FACTORS AFFECTING THE RESISTANCE AND SUSCEPTIBILITY OF *PHASEOLUS VULGARIS* TO RACES OF *COLLETOTRICHUM LINDEMUTHIANUM*

by

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ABSTRACT

Races of Colletotrichum lindemuthianum on excised French bean hypocotyls formed appressoria and penetrated epidermal cells regardless of the resistance and susceptibility of varieties used. Following penetration, many cells of susceptible hypocotyls were colonized by hyphae but remained apparently unaffected for three or more days. Necrosis then occurred and macroscopic brown lesions appeared. Infections of resistant varieties were normally restricted to single cells which became necrotic soon after penetration; symptoms were minute orange flecks. Two types of cellular response were distinguished in infected resistant varieties; the type found depended on the fungal race used. Hyphal growth (race beta on resistant cultivar Immuna) slowed soon after infected cells became necrotic, but continued for several days. Phaseollin was detected in resistant and susceptible reactions when cell damage occurred and increased as browning intensified. In varieties giving an intermediate response, necrosis occurred after a few cells were infected; symptoms were obvious brown flecks.

Avirulent races of C. lindemuthianum caused localized protection from a virulent race applied later, or up to one day earlier. Protection affected only the growth of intracellular hyphae, not spore germination or penetration. It was not caused by direct antagonism between races. "Protected" host cells seemed abnormally sensitive to the presence of hyphae of the virulent race, and underwent earlier necrosis than did comparable "unprotected" cells. Protectio: was induced specifically

by spores of avirulent races but non-specifically by culture filtrates of all races. The active components in these filtrates were water soluble, dialysable and heat stable.

Tissue was rendered susceptible to avirulent races by prior treatment with chloroform or heat (44-50°C) and also after excision from a large bulk of supporting tissue.

In some types of resistant and susceptible tissue a dense deposit, "reaction material", formed in cells at the site of penetration and hyphae developed no further.

These findings are discussed in relation to different hypotheses concerning processes of resistance.

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INTRODUCTION

The use of varieties which are resistant to attack by particular pathogenic organisms has become an important method of controlling plant disease. Frequently very high levels of resistance resulting in the almost complete absence of symptoms are exhibited by varieties in which the capacity to resist is controlled by a small number of resistance genes. Such resistance is commonly termed varietal or major gene, resistance. The operation of this relatively simple genetic system has probably been a major factor in the successful application of plant breeding methods to disease control.

Widespread use of resistant varieties led to the discovery that within many species of plant pathogen, particularly those which are obligate parasites, there exist distinct physiologic races, identical in their morphological characteristics but which vary in their ability to attack different varieties of host. This means that an individual race is able to attack some varieties (i.e. is virulent on susceptible varieties) but fails to cause disease on others (i.e. is avirulent on resistant varieties); each race differs from all others in the result of its interaction with at least one host variety. It follows that the racial identity of a particular isolate of pathogen can be determined by its characteristic pattern of virulence and avirulence on a group of differential varieties.

Clearly, there exists a very high degree of specificity in the ability of races to infect host varieties, and of different varieties to resist physiologic races. The genetic basis of specificity has

been established for some diseases, the most famous example being that of Flax rust (Flor 1942 and 1955) where a set of virulence genes in the pathogen, Melampsora lini appeared to complement the resistance genes of the host; susceptibility only occurred with certain combinations of these genes. In this case, and probably in many others where a relatively simple "gene for gene" system although not established is usually inferred, specificity would appear to depend on the genetic compatibility of race and variety. To understand the mechanism by which these genetic factors are ultimately expressed two major questions must be answered:-

1 What prevents an avirulent race of pathogen from developing in a resistant host? (i.e. what causes resistance?)

2 What events occur during the initial interaction of race and variety which determine whether resistance or susceptibility will result? (i.e. what is the cause of specificity?)

