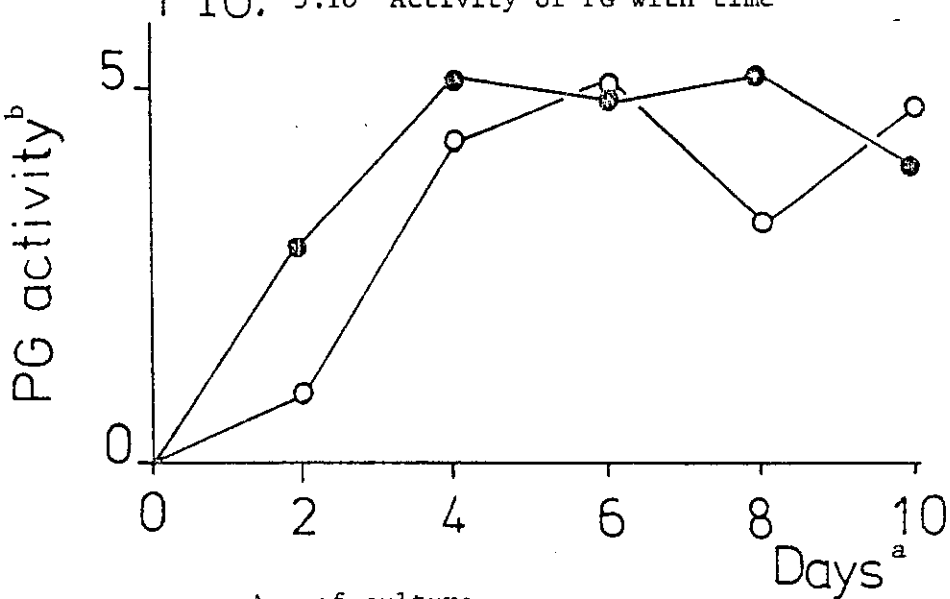


FIG. 5.10 Activity of PG with time



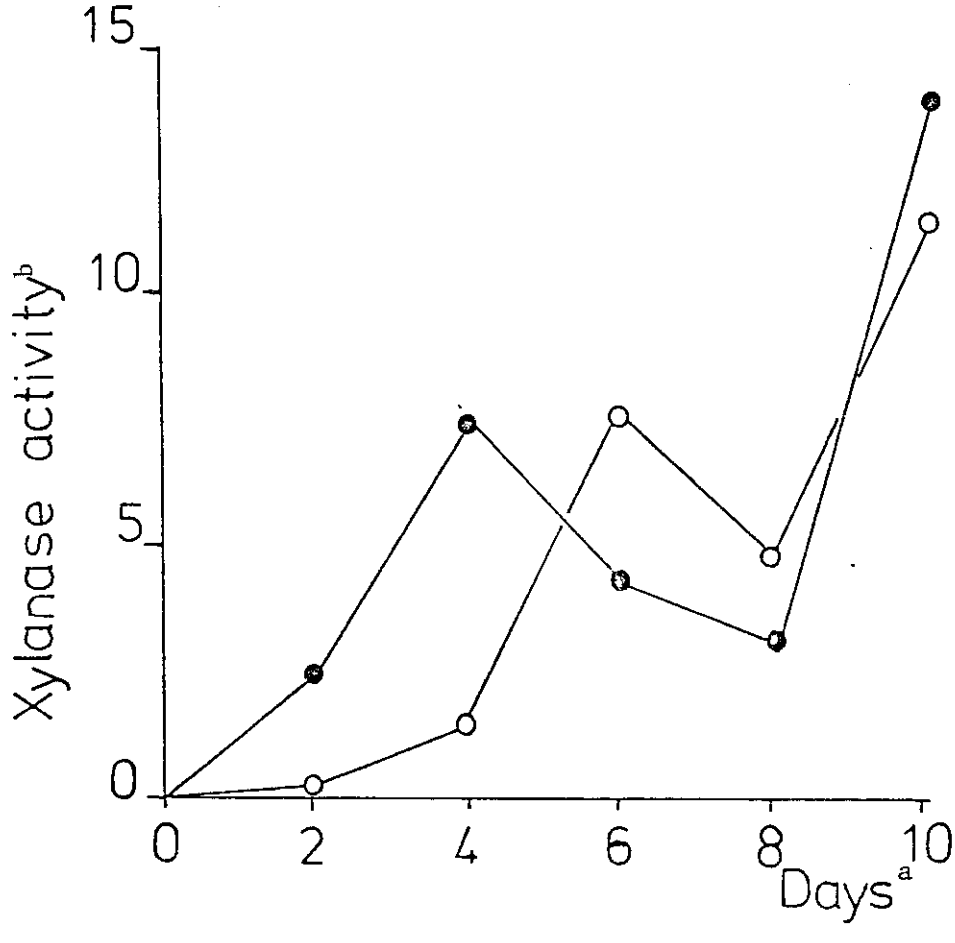
a Age of culture

(●) Healthy cell wall material as carbon source

(○) Protected cell wall material as carbon source

b Enzyme activity expressed as µg GA/mℓ/min.

FIGURE 5.11 Activity of xylanase with time



a Age of culture

(●) Healthy cell wall material as carbon source

(○) Protected cell wall material as carbon source

b Enzyme activity expressed as µg xylose/mℓ/min.

FIGURES 5.9, 5.10, 5.11 show the increases in polysaccharidases when grown on healthy unprotected or protected cell wall material.

PG and TE activity are present after 2 days, PG reaching a plateau at 4 days, TE two days later. Xylanase activity, although present after 2 days increases later than the pectic enzymes at 10 days.

Results were means of four flasks per treatment. Variation between flasks was sometimes considerable and, therefore, no significant difference was noted between enzymes induced by healthy or protected cell wall material.

11. Degradation of healthy unprotected and protected cell wall material

Pectic material was extracted from healthy unprotected and protected cell wall material with a Ca^{2+} chelating oxalate/citrate buffer under conditions of minimal degradation (as described in Materials and Methods).

Following extraction of pectic material, the cell wall residue was resuspended in mild alkaline conditions for 12 hours to solubilise remaining pectic substances. The insoluble residue was then resuspended in 4.3 M KOH for 24 hours. Solubilised polysaccharides were precipitated by addition of 50% acetic acid to pH 4.8 and methanol (3 volumes). The precipitate was washed extensively with acetone and dried for 24 hours at 50°C. This method gave a fraction rich in xylose and glucose with only small amounts of arabinose, mannose, galactose, rhamnose and fucose (Doux-Gayat et al. 1978).

Table 5.3 shows the quantities of wall material from unprotected and protected cucumber plants.

Table 5.3: Relative quantities of cell wall material

		Unprotected	Protected
A	Fresh weight	576.16g	421.26g
B	Cell wall dry weight	22.09g	14.51g
C	B as % of A	3.83%	3.44%
D	extracted pectic substances	5.72g	3.95g
E	D as % of B	25.90%	27.20%
F	extracted hemicelluloses	1.88g	1.14g
G	F as % of B	8.51%	7.8%

Due to the extensive washing and filtering during the extraction of wall material, the differences in quantities of various components are not considered to be significant. The figures serve as a guide to the approximate yield obtainable.

a. Pectic material extracted from unprotected and protected cell walls was dissolved at various concentrations and assayed against trans-eliminase, produced in shake culture of C. lagenarium grown on healthy cell walls, by absorbance of reaction products at 232 nm.

FIGURES 5.12 and 5.13 show the double-reciprocal (Lineweaver-Burk) plot for healthy and protected pectic substances respectively.

K_m values for pectic substances from the two sources were very similar.

b. Xylan rich fractions from unprotected or protected cell walls were dissolved and used as substrates for xylanase activity of culture filtrates from shake cultures of C. lagenarium grown on healthy cell

FIG. 5.12 Lineweaver-Burk plot of healthy pectic substances as substrate

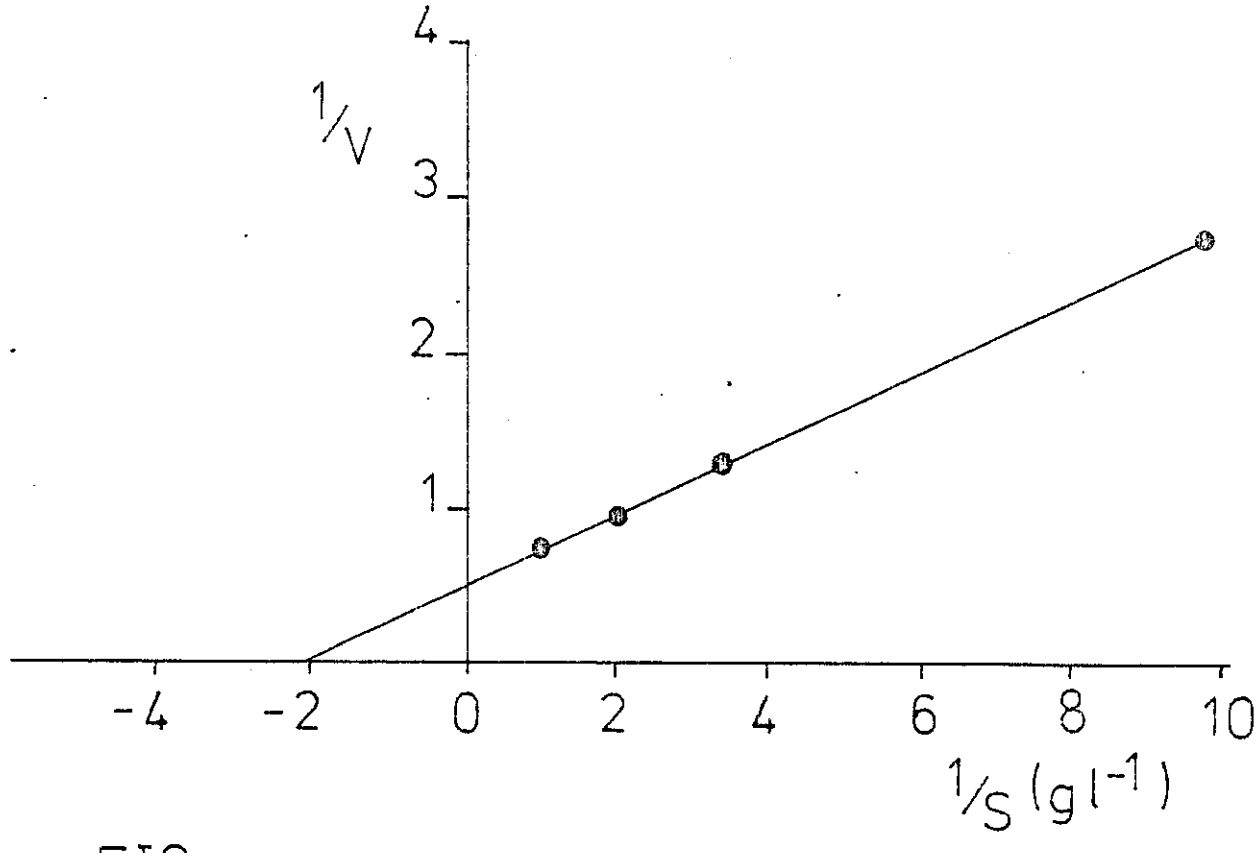
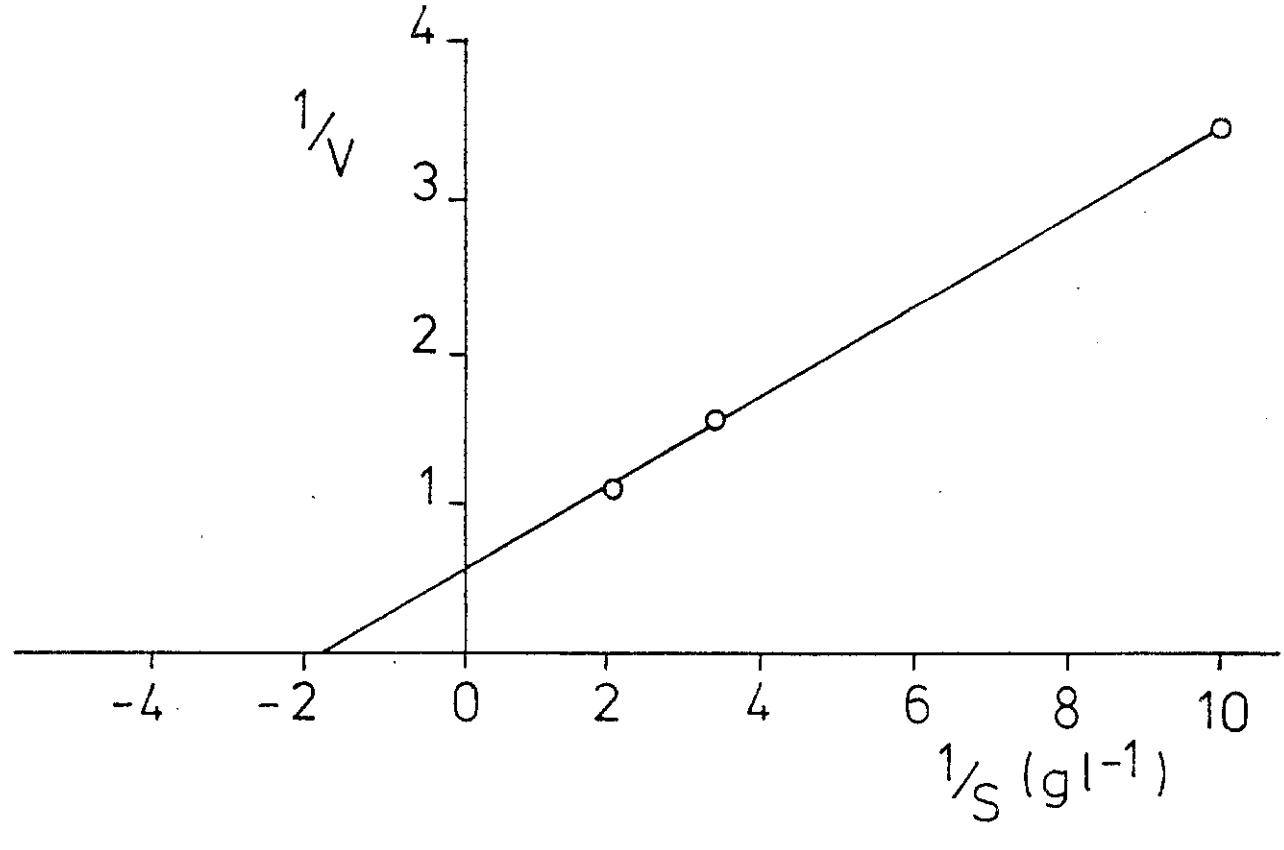


FIG. 5.13 Lineweaver-Burk plot of protected pectic substances as substrate



wall material. Enzyme activity was assayed using the Nelson/Somogyi method for reducing groups.

FIGURES 5.14 and 5.15 show the double-reciprocal (Lineweaver-Burk) plot for healthy and protected xylan-rich cell wall material.

K_m values for the xylan-rich fractions from the two sources were very similar.

c. Chromatography of reaction products

The composition of reaction mixtures was examined at intervals by thin layer chromatography (tlc) or descending paper chromatography (as described in Materials and Methods), to determine how substrates were degraded.

Aliquots of 50 $\mu\ell$ or 80 $\mu\ell$ were taken from reaction mixtures for t.l.c. and paper chromatography. Aliquots were boiled at 100°C for 5 minutes prior to loading.

FIGURE 5.16 shows the reaction products when culture filtrates of C. lagenarium were incubated with NaPP and pectic substances extracted from the cell walls of unprotected and protected cucumber plants. The product of all three substrates co-chromatographed with galacturonic acid. No oligomers were detected.

FIGURE 5.17 shows the reaction products of culture filtrates of C. lagenarium acting on xylan (ex larch) and unprotected and protected hemicellulose material extracted from cucumber plants.

FIG. 5.14 Lineweaver-Burk plot of healthy hemicellulosic material as substrate

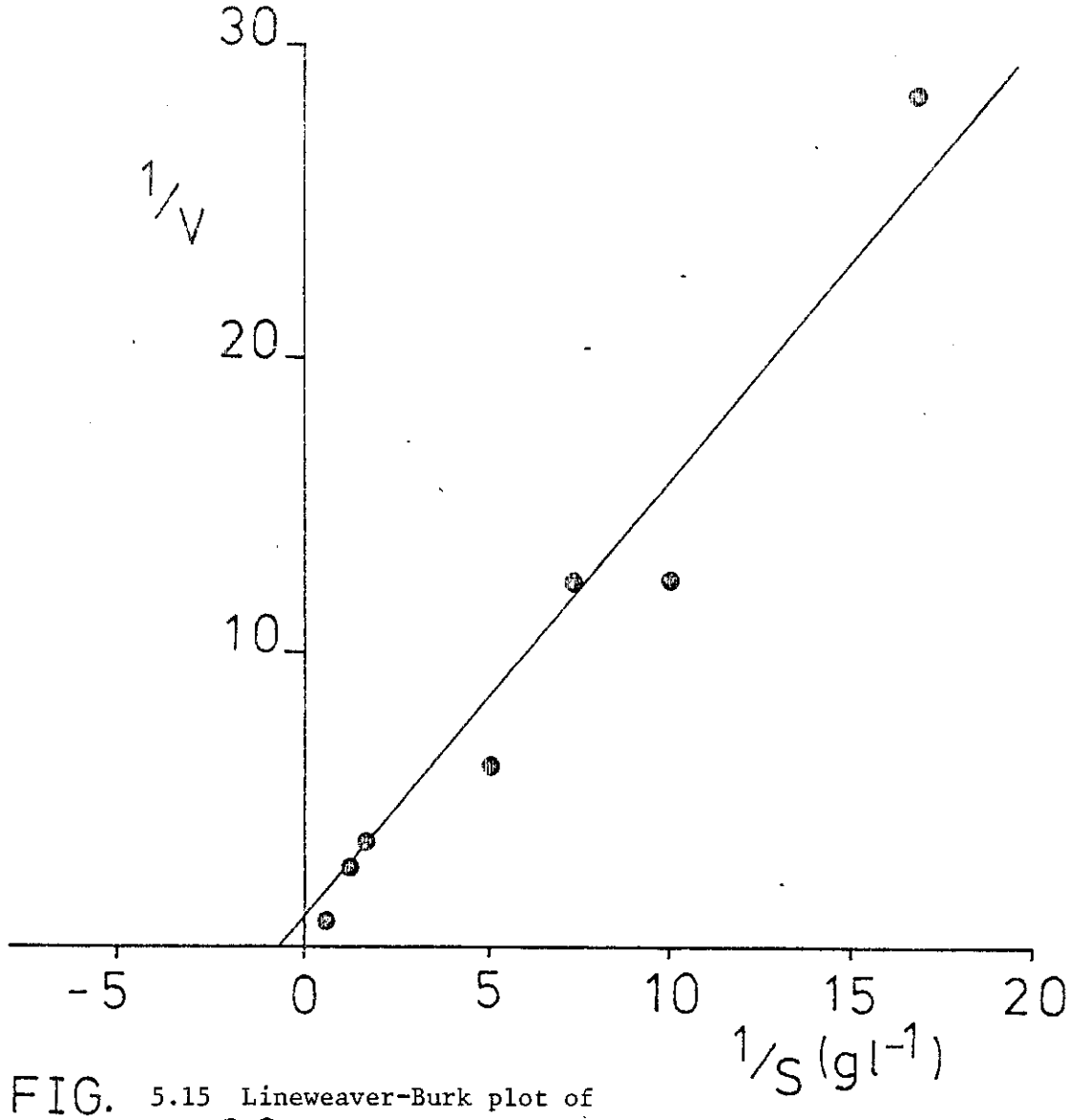


FIG. 5.15 Lineweaver-Burk plot of protected hemicellulosic material as substrate

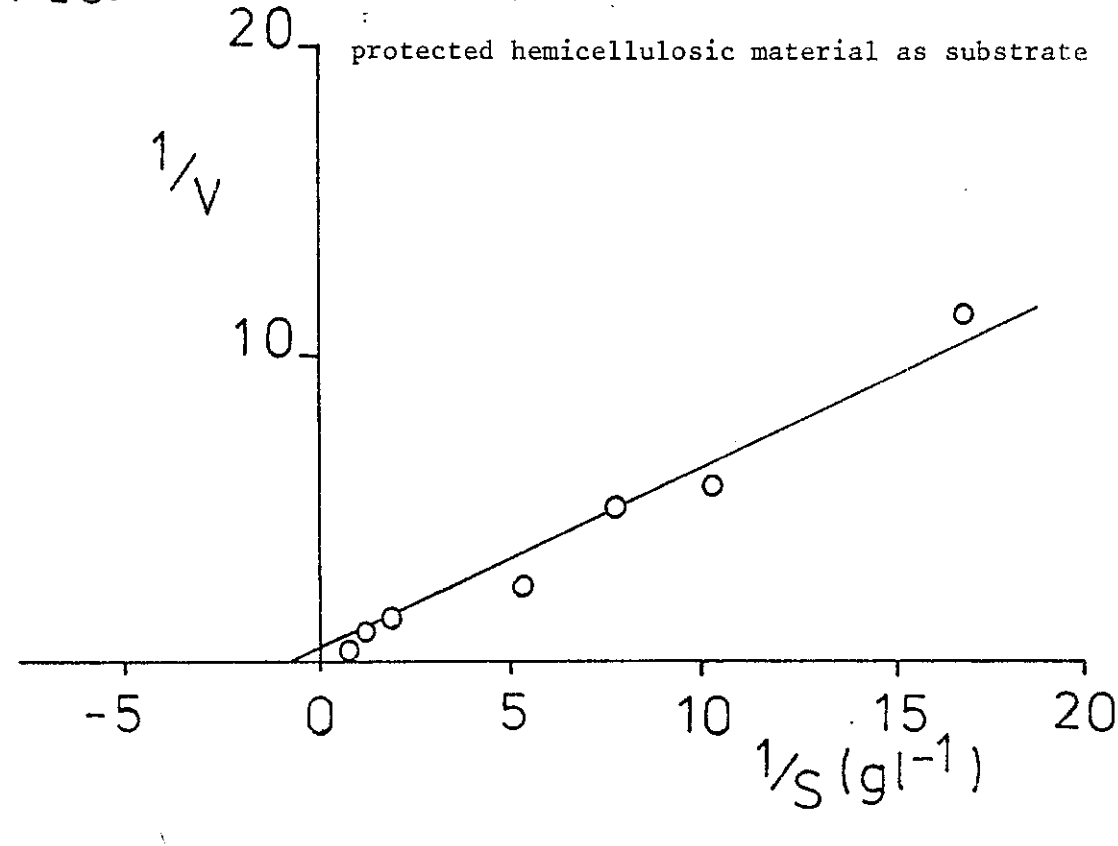


FIG. 5.16 Reaction products of enzymes on NaPP and cucumber pectic substances

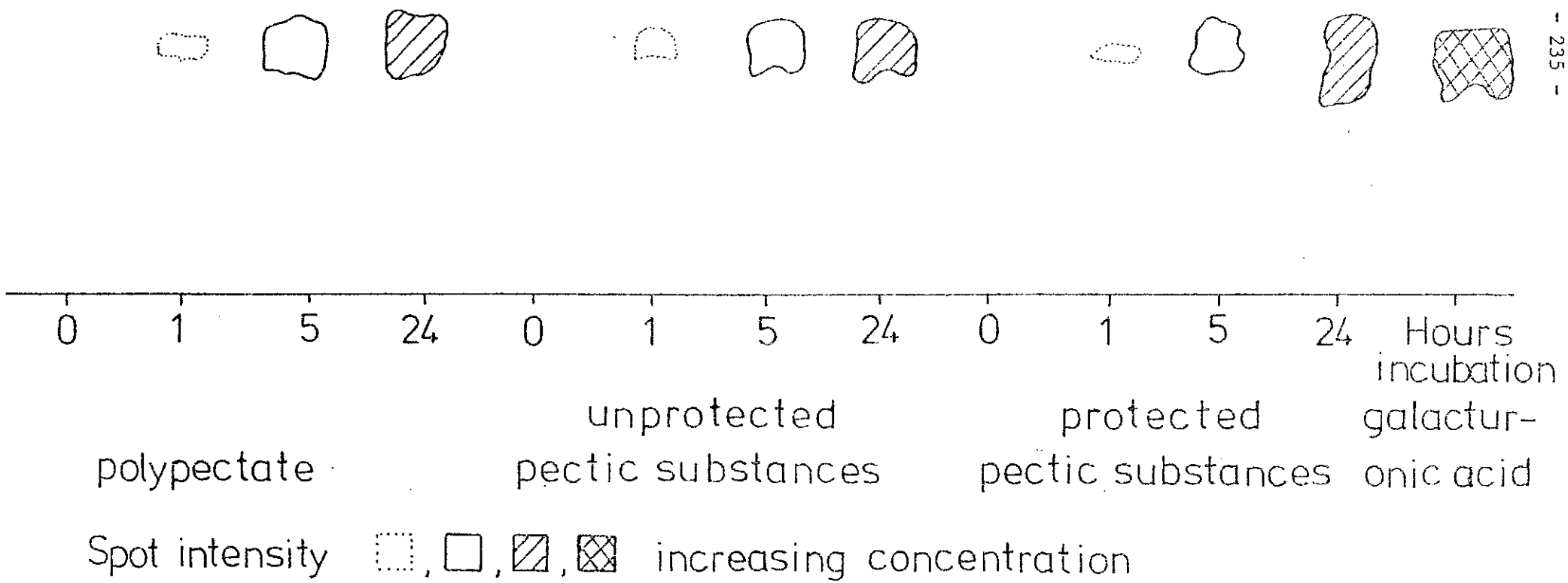
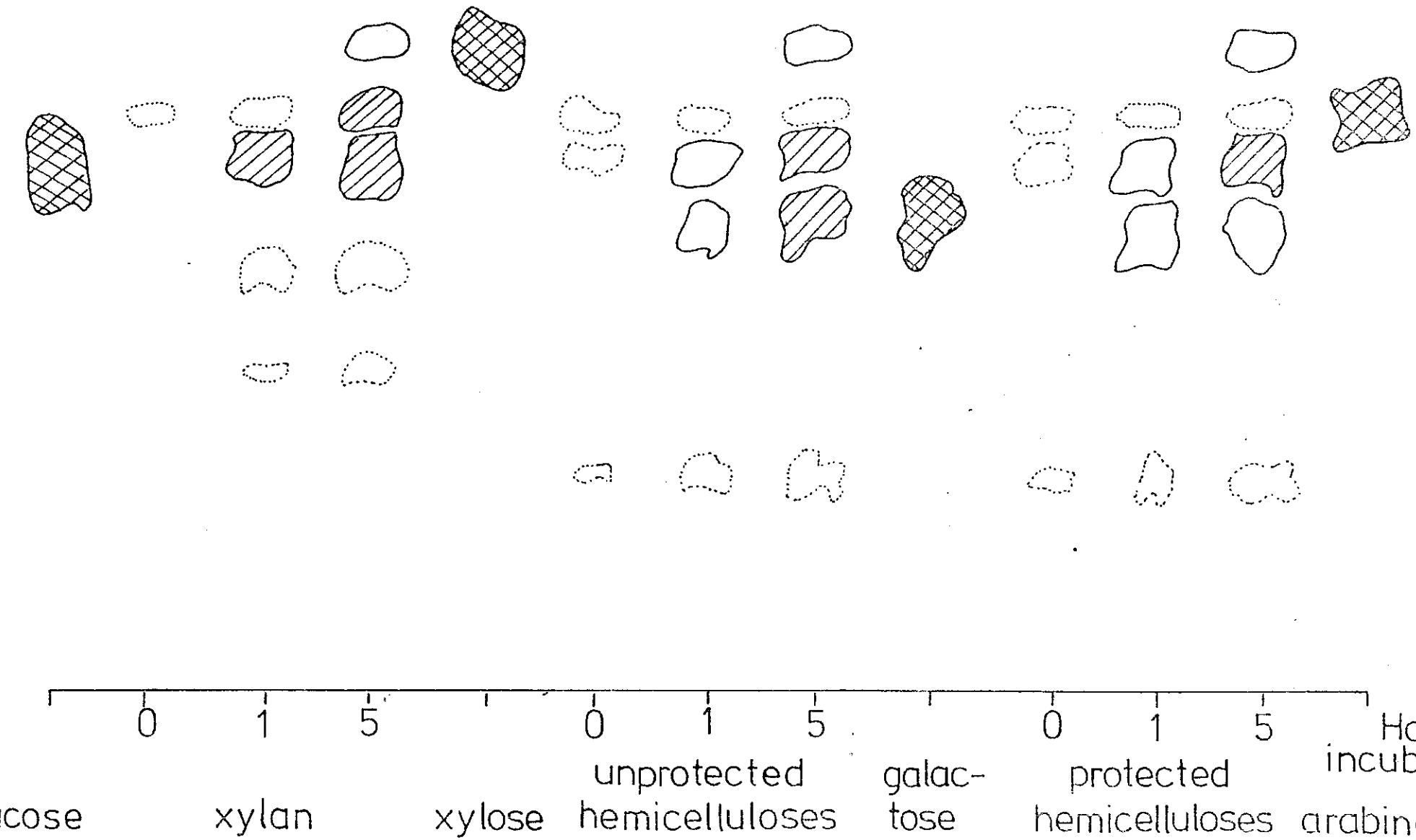


FIG. 5.17 Reaction products of enzymes on xylan and cucumber hemicellulosic substances



Degradation products of xylan appear to be mainly glucose, arabinose and xylose with traces of slower moving substances, possibly oligomers.

Degradation products of extracted cucumber hemicelluloses were different to xylan from larch in that the main products were glucose, galactose and xylose with only trace amounts of arabinose. A slower moving spot was thought to be galacturonic acid.

Although the extracted hemicelluloses contained xylose, glucose and arabinose, it seems likely that there was a residual quantity of pectic substances still present.

There was no significant qualitative or quantitative difference between unprotected or protected hemicellulose degradation products.

12. Other enzymes

Culture filtrates of C. lagenarium were tested for activity on a number of p-nitrophenyl substrates. Reaction mixtures containing 2.0 mℓ substrate (0.1%) in buffer and 1 mℓ of dialysed culture filtrate were used.

Table 5.4 shows the activities expressed as µg of p-nitrophenyl released per minutes per mℓ of enzyme.

Table 5.4: Enzyme activity on p-nitrophenyl substrates

Substrate	Enzyme activity ($\mu g\ min^{-1}\ m\ell^{-1}$)
α -D-galactopyranoside	0.963
β -D-galactopyranoside	0.923
α -L-arabinofuranoside	0.593
β -D-xylopyranoside	0.056
β -D-glucopyranoside	4.2
β -D-glucuronide	0
β -D-mannoside	0

Table 5.4 shows that C. lagenarium produces a wide range of enzymes in culture capable of degrading a large number of substrates.

DISCUSSION V

Enzyme preparation from C. lagenarium culture filtrates degraded sodium polypectate, citrus pectin, xylan, araban and carboxymethylcellulose. From this it is assumed that culture filtrates contain pectic enzymes, xylanase, arabanase and carboxymethylcellulase. Citrus pectin and sodium polypectate were both degraded by trans-eliminative cleavage, it was not determined whether this was by the same or different enzymes.

Pectate trans-eliminase was the major pectic enzyme produced although pectin trans-eliminative activity and small amounts of polygalacturonase (PG) were detected. This may or may not reflect the in vivo synthesis of these enzymes.

PG has been shown to be the major enzyme produced by Colletotrichum lindemuthianum cultured on Red Kidney bean hypocotyl cell walls (English et al. 1971) and by Fusarium oxysporum f. sp. lycopersici (Jones et al. 1972).