To date, most work has been concerned with the former of the questions. Much of the evidence has suggested that growth of the avirulent race is inhibited during an "active" host response indicated by changes in the appearance, chemical composition or physiological activity of infected host tissues. The causal role in resistance of such changes, can only be finally established when the exact relationship between a specifically defined process and observed inhibition has been determined.

Detailed studies on the mechanism of resistance to an avirulent race have often been made using a "model system", composed of a number of varieties of a single host species and several races of a pathogen.

One such system, C. lindemuthianum on P. vulgaris has been used extensively in this laboratory for the past six years. This was originally chosen by Smith (1967) for a study of the possible role in resistance of the host-produced inhibitor which appeared in drops of spore suspension applied to the cavities of bean pods. There was an added advantage that as C. lindemuthianum is not an obligate parasite it could easily be used in assays of fungitoxicity and grown in axenic culture to yield products of specifically fungal origin. More recently, Dr. J.A. Bailey, Mr. G. D. Lyon and I have studied the interaction between races of C. lindemuthianum and bean hypocotyl tissue. The work of Bailey has been mainly concerned with the detection and quantitative determination of phaseollin, an antifungal compound produced in bean tissue after infection, during the development of resistant and susceptible responses. Lyon has mainly studied the mechanisms of host synthesis, and fungal breakdown, of phaseollin.

Bearing this other work in mind the author has investigated the subject from a biological point of view with four main objectives, treated separately in the four sections of the thesis. These were:-

1) To give a complete picture of microscopic events which occurred during the development of resistant and susceptible responses in terms that could be related to biochemical and other studies

2) To define the effects of normal experimental variables

3) To determine, in tissue treated with spores of an avirulent race ("cross-protection") or fungal products ("acquired resistance"), whether the development of the virulent race in the same tissue was reduced.

4) To determine whether certain treatments could cause a reduction in host response of resistant tissue and thus allow an increased development of the normally resisted race ("acquired susceptibility").

In the past the demonstration of acquired susceptibility, cross protection and acquired resistance was considered to be evidence that the mechanism of resistance involved an active host response. Detailed studies of the factors affecting resistance and susceptibility could possibly provide more information and perhaps suggest the basis of a critical test of an hypothesis concerning the mechanism of resistance. A greater insight into the mechanism of specificity, about which little is known, might also be obtained.

In an attempt to summarize current knowledge concerning the mechanism of resistance of bean to C. lindemuthianum, and cross protection, acquired resistance and acquired susceptibility studies, where these have given information concerning the cause of resistance to this and other organisms, the following Review of Literature has been included.

REVIEW OF LITERATURE

1. The Anthracnose disease of French Bean

a) Symptoms of infection

C. lindemuthianum causes dark brown sunken lesions on infected stems and hypocotyls of P. vulgaris (Walker, 1950). These are limited in size in older tissue but young susceptible hypocotyls can suffer complete collapse. Lesions on pods are light brown and are formed by the enlargement of small necrotic spots to a diameter of a centimetre or more, the fungus often growing through the pod to infect the seeds. Pod and stem lesions are characteristically sunken when mature and bear erumpent acervuli, from which the spores are produced. Leaves show blackening in small spots along the line of the veins surrounded by green to pale brown areas of collapsed lamina. In resistant varieties symptoms are much reduced, often to microscopic red-brown spots, or absent.

b) Races of C. lindemuthianum

Following inoculation tests on different bean varieties, isolates of C. lindemuthianum can usually be assigned to one of four races (alpha, beta, gamma and delta). This race system is thought to define the various forms of the pathogen in Europe and the United States (Hubbeling, 1957) but not in Australia (Cruickshank, 1966) or

Mexico (Yerkes and Ortiz, 1956). Even in Europe some isolates, behaving as a single race on a whole series of varieties, may differ in the result of their interaction with one or two others. For instance, the variety Kievit Koekoek was originally thought to be resistant to all four races and thus was of commercial importance. However some isolates considered to be of races beta and gamma have been found to attack this variety (Bannerot, 1965). Its commercial production has now ceased.