Trans-eliminase activity was the major component of the enzymes produced by Colletotrichum musae on bananas in storage (Shillingford and Sinclair, 1980).

The pH of the environment in which pathogens grow may influence the stability of the enzymes produced. Trans-eliminase production is affected in this way (Bateman, 1966; Bateman, 1967; Perley and Page, 1971). Hancock (1966) reported synthesis of pectate lyase by Colletotrichum trifolii at alkaline pHs. Diseases caused by various Fusarium species are reported to be associated with a shift to alkaline

pHs and pectic lyase production (Bateman, 1966; Hancock, 1968a; Mullen and Bateman, 1971). This work shows production of lyase to be greater than that of polygalacturonase. One possible explanation may be the alkaline pH of cucumber sap.

The pH optima for pectic hydrolases are generally acidic whereas those of trans-eliminases are normally alkaline (Bateman, 1966).

Pectic enzymes produced by C. lagenarium had hydrolase activity optimal at c pH 5.0 and lyase activity optimal at c pH 9.0.

Xylanase produced by C. lagenarium had an acidic pH optimum (pH 5.7) similar to the optima described for other xylanases (Cooper et al. 1978; Inaoka and Soda, 1956; King and Fuller, 1968).

Arabanase exhibited a wide pH optimum from 4-6, similar to that described for Verticillium albo-atrum (Cooper et al. 1978), as did cellulase, between pH 3-9 with an optimum of pH 6.3.

Reduction in polygalacturonase activity by Ca^{2+} has been reported (Bateman, 1964; Rombouts and Pilnik, 1972). Conversely, trans-eliminase activity is normally activated by Ca^{2+} (Bateman, 1966; Rombouts and Pilnik, 1972). Results of this work agree with these findings with hydrolase activity on NaPP reduced by Ca^{2+} (possibly due to the formation of insoluble calcium pectate) and lyase activity on both NaPP and pectin increased.

Optimal Ca^{2+} concentration was about 0.02 M which is of a similar magnitude to that reported for pectin lyase produced by Verticillium albo-atrum (Cooper et al. 1978).

C. lagenarium pectate lyase reduced substrate viscosity by 50% corresponding with approximately 1% release of reducing sugars. 'Endo' enzymes usually reduce substrate viscosity by 50% by cleavage of c 0.5-2% of the available bonds whereas 20% cleavage or more may be necessary for an 'exo' enzyme to induce similar viscosity loss (Rombouts, 1972). This enzyme behaved in a typical 'endo' manner.

The Michaelis constant (k_m), generally assumed to approximate a substrate constant (see Results), showed pectate trans-eliminase of C. lagenarium to have a greater affinity for NaPP than pectin trans-eliminase for pectin.

The major enzyme present in filtrates of liquid cultures of C. lagenarium grown on cucumber cell wall material is an endo-pectate trans-eliminase which is highly sensitive to Ca^{2+} concentration and has an optimum pH of 9.1.

English et al. 1971 found that cell wall material isolated from tissues susceptible to Colletotrichum lindemuthianum stimulated the fungus to secrete relatively large quantities of α-galactosidase. Material extracted from roots resistant to infection by C. lindemuthianum failed to induce high levels of the same enzyme. The amount of α-galactosidase secreted by the fungus cultured on different types of cell walls was not proportional to the amount of galactose in the cell wall polysaccharide from the two sources. It was suggested that the differential ability to stimulate α-galactosidase may have reflected differences in the structures of the galactose - containing cell wall polymers.

In this work, no differences were detectable in the enzymes induced by cell wall material from protected or unprotected cucumber plants, neither was there any difference in the ability of enzymes of C. lagenarium, induced on healthy cucumber cell walls, to degrade polysaccharides from either source.

Similar amounts of pectic and hemicellulosic material were extracted from unprotected and protected cucumber cell walls. The Michaelis constant (k_m) was also similar for material from both sources when used as substrates, and analysis of degradation products by t.l.c. or paper chromatography showed no qualitative difference or difference in rate of appearance of products.

It is likely that enzymes degrading polysaccharides in vivo would be restricted by the availability of their particular substrate. Early degradation by pectic enzymes would open the way for hemicellulases and cellulases. This may be reflected by the sequential synthesis of polysaccharidases on cell walls in vitro (Cooper and Wood, 1975; English et al. 1971; Mullen and Bateman, 1976) and in vivo (Heale and Gupta, 1972; Heath and Wood, 1971; Weste, 1970). Results of this work show pectic enzymes to be detectable after 2 days, reaching a plateau at 4 and 6 days for PG and trans-eliminase respectively. This was followed by increases in xylanase after 10 days. These findings agree with results of other workers.

Deese and Stahmann (1962a, b) reported that substantially more PG is produced by Fusarium oxysporum when the organism is cultured on intact stem tissue of susceptible tomato plants than on resistant tissue. Results supporting these were obtained by Mussel and Green (1970). In

contrast, a number of authors have been unable to correlate varietal resistance or susceptibility of an host to the cell wall composition (English et al. 1972; Langcake and Drysdale, 1975).

The results of this section suggest that the composition of cell walls from protected and unprotected cucumber plants are similar as they were degraded at the same rate and produced similar decomposition products. In addition, they induced similar amounts of wall degrading enzymes when used as a carbon source for C. lagenarium in culture.

It is unlikely that systemically induced resistance to C. lagenarium in cucumber is due to changes in cell wall composition, preventing degradation by either pectic or hemicellulosic enzymes.

Section 6. Histology

Cucumber plants were grown in John Innes (No. 2) compost and their first leaves were inoculated with a suspension of C. lagenarium spores (1 x 10^7 $m\ell^{-1}$) after 15 days. Second leaves were challenge inoculated with C. lagenarium, as described in Materials and Methods, after a further 9 days. Leaf disks (4 mm) were taken from systemically protected and unprotected challenged sites at various intervals following the challenge. Disks were cleared with methanol-lactic acid-chloroform (1:1:1) or 95% ethanol and prepared for microscopy as described earlier.

To assess germination of C. lagenarium in cucumber sap, exudates from systemically protected and unprotected plants were collected. Stems were cut above the inoculated first leaves and a plastic 'auto-pipette' tip placed over the cut end to prevent drying out. Sap which accumulated in the pipette tip could be easily collected. Between 0.5-1 $m\ell$ could be collected from 4 plants in this way in approximately 1 hour. Exudates were filter sterilised and used for spore germination tests as described in Materials and Methods, and compared to water.

In spore germination tests on slides, spores of C. lagenarium began to germinate after 4 hours. This was seen as a slight protrusion from one end of the conidium. After 8 hours, spores in water had formed appressoria. Spores in sap, however, had formed a small germ-tube but no appressoria. These germ-tubes continued to grow in both protected and unprotected sap. After 20 hours a highly branched mass of mycelium covered the slide, but still no appressoria had formed; (see Plate 1). Sap from protected cucumber plants did not inhibit fungal growth.

Germination on leaf tissue was similar to that in water on slides. Appressoria were formed immediately at the end of conidia, or at the end of a short germ-tube (see Plate 2). Penetration occurred in unprotected tissue after 48 hours (Plate 3). Over the following 72 hours, secondary hyphae grew through adjacent cells until after 5 days the entire inoculated area contained hyphae (Plates 4, 5, 6 and 7). Seven days after challenge inoculation the unprotected tissue had many acervuli with spore masses and dark brown setae pushing through the leaf surface (Plate 8).

On protected leaf disks, appressoria formation and penetration occurred at the same time as on unprotected disks (Plates 9 and 10). Far fewer penetrations were seen (only 7% of conidia compared to 25% in unprotected disks). Further growth after penetration was severely restricted.

When tissue was stained with toluidine blue for lignin after 3 days individual cells, and small groups of cells, in both protected and unprotected disks, took up the stain (Plates 11 and 12). Four days after inoculation unprotected disks stained diffusely whereas staining in protected disks remained restricted to groups of cells (Plates 13 and 14).

In unstained disks, clear haloes were observed around some appressoria on protected disks, 4 days after inoculation (Plates 15 and 16). When stained with phloroglucinol/HCℓ (as described in Materials and Methods) the haloes stained mauve indicating that they probably contained lignin. Haloes were more evident 5 days after inoculation (Plates 17-20). Unprotected tissue after the same period was well colonised and

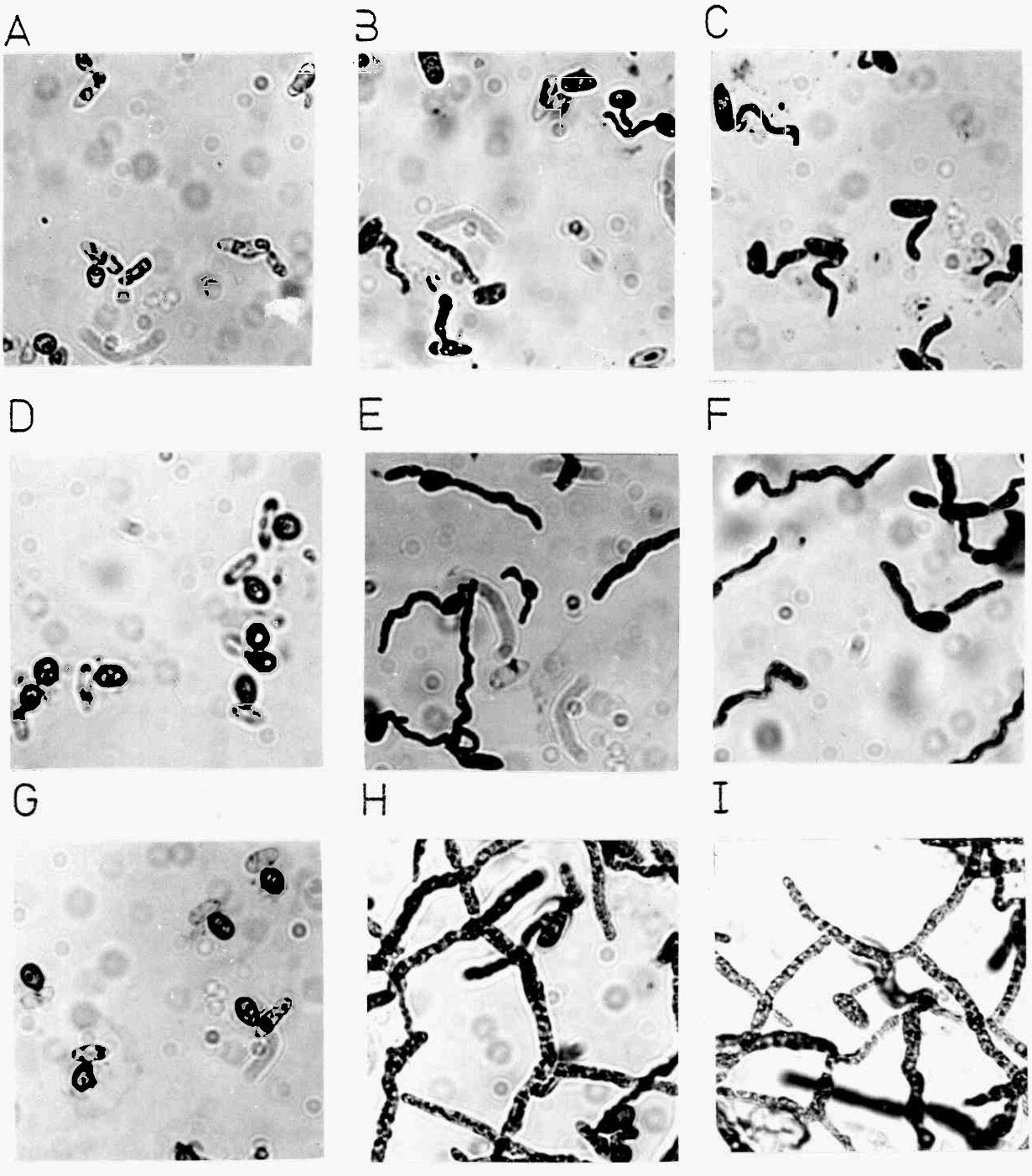
staining with phloroglucinol/HCℓ was very diffuse. It was not possible to find similar haloes. When protected disks were stained with aniline blue and observed by epifluorescent microscopy, a number of haloes were seen to fluoresce indicating the presence of callose material (Plates 21 and 22).

Protected tissue also showed granulation and browning indicating possible hypersensitive death of individual cells (Plate 23). These cells also stained with phloroglucinol/HCℓ (Plate 24). There was not always obvious evidence of penetration of these cells and granulation did not always occur where there was penetration.

Plate 1: Germination of C. lagenarium spores in sap from healthy and protected plants.

Frames A, D and G show germination of C. lagenarium spores in water after 8, 12 and 20 hours.

Frames B, E, H and C, F, I show germination of spores in sap, collected from unprotected and protected cucumber plants, after 8, 12 and 20 hours.



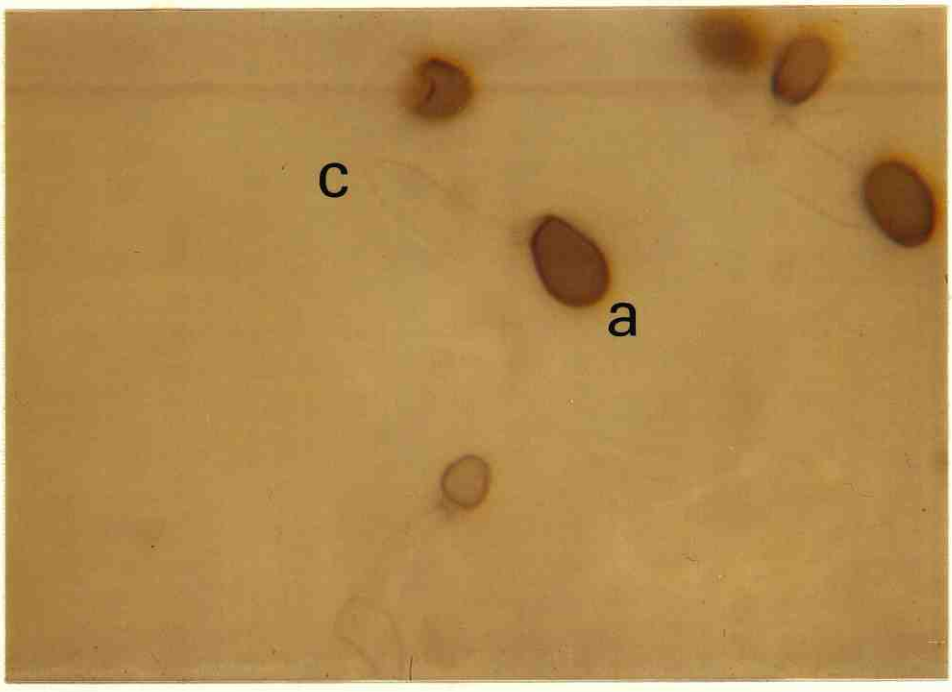


Plate 2

Appressoria formed at the end of a conidium 24 hours after inoculation of unprotected tissue. x 1000