Bean varieties may be susceptible to all races or resistant to one or more. Only the variety Cornell 49-242 and some of the Mexique series are resistant to the four major races (Bannerot, 1965). Resistance in these varieties is controlled by a single dominant gene (Mastenbroek, 1960); the genetics of resistance in others is more complex and involves more than one gene to control resistance to individual races.

c) Histological studies on the infection of susceptible and resistant bean varieties

(i) Germination and penetration

Dey (1919) found that spores normally germinated by means of a single germ tube, the end of which formed a spherical vesicle separated from the remainder by a septum. As this round body, the "appressorium" matured the walls became thicker, darkly pigmented and the base, attached to the cuticle of the host epidermis. Rahe, Kuc, Chuang and Williams (1969a) found no difference in the process of spore

germination on resistant and susceptible varieties. The extent of germ tube growth was influenced by nutrients added to the infection drop (Mercer, Wood and Greenwood, 1970). Increasing concentrations acted to suppress appressorium formation in favour of greater germ tube growth and when the stimulatory effect was very marked very few lesions developed on hypocotyls.

The epidermal cells of bean hypocotyls were entered by means of a fine infection hypha passing between the base of the appressorium and the cell lumen (Dey, 1919 and Mercer, 1970); stomatal penetration occurred very rarely. Dey (1919) could find no evidence of enzyme action on the cuticle but penetration of the cellulose layers was accompanied by their swelling and dissolution suggesting that enzymes were active. The localized action of enzymes around the infection hypha was indicated by the electron microscope studies of Mercer (1970). Penetration of epidermal cells took place in both resistant and susceptible varieties (Leach, 1923 and Sinden, 1937).

(ii) Infection of susceptible varieties

Sinden (1937) suggested that, on reaching the cell lumen, the infection hypha expanded and caused the host plasmalemma to be invaginated around it. Leach (1923) noticed that the hyphae found in cells after infection were of wide diameter and named them "primary hyphae". Primary hyphae characteristically colonized cells intracellularly and became constricted when passing through a cell wall, only to swell again on reaching the next cell. The fungal protoplasm at the tip of the hypha was thought to be "naked" during the process of wall

penetration (Sinden 1937 and Mercer 1970). Penetration was thought to be mechanical by Leach, but Sinden and Mercer found evidence of enzyme action.

Leach (1923) and Sinden (1937) found that cells containing actively growing primary hyphae showed no signs of damage for two or more days after they were first penetrated. Deterioration of cell contents and loss of turgor occurred at about the time when narrower hyphae, which passed through cell walls without any constriction, were first seen. These were termed "secondary hyphae" by Leach. Both workers suggested that wall degrading enzymes were produced by hyphae during their passage through cell walls. Rapid colonization by secondary hyphae of the remaining uninvaded tissue was found to take place and the hypocotyls eventually collapsed and went brown. The death of cells in advance of hyphal penetration was noticed in leaf lamina tissue (Sinden, 1937). Sinden considered the effect to be due to the action of a toxin.

Thus a major feature of growth within a susceptible variety appeared to be the failure of the intracellular hyphae to cause any noticeable effects on host cells until fairly extensive colonization had taken place.

(iii) Infection of Resistant Varieties

Leach (1923) and Sinden (1937) found that soon after penetration, cells of a resistant variety became granular and brown. Hyphae did not grow to colonize other cells. Leach suggested that growth stopped because the fungus was unable to find a specific, essential nutrient, death of the host cell being caused by products of lysis of the starving hypha.

Sinden found that the following sequence of events occurred in penetrated epidermal cells of resistant hypocotyls :-

1 The rate of protoplasmic streaming increased

2 Hyaline granules appeared in the cytoplasm, accumulated round the hypha and became yellow,then brown

3 Movement stopped near the hypha

4 Coagulation, and later browning, of the cytoplasm spread outwards from the site of hyphal penetration.

Coagulation of cytoplasm in the hypha did not occur until after that of the host cell,but growth was inhibited. The rate of cell reaction and the extent of hyphal growth varied from cell to cell and with age, variety, race and the tissue; occasionally small lesions were formed.