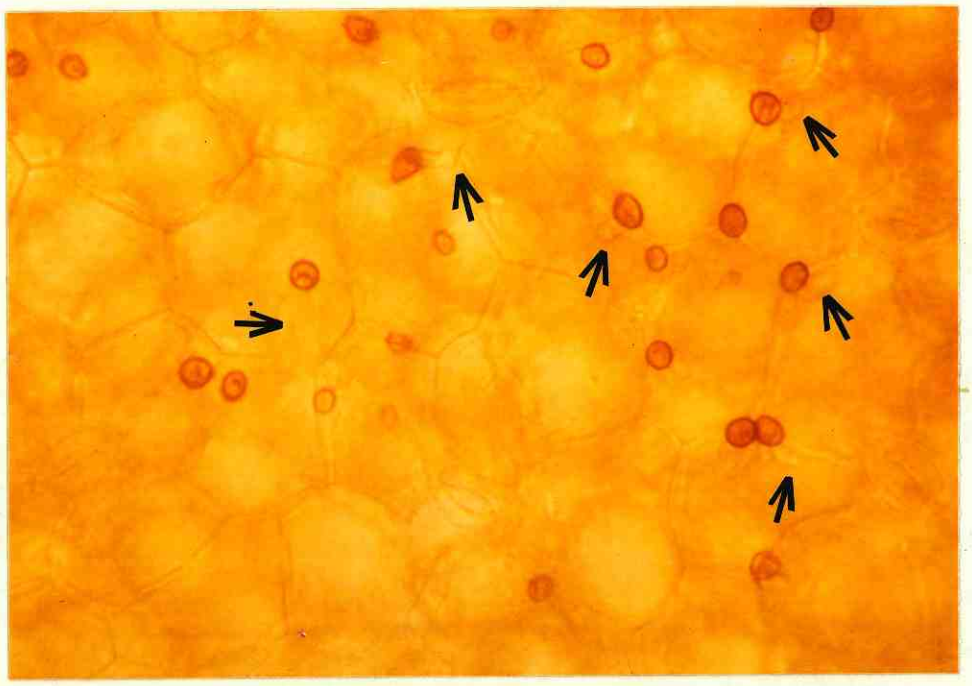


Plate 3: Penetration by infecting hyphae (arrowed) of unprotected tissue 48 hours after inoculation. x 400

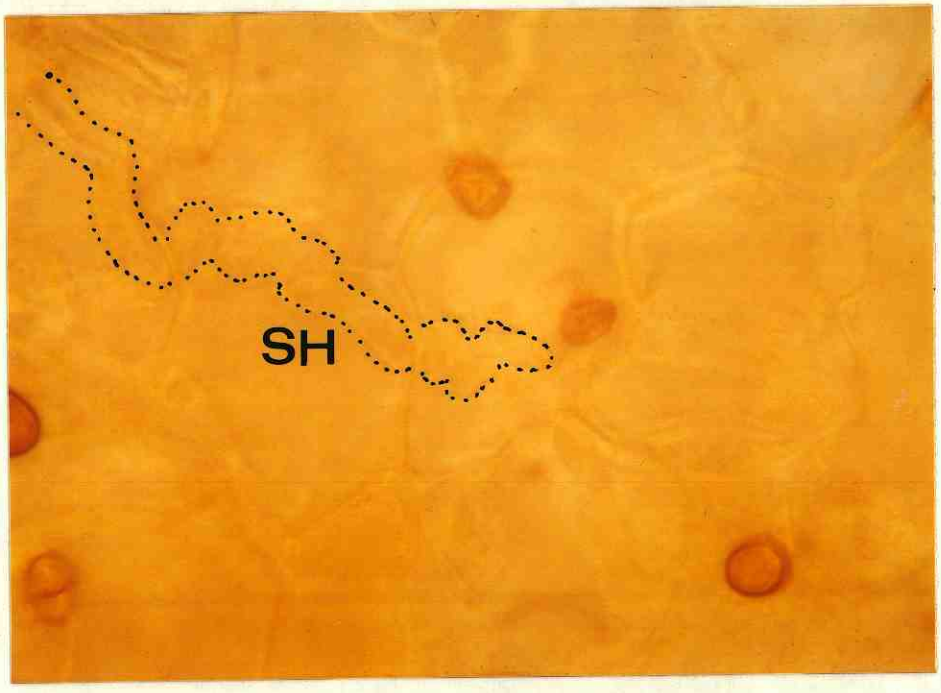


Plate 4: Secondary hyphae (SH) ramifying through epidermal cells of unprotected tissue 72 hours after inoculation. x 1000

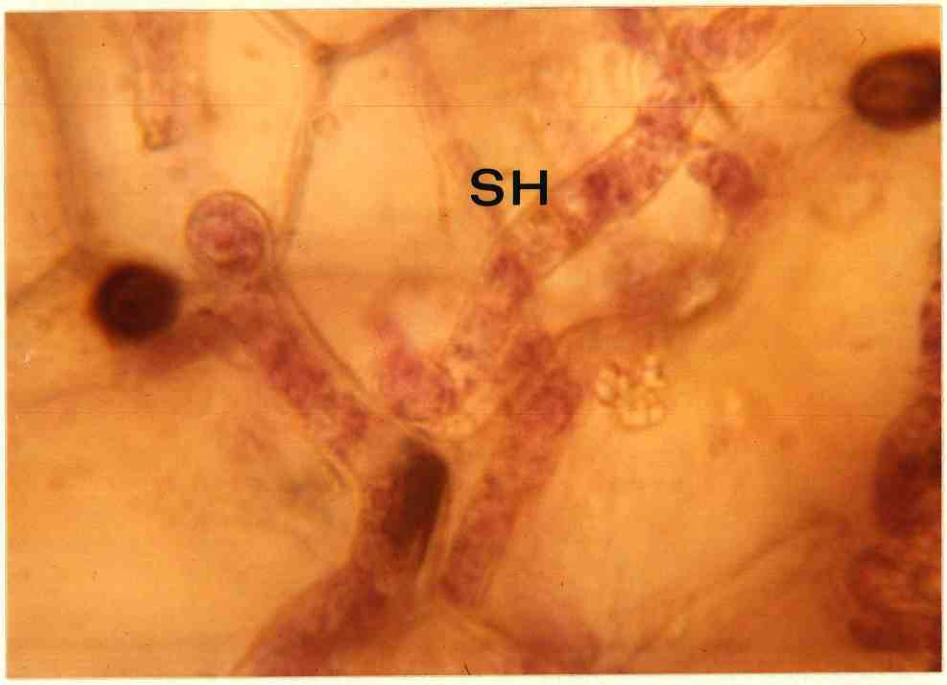


Plate 5: Secondary hyphae (SH) ramifying through epidermal cells of unprotected tissue 96 hours after inoculation. x 1000

Plate 6: Unprotected tissue 120 hours after inoculation. x 400

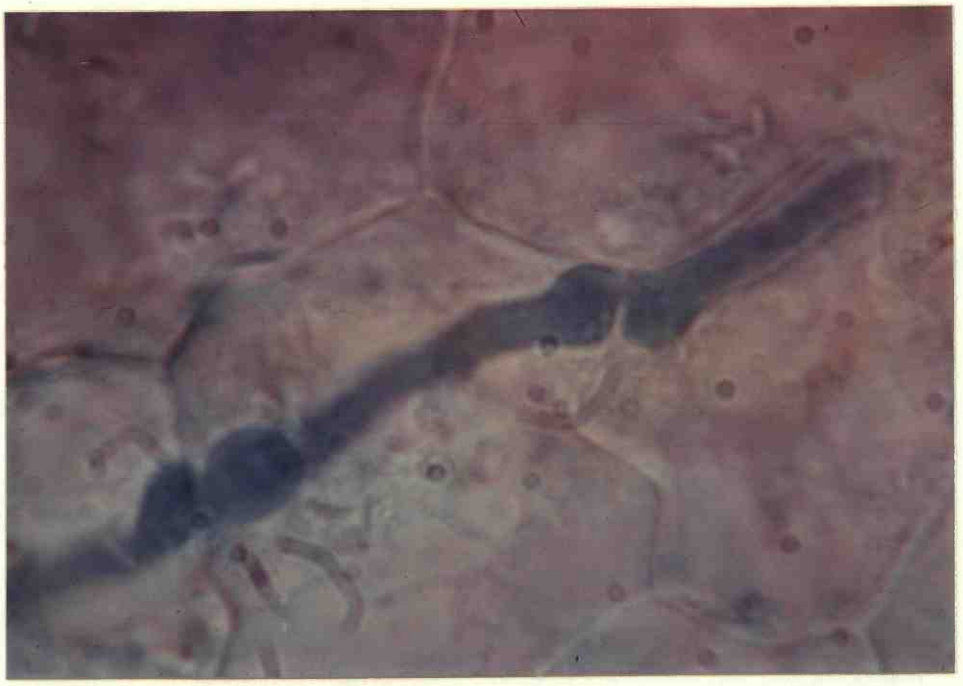


Plate 7: Secondary hyphae constricting to pass through epidermal cell wall. x 1000

Plate 8: Unprotected tissue 7 days after inoculation showing acervuli with spore masses and dark brown setae. x 100

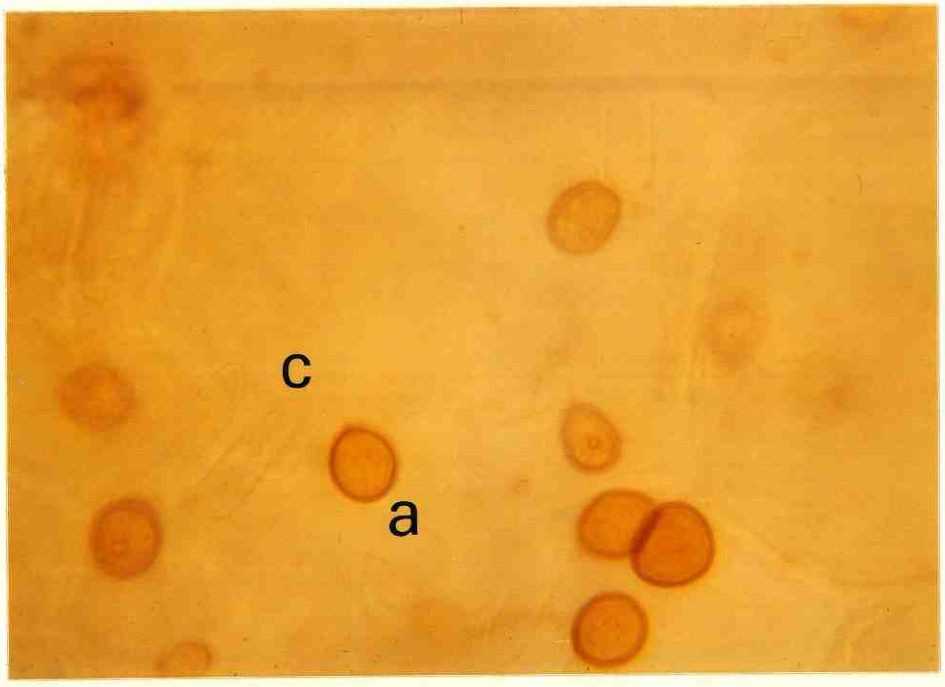


Plate 9: Appressoria formation 24 hours after inoculation of protected tissue. x 1000

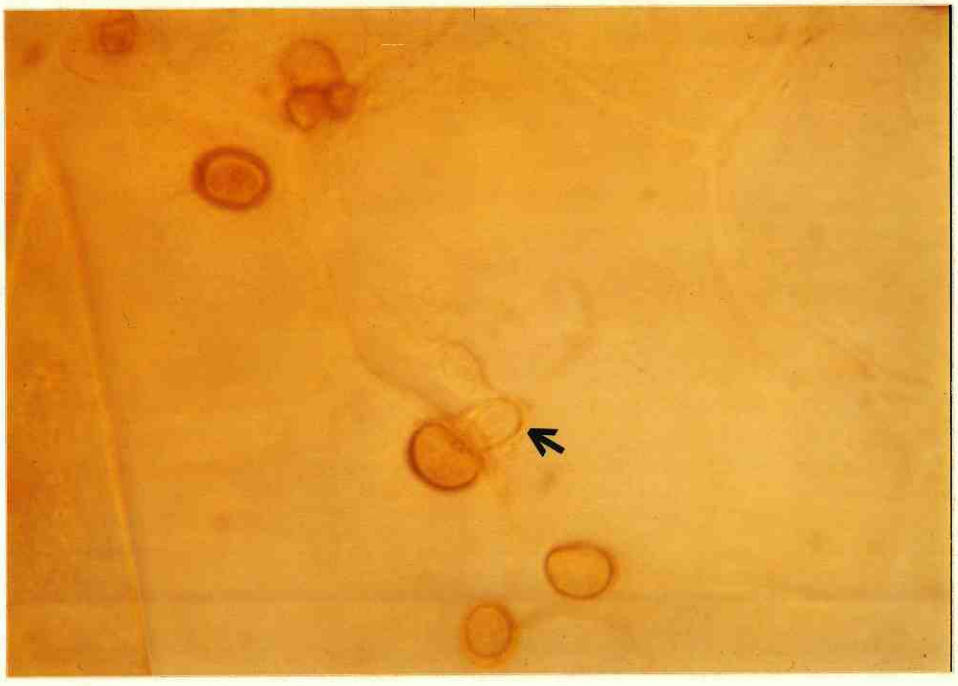


Plate 10: Penetration by infecting hyphae (arrowed) 48-72 hours after inoculation of protected tissue. x 1000

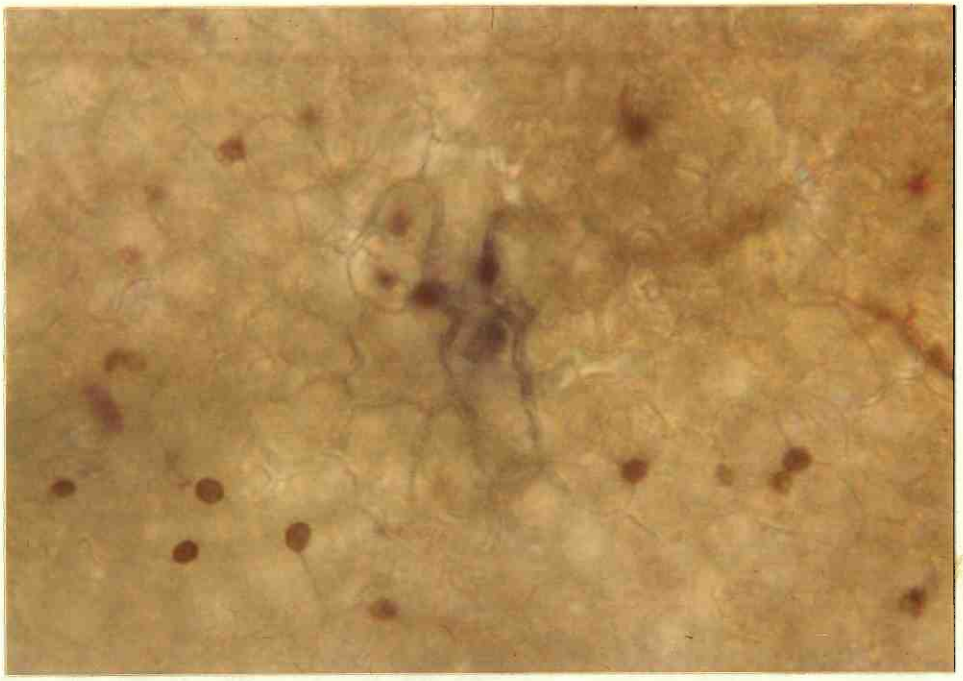


Plate 11: Unprotected tissue stained with toluidine blue 3 days after inoculation. x 400

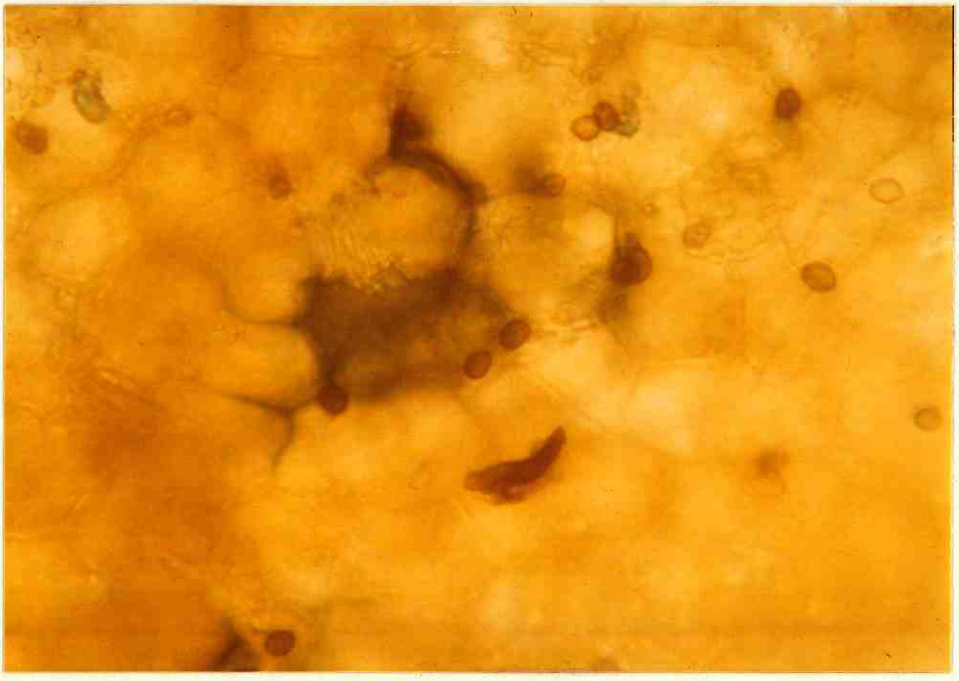


Plate 12: Protected tissue stained with toluidine blue 3 days after inoculation. x 400

Plate 13: Diffuse staining of unprotected tissue with toluidine blue 4 days after inoculation. x 400

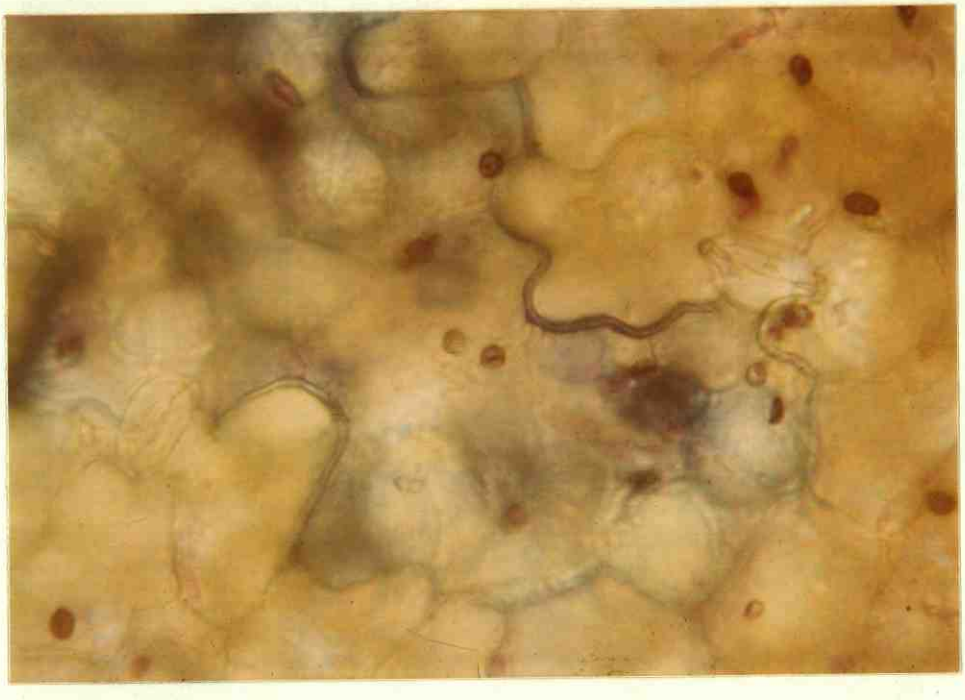


Plate 14: Staining of protected tissue with toluidine blue 4 days after inoculation restricted to groups of cells. x 400

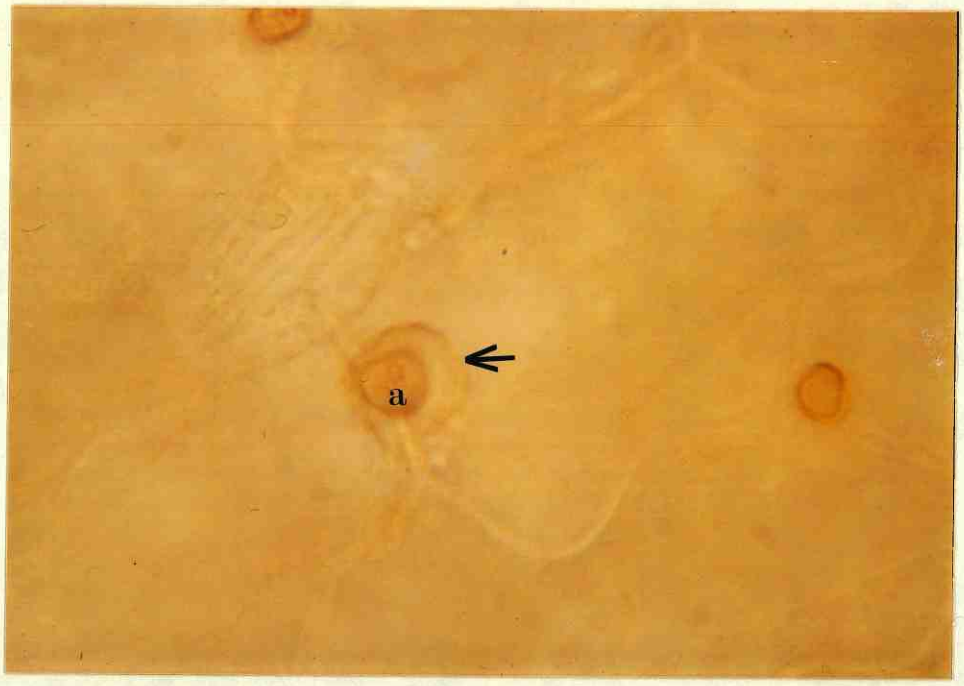


Plate 15: Protected tissue 4 days after inoculation showing a halo around an appressorium. x 1000

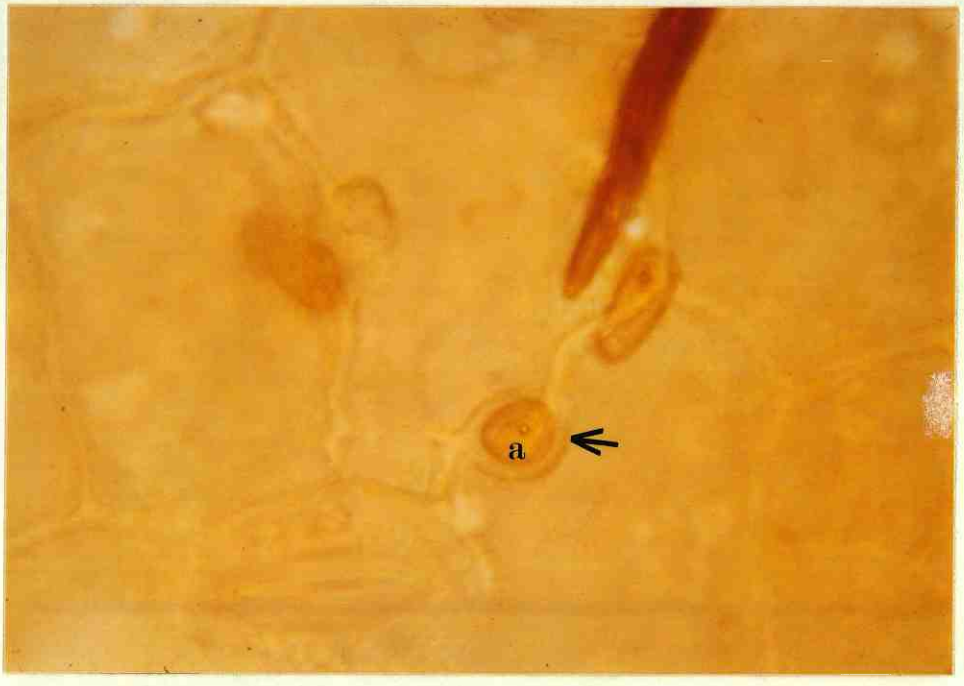


Plate 16: Protected tissue 4 days after inoculation showing a halo around an appressorium. x 1000