A decrease in pH (detected with methyl red), a strong oxidizing reaction (detected with rosaniline) and the presence of catechol tannins (detected with ferric alum - in walls and granules) were found in responding cells. Diffuse browning and phenolic compounds were detected in adjacent cells.

Sinden concluded that the fungus died, after the host cell, because of the action of some phenolic substance and not through starvation. This phenol was thought to be produced from a precursor which was present in high concentrations (to explain the very rapid cell reaction) possibly in the vacuole, the role of the fungus being in "irritation" rather than directly synthesizing the inhibitor. The incompatibility of host and pathogen proteins,

or metabolic products of the fungus, were considered likely causes of irritation. It was thought that virulence and avirulence might be determined by the ability or failure of a race to produce an irritant or by differences in the sensitivity of races to this fungitoxic substance.

The hypothesis, that resistance might be due to an active response of the host cell, in which both the production of an antifungal compound and the death of the cell takes place, was not new. Observations on similar reactions in plants infected by rust fungi (Gibson 1904, Ward 1905, Marryat, 1907 and Allen, 1923) led to similar conclusions.

Stakman (1915) referred to the "abnormally rapid death of host plant cells" as "hypersensitivity". This definition for a term which is in common use, often to imply an actual mechanism of resistance, still seems to be the most useful. It would apply to the response of cells of a resistant variety in the work of Leach (1923) and Sinden (1937).

d) Biochemical studies

More recently attempts have been made to isolate fungitoxic compounds from beans infected with C. lindemuthianum. Six slightly inhibitory, UV fluorescent compounds were detected in infected bean seedlings by Romanowski, Kuc and Quackenbush (1962). These were thought to be phenols of plant origin (none was found in extracts of mycelium), and

were produced in both resistant and susceptible reactions. Three, including the compound which gave the largest zone of inhibition, were also found in healthy and damaged seedlings. Thus merely the production of fungitoxic phenols could not explain why some varieties were resistant and some susceptible.

Rahe, et al (1969a) studied the relationship between changes in phenol metabolism and the appearance of visible symptoms of host response. Total phenol content, the activity of phenylalanine ammonia lyase (P.A.L.) and the presence of specific phenols, separated by thin layer chromatography, were used as an indication of these metabolic processes. The enzyme P.A.L. is involved in the biosynthesis of phenolic compounds.

In hypocotyls, inoculated with either the virulent or avirulent race, P.A.L. activity and total phenol content were found to increase after infection. In addition a number of phenolic compounds, not present in uninoculated tissue, were detected on the chromatograms. Although these changes occurred during both resistant and susceptible reactions, the times at which they were first detected differed (36 - 48h in the resistant and 72 - 84h in the susceptible). Histological studies showed that these times roughly corresponded to the death of infected cells, and the period of increase, to the gradual appearance of visible symptoms (minute brown flecks in the resistant and dark brown collapsed areas of hypocotyl in the susceptible). In susceptible hypocotyls, the apparent lag between penetration (at ca 48h) and stimulation of phenol synthesis (72h onwards) corresponds to the period when intracellular colonization by primary hyphae is taking place. As

noted earlier host cells at this stage rarely show any signs of damage. Thus it was concluded that major changes in phenol metabolism take place at about the same time as necrosis develops and that these changes occur in response to a virulent race only after substantial tissue colonization has taken place. Similar trends were found in young tissue.

No data was available concerning the fungitoxicity of the unidentified phenols although the same workers (Rahe , et al 1969b) found zones of inhibition around spots on chromatograms that had been sprayed with spores. One known inhibitor of C. lindemuthianum, phaseollin (Cruickshank and Perrin, 1963), was detected. Judging from the intensity of spots on chromatograms at different times, high concentrations of this substance may have been produced after some other major phenolic components and after the onset of cell reaction. However no quantitative data were available.