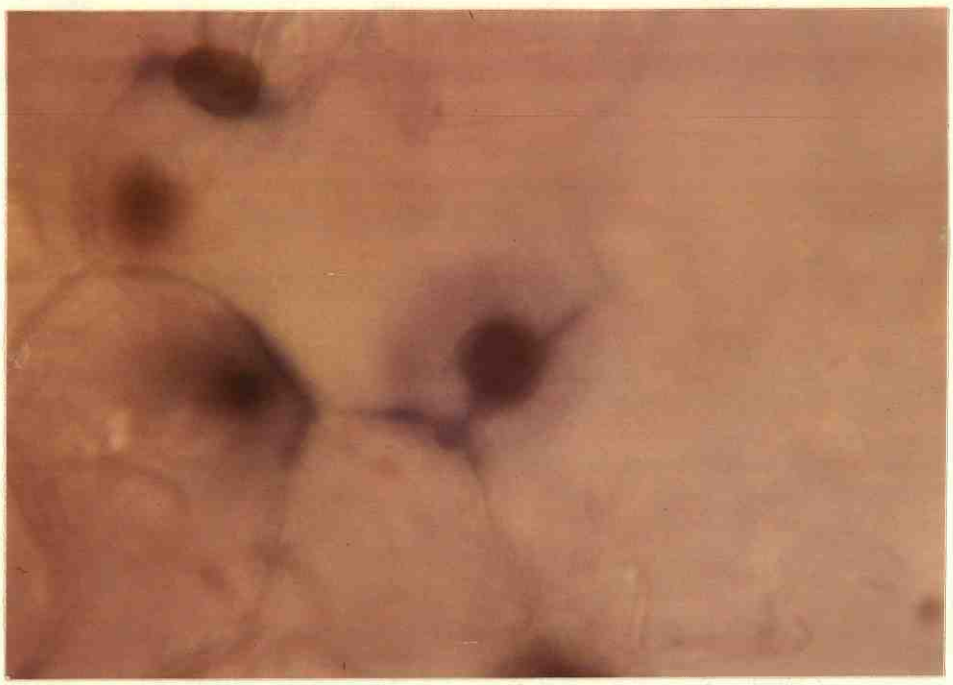


Plate 17: Protected tissue stained with phloroglucinol/HCℓ 5 days after inoculation showing a possible lignified halo. x 1000

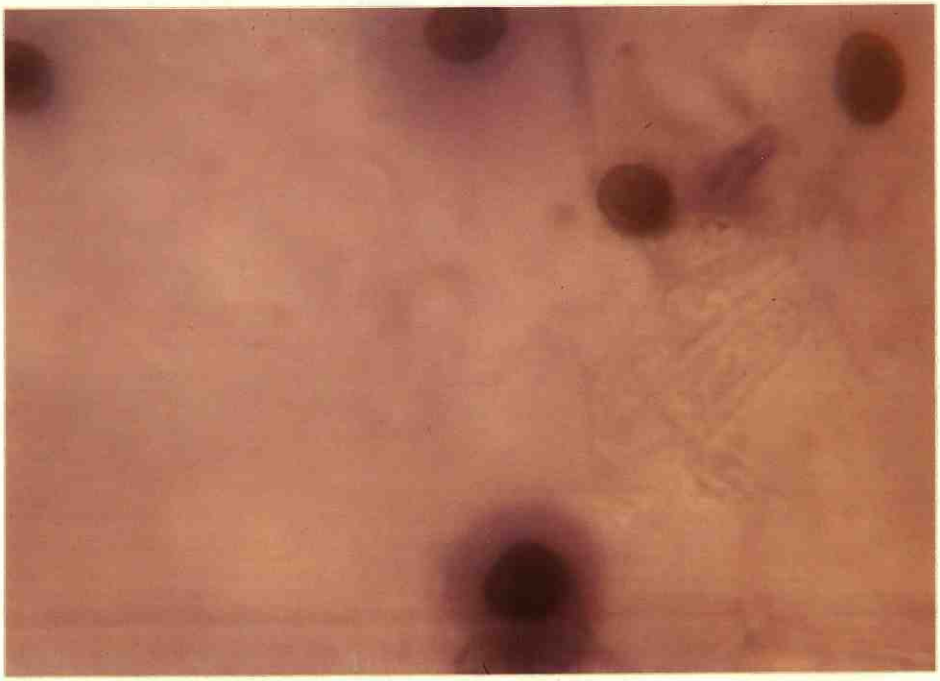


Plate 18: As plate 17. Two appressoria with haloes, two without. x 1000

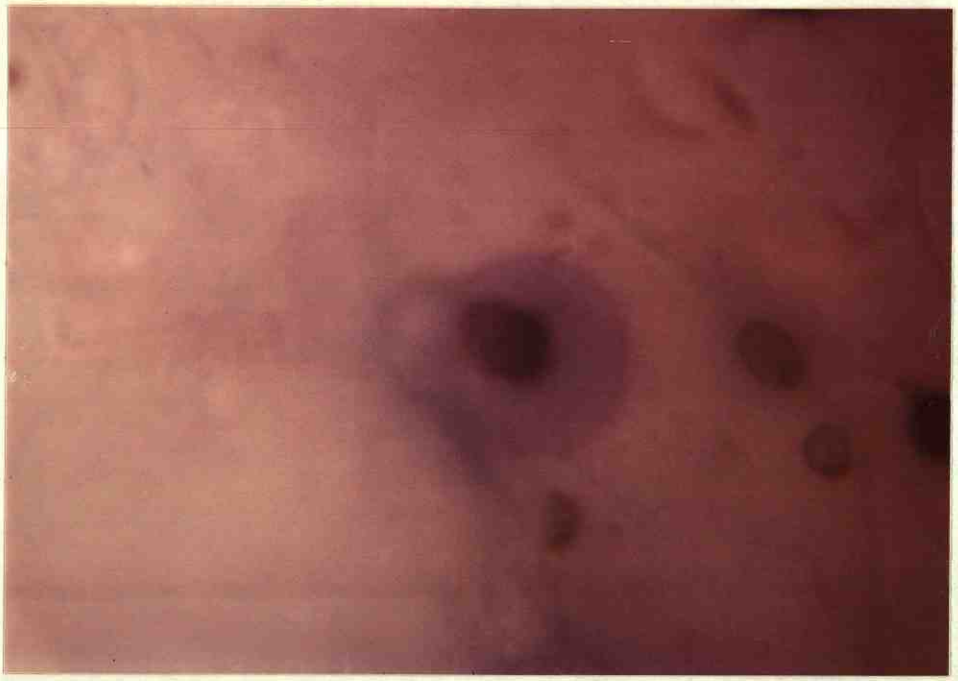


Plate 19: Large phloroglucinol/HCℓ staining halo around appressorium 5 days after inoculation of protected tissue. x 1000

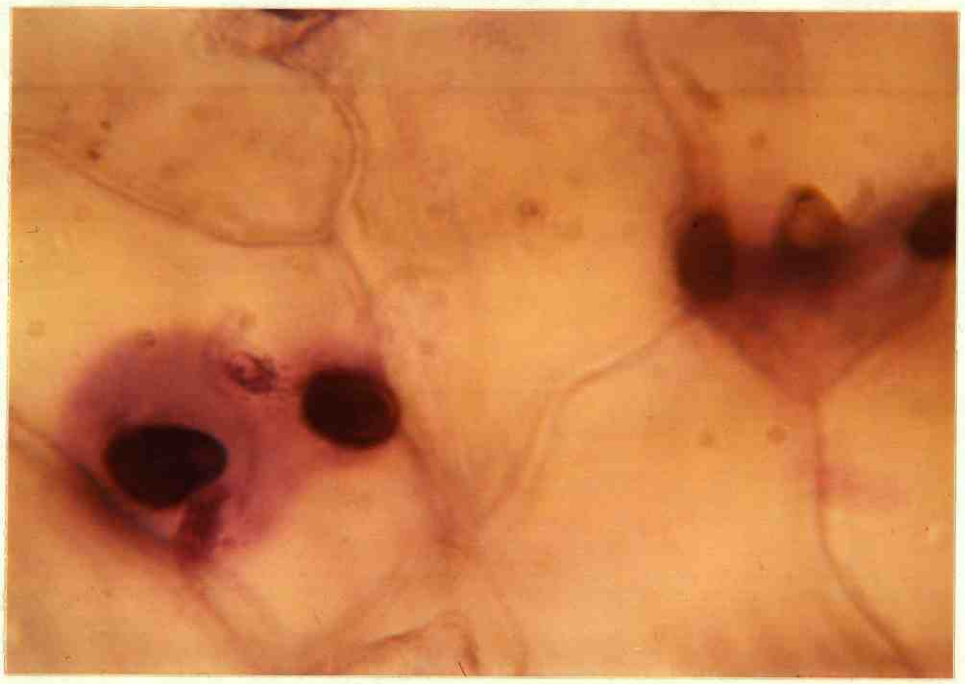


Plate 20: As plate 19. x 1000

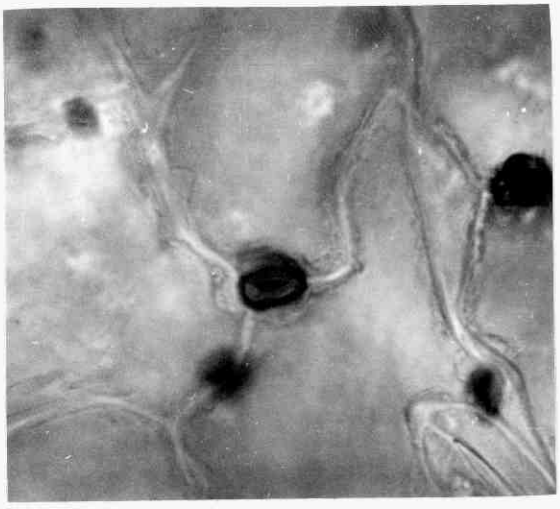


Plate 21: Protected tissue 5 days after inoculation showing clear haloes (arrowed). x 1000

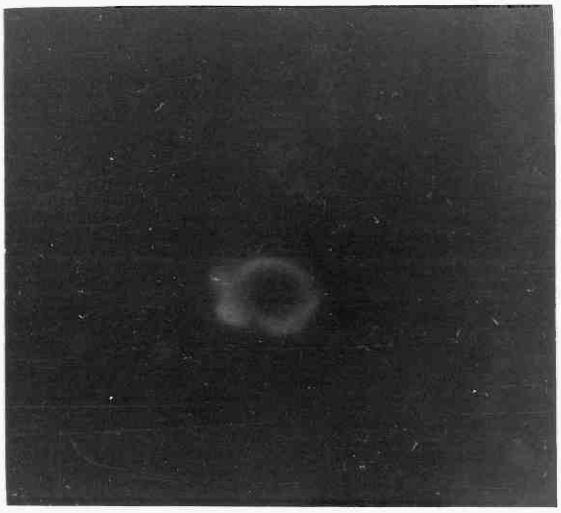


Plate 22: Plate 21 stained with aniline blue and observed by epifluorescent microscopy indicating the presence of callose in the haloes. x 1000