Phaseollin is an antifungal compound produced specifically (as far as is known) by plants of the genus Phaseolus. Cruickshank (1963) indicates that this compound may be termed a "phytoalexin" (according to the definition of Muller and Borger, 1940) and suggests that such post-infectionally produced, antifungal compounds might determine the course of varietal resistance if either of two situations applied:-

a) they were produced in inhibitory concentrations only in response to an avirulent race,

b) races showed differential sensitivity.

The original hypothesis of Muller and Borger (1940), based on work with

Phytophthora infestans infections of potato suggested that the speed of formation (i.e. the time required for the appearance of inhibitory concentrations) might be important. This therefore would suggest a third possibility of the role of a phytoalexin.

The two hypotheses of Cruickshank (1963) were examined by Smith (1967) with diffusates from inoculated bean pods. Although neither phaseollin nor a second antifungal compound from bean (Pierre and Bateman, 1967) was specifically identified, these diffusates were inhibitory. Spore germination of the four races was equally sensitive to the inhibitor(s) and there were no consistent differences in the levels produced by resistant and susceptible varieties. Inhibition occurred with diffusates from both types of variety. Although these results indicated that the above hypothesis could not explain differential resistance and susceptibility, Smith (1967) suggested that pods might not have given the correct response to virulent and avirulent races by the time diffusate was collected. Thus information concerning inhibitor production in other bean tissues was required.

Using a similar theoretical approach, Bailey (1970) examined the possible role of phaseollin in the resistance of bean hypocotyls to C. lindemuthianum. Phaseollin was extracted from infected tissue, purified by solvent partition and chromatography then assayed quantitatively by UV spectrophotometry. Later gas/liquid chromatography was used to try to detect very small amounts present during the early stages of a reaction.

With respect to the differential sensitivity of races, it was found that races were inhibited equally by the same concentrations

of purified phaseollin.

As with the studies of Rahe, et al (1969a) phaseollin was found to accumulate during both resistant and susceptible reactions and thus was not produced only in response to the avirulent race. Greater amounts on a μ g phaseollin per g fresh wt. of tissue basis, were found in hypocotyls showing a susceptible than a resistant reaction. However in tissue showing a susceptible reaction nearly all cells were infected and necrotic compared with only a few scattered cells in resistant tissue responding to the avirulent race. Thus in the latter any compound produced exclusively in brown cells would be "diluted" when an extract of all the tissue was made. In an attempt to overcome the difficulties caused by unequal amounts of affected tissue, Bailey adapted a formula used by Muller (1958a) to calculate the phaseollin content of the individual brown cells in infected resistant tissue. Values as high as 4250μg phaseollin/cc of cell were obtained compared with 57.5/cc for the susceptible reaction. Bearing in mind that purified preparations of this substance completely inhibited the growth of germ tubes of C. lindemuthianum at a concentration of 5 μg/ml, and established mycelial growth at 65μg/ml (Bailey, 1970), it is clear that phaseollin could well be responsible for the inhibition of hyphal growth in cells of a resistant variety. It is also evident that at a time when resistant and susceptible responses have been more or less completed, more phaseollin was found in cells showing the resistant response.

It has not yet been firmly established whether phaseollin is confined to the brown infected cells or is present in other cells of the extracted tissue. As pointed out by Bailey (1968) the position of the site of phytoalexin production is important in a consideration of its role. Clearly if phaseollin only accumulated in adjacent healthy cells and not in those where fungal growth is actually found to be inhibited, the production of this compound would be of little significance.

Bailey (1968) has also emphasized the importance of the relative times of inhibitor production and observed fungal inhibition. This temporal relationship is obviously critical in deciding whether a single chemical compound is responsible for resistance, particularly in a situation where large numbers of compounds are likely to be produced and to accumulate in high concentrations. As mentioned earlier several phenolic compounds have been detected in extracts from infected beans and some or all may have had antifungal activity. It is probable that others may have remained undetected because of low concentration, further metabolism, destruction during extraction, poor separation on chromatograms or failure to react with locating agents.