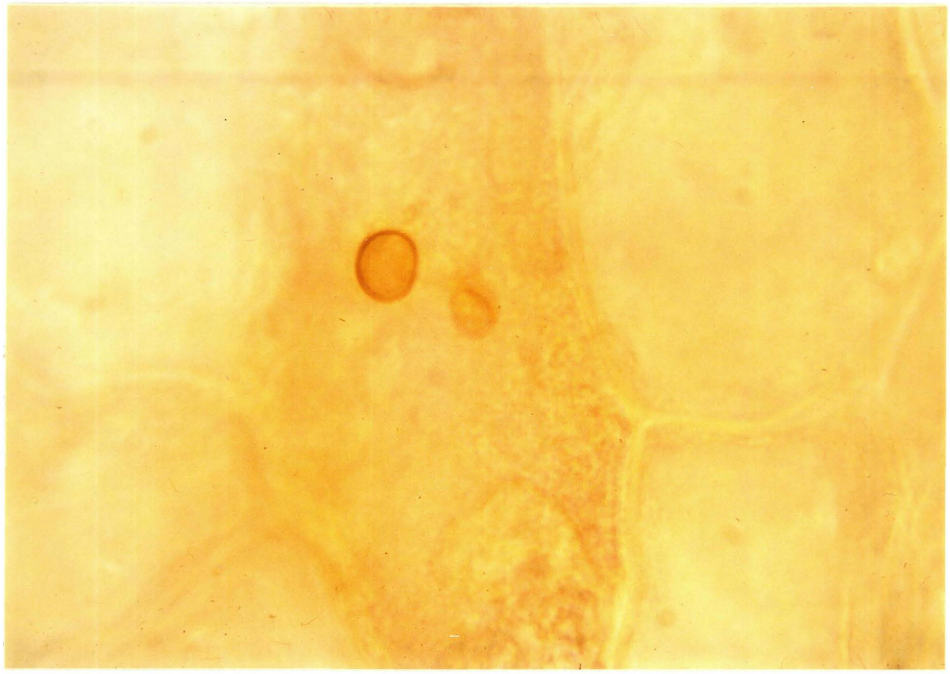


Plate 23: Granulation of protected cell 5 days after inoculation x 1000

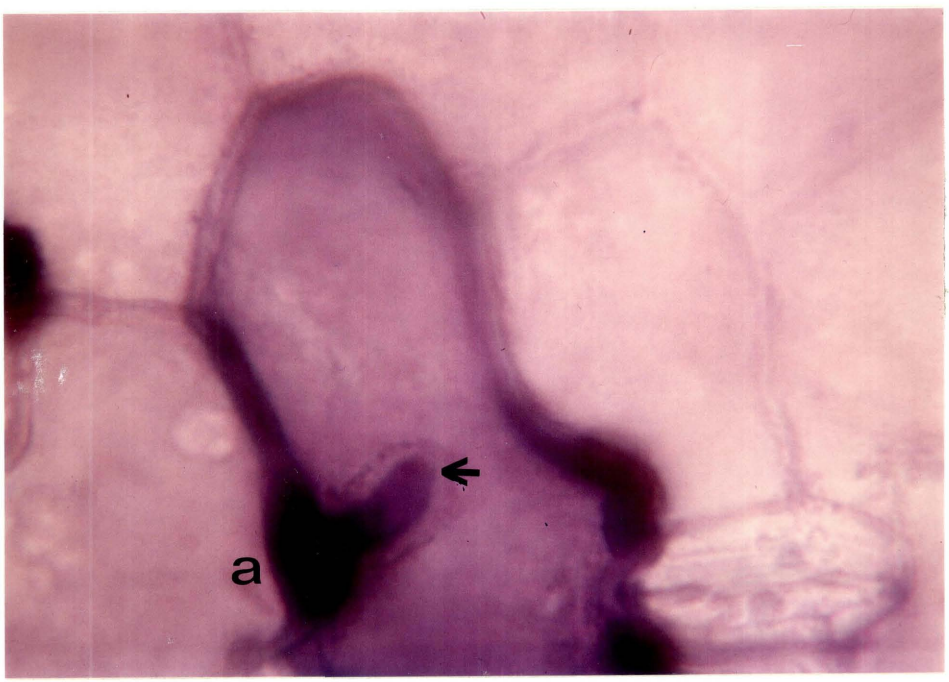


Plate 24: Protected tissue with appressorium (a) and infection hyphae (arrowed) stained with phloroglucinol/HCℓ 5 days after inoculation x 1000

DISCUSSION VI

Appressoria formation by C. lagenarium was not significantly different between protected and unprotected cucumber tissue.

Direct penetration of leaves was observed after approximately 48 hours. Spores often settled in the depressions between epidermal cells and formed appressoria above vertical cross-walls. No stomatal penetration was observed even when spores and appressoria were attached to guard cells. Visible lesions appeared between 4-7 days on unprotected cucumber tissue. This infection process was similar to that described for Colletotrichum orbiculare (= C. lagenarium) on water-melon by Anderson and Walker (1962).

Busch and Walker (1958) in their studies of cucumber anthracnose found that penetration occurred equally well in both resistant and susceptible leaves, but that the progress of the hyphae was much slower in resistant tissue. It was found in the present work that penetration by C. lagenarium was reduced to c. 7% in systemically protected tissue compared with c. 25% in unprotected tissue. This confirms the findings of Richmond et al (1979). Protected tissue rarely showed more than one or two cells of the epidermis infected by hyphae from one appressorium. Usually, the infection hyphae stopped before reaching the opposite wall of the penetrated cell. This is similar to the infection process described by Anderson and Walker (1962) in genetically resistant water melon varieties and by Busch and Walker (1958) in resistant cucumber.

The initial stages of infection of protected and unprotected cucumber tissue, ie appressoria formation and time of penetration, were the same

for both tissues. The number of penetration sites and mycelial development was much reduced in protected tissue.

Formation of papillae is thought to be one of the mechanisms by which plants may resist penetration by fungal hyphae (Aist and Israel, 1977). Ride (1980) showed that lignified papillae and haloes from Botrytis cinerea inoculated wheat leaves were highly resistant to in vitro degradation by fourteen fungal species. Henderson and Friend (1979) showed that a resistant, but not a susceptible, reaction of certain potato disks was accompanied by a relatively rapid rise in PAL activity and deposition of lignin-like material. Hijwegan (1963) showed that lignification occurred after inoculation of resistant but not susceptible cucumber plants with Cladosporium cucumerinum. He suggested that lignification could be an active form of resistance in host-parasite relationships. Nambu, (1974) (sited in Kunoh and Ishizaki, 1975) found that C. lagenarium induced a clear halo around the penetration points on Ipomoea nil cotyledons suggesting physiological changes in the host cell around the penetration sites.

Histological observations in the present work suggest that lignification occurs in both unprotected and protected cucumber tissue. Lignification appears to lag behind cell penetration as individual cells or groups of cells stain with toluidine blue or phloroglucinol/HCℓ 3 days after inoculation, one day later than the initial stages of cell penetration. Four days after inoculation, unprotected tissue shows a diffuse staining with toluidine blue over most of the inoculated area whereas staining in protected tissue is still restricted to individual cells or groups of cells. Although no quantitative assessment of lignin accumulation was undertaken, histological observations suggest that more stain was taken

up by the unprotected disks. This is similar to recent work by Kuć (1982) in which lignification occurs in susceptible foliage infected with C. lagenarium but only after the fungus has developed in the tissue.

Distinct haloes were evident 4 days after inoculation of protected tissue beneath certain appressoria. These appressoria did not appear to have formed infection hyphae and therefore had not penetrated the cell wall. The haloes stained with aniline blue indicating the presence of β-(1-3) glucan and also with toluidine blue or phloroglucinol/HCℓ for lignin like compounds. Haloes were most obvious 5 days after inoculation in protected tissue. It was not possible to detect similar haloes in unprotected tissue which by the same time was completely colonised by secondary hyphae and producing a diffuse staining reaction.

It was not possible to determine whether the lignin-staining papillae were preventing penetration. They appeared at a later stage than in normal penetration and seemed to be beneath non-developing appressoria. It is not clear whether the haloes are the cause or the effect of non-development. Lignification may however prevent further growth of some infection hyphae due to apparent lignification of individual cells (Plate 24). Not all penetrated cells on protected tissue take up the phloroglucinol/HCℓ stain and not all cells that take up the stain appear to be penetrated.

Lignification may be one of several defence mechanisms operating in protected cucumber tissue against C. lagenarium. Lignification of penetrated cells prevents further development of secondary hyphae and lignified haloes probably prevent later penetration by infection hyphae at that site.

E. SUMMARY

Induction of resistance in cucumber leaves has been shown to occur following necrosis on lower leaves caused by a pathogen. Non-pathogenic organisms, heat-killed conidia or cell-free components of pathogenic fungi do not induce the same response. Induction seems to be triggered by a certain type of necrosis on lower leaves as necrosis caused by mechanical or chemical injury has no effect.

Colletotrichum lagenarium, as the inducer organism, requires as few as three lesions on leaf 1 to cause a reduction in lesion size on leaf 2. Induced resistance could, however, be overcome by increasing the spore concentration of the challenge inoculum.

The alteration in response of systemically protected leaves does not appear to be restricted to epidermal cells as removal of the epidermis prior to the challenge inoculation does not significantly decrease the number of lesions which subsequently develop.

Induction of resistance within a single leaf, following inoculation of the tip, and on second leaves following inoculation of first leaves both require a lag period of about five days. The distances between inducer and challenge inoculations were approximately 2 and 20 cm respectively. One possible explanation is that the inducing inoculation requires five days to establish sufficient infection to stimulate production of a theoretical 'signal' substance.

'Movement' of resistance was mainly from tip to base within a leaf and bottom to top in a plant. Some resistance was apparent in first leaves

following prior inoculation of second leaves. This suggests a phloem-borne 'signal'. Further evidence for this was obtained by shielding light from the inducer first leaf, thereby reducing the movement of substances out of that leaf, preventing the induction of resistance in the second leaf. An alternative explanation of this may be that light is necessary for the production of such a 'signal'.

A detailed chemical analysis of phloem content may help elucidate the nature of a theoretical signal.

Histological studies showed that the initial stages of infection were the same in both protected and unprotected leaves. Formation of appressoria and penetration occur at the same time, although penetration of protected leaves is much reduced. Lignin staining haloes were found in protected tissue five days after challenge inoculation. It was not clear, however, whether these haloes were actually preventing penetration or merely forming under unsuccessful penetration sites. Not all appressoria had lignified haloes around them. Further study on the exact timing of halo formation in relation to penetration is necessary.

C. lagenarium produced many extracellular enzymes in vitro, the main one being an 'endo'-pectate trans-eliminase. This enzyme was able to degrade cell-wall material from both healthy and protected cucumber plants equally well in vitro. This may or may not reflect the situation in vivo.

Investigation of changes in host enzymes showed that levels of peroxidase, ribonuclease and chitinase did not alter significantly in protected tissue. β-(1-3) glucanase activity increased slightly but not

enough to constitute a defense mechanism. Phenylalanine-ammonia lyase (PAL) activity in protected tissue increased by up to 50%. In view of the possible role of lignin as a defense mechanism and the involvement of PAL in lignin synthesis, these increases in activity should be investigated further.

The effects of cycloheximide and ultra violet light on infection by C. lagenarium suggest that both the normal susceptible response and induced resistance in cucumber, not surprisingly, involve some active metabolic process.

6-Benzylaminopurine sprayed on to cucumber leaves reduced lesion size but not the lesion number. Metabolic changes observed in sprayed leaves were not detected in systemically protected tissue. It seems unlikely, therefore, that induced resistance is mediated via the action of the plant growth regulator.

Resistance can be induced locally and systemically (over very short distances) by injection of polyacrylic acid, acetylsalicylic acid and various related compounds. Resistance to C. lagenarium was not evident 24 hours after injection but was after 96 hours. Test substances were not fungitoxic when included in liquid shake cultures. Resistance was not detected in first leaves following injection of cotyledons so it seems likely that the mechanism differs from that observed following infection of cotyledons with a pathogen. It is possible, however, that test substances may in some way mimic the effect of a theoretical 'signal' substance although not being as mobile as a 'signal' substance inevitably must be. Other methods of application of test substances were not so effective which must exclude their use as potential fungicides.

It is clear, though, that further investigation into plant immunisation by prior inoculation or by chemical stimulation of defense mechanisms will indicate ways in which host defense mechanisms can be manipulated to control plant disease.

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