Bailey (1970) carried out exact, quantitative studies on the course of appearance of phaseollin in infected resistant and susceptible tissue. In general his results agreed with those of Rahe et al (1969a) in that phaseollin first appeared at about the time when cell damage was evident and increased thereafter. Bands giving a positive reaction with phenol locating agents were rarely found on chromatograms of extracts from uninoculated tissue or susceptible tissue invaded with

primary mycelium. In these tissues no cell damage was apparent. Phaseollin was not detected in eluates of these bands subjected to UV spectrophotometry or the more sensitive, gas/liquid chromatography. Thus phaseollin production and the development of necrosis were closely associated.

The development of a completed hypersensitive response or mature dark brown lesions, is not an instantaneous process. As was suggested earlier, biochemical changes probably take place after cell death and lead to the accumulation of a number of fungitoxic substances. It is likely that some substances achieve maximum concentration soon after cell death and others much later. If a substance is to be shown to be responsible for resistance then its appearance in inhibitory concentrations must be shown to correspond to the time of inhibition of hyphal growth. The techniques developed by Bailey gave very accurate information about the levels of phaseollin in tissues at different times after inoculation and his *in vitro* studies with purified phaseollin clearly defined the inhibitory action of this substance against *C. lindemuthianum*.

The development of some fairly accurate quantitative methods for defining the interaction of host and fungus *in vivo* was required to complement this work. This was needed to show the relationship between the time course of biological and biochemical events. The first section of the thesis is mainly concerned with the development and use of such methods. These methods were also used as a tool for the study of induced susceptibility and cross protection, for which they were first instituted.

e) A proposed mechanism of resistance based on cell wall polysaccharides

Recently Albersheim, Jones and English (1969) have suggested that the major metabolic changes which take place during infection of a resistant variety are only secondary effects, resistance and specificity being determined by the action of extracellular fungal enzymes on polysaccharides of the host cell wall.

The importance of cell wall compounds and fungal enzymes was suggested because of their possible role in age resistance. It is well known that old tissue is more resistant to a virulent race of C. lindemuthianum than young tissue (Sinden, 1937 and Griffey and Leach, 1965). Nevins, English and Albersheim (1968) found that marked changes took place in the content of trifluoracetic acid extractable neutral sugars of hypocotyl cell walls during their extension. Glucose, galactose and arabinose declined to a basal level, reached at the end of the extension period, while xylose increased and became constant. Although some of the decreases may have been due to dilution because of the addition of cellulose, lignin etc. to the cell walls, the actual content of non-cellulosic glucose, galactose and arabinose was thought to be less in older walls. The basal level of these three sugars was reached after about 8 - 12 days. After 14 days, varieties were resistant to all races of C. lindemuthianum.

When purified cell wall material was treated with culture filtrates from C. lindemuthianum, grown with similar cell wall material as its sole carbon source, galactose was removed more than any other sugar

(English and Albersheim, 1969). This suggested that alpha galactosidase might be a major cell wall degrading enzyme of C. lindemuthianum. Clearly if the ability to colonize bean tissue depended on a high substrate (galactoside) level in cell walls then the decline in galactose in old tissue could explain the observed increase in resistance.

With regard to varietal resistance, English and Albersheim (1969) found that over incubation periods of 16 - 20 days more alpha galactosidase was produced when races were grown on isolated cell wall material from susceptible than from resistant varieties (initial rates of production were often very similar). It was suggested that the "affinity" of individual races for different varieties might lie in properties of the cell wall that cause induction of alpha galactosidase in susceptible varieties, and repression in resistant. Albersheim et al (1969) stated that glucose and xylose suppress the production of C. lindemuthianum alpha galactosidase and suggested how such a suppression could cause differential resistance and susceptibility. Resistance to each race was assumed to be controlled by a single dominant gene.

- If avirulence were also a dominant character then it was expected that one fungal enzyme additional to those formed by the virulent race was produced. An enzyme liberating glucose from the cell wall could cause resistance by suppressing alpha galactosidase production.

- If avirulence were recessive, resistance would result if the alpha galactosidase produced were an exo-enzyme, thus only removing galactose residues slowly from the end of a chain. Another enzyme, produced by the virulent race, was required to cleave long chains.

If processes as outlined above determined whether the result of

infection were a resistant or susceptible reaction, then one would expect (i) penetration of cell walls to be uncommon in resistant varieties, (ii) treatments reducing host response to fail to increase hyphal colonization and (iii) for there to be a poor correlation between host response and inhibition of hyphal growth in host tissues.

2. Cross Protection and Acquired Resistance

The term "cross protection" will be confined to the effect of an avirulent or less virulent race or strain of fungus, bacterium or virus on the development of disease caused by a related virulent race. It is being used in this restricted sense in order that a term widely used in virology (Price, 1964) is not given a different meaning when applied to fungal and bacterial diseases. No allusion to mechanism is implied. "Acquired resistance" will be used as a general term to describe protection brought about by related or unrelated organisms, microbial extracts (or products) or other chemicals which do not directly cause the protection. This avoids the use of the words "induced" and "immunity" which have different meanings in different biological situations. Where a micro-organism or a microbial product has been shown to affect the pathogen directly, "antagonism" is used.

In 1940, Muller and Borger reported that when sporangia of an avirulent race of P. infestans were applied to the cut surface of a potato tuber some time before inoculating with a race which would normally cause a rot, the amount of tissue damage caused by this second race was much reduced. Effective cross protection was only observed in areas inoculated with the avirulent race and was thus thought to be a localized phenomenon. A series of experiments (reviewed by Cruickshank, 1963 and Wood, 1967) led them to suggest that this effect was probably due to the production of an antifungal compound(s) (or Phytoalexin), after and in response to infection by an avirulent

race, and which inhibited both races. Since then, and after the initial success of Cruickshank and Perrin in isolating and characterizing phytoalexins from pea (1961), and bean (1963b), the study of fungal inhibitors produced after infection has become one of the most active areas of current research on mechanisms of disease resistance.

Although of major theoretical importance, because it enabled Muller and Borger to put forward the Phytoalexin Theory, this was not the first report of cross protection. The first to report that cross protection had been obtained were: with fungi - Ray (1901) and Beauverie (1901), with related strains of virus - McKinney (1929) and with bacteria - Ray (1901).

Ray (1901) found that injection of weakly virulent cultures of Botrytis cinerea and certain pathogenic bacteria into their legume hosts before inoculating with virulent strains prevented the development of disease. Extracts of cultures of the virulent strain also had this protective effect when applied to the soil round the host plant. Beauverie (1901) applied attenuated strains of B. cinerea to the soil containing Begonia plants and found that, compared with untreated plants they developed less disease when transferred to soil containing virulent strains. McKinney (1929) found that tobacco plants infected with a light green mosaic virus showed no change in symptoms when inoculated with yellow mosaic virus (T.M.V.) although normal "yellow" symptoms developed when plants with a dark green mosaic infection were used.

Early work was reviewed critically by Chester (1933) and Vavilov (1935 translated Chester, 1949). Many of these reports were

considered to be unproven because of criticisms of technique or the failure of other workers to confirm previous results.

Little conclusive information about the operation of active resistance mechanisms is available in these early reports and the absorption of fungal antibiotics, or growth effects, could also be likely explanations (Wood, 1967). Table 1 lists some recent reports of acquired resistance where antagonism or direct effects of chemical compounds have not been definitely shown. Of these reports of acquired resistance, those which give the greatest information about the operation of disease resistance mechanisms are those concerning sweet potato, tobacco and French bean.

Fungitoxic compounds are often produced in plant tissue after treatment with an avirulent strain or chemicals known to cause cross protection. However protection and the production of these compounds are not always found to coincide thus casting doubt on their role in protection and possibly in the mechanism of resistance as a whole. For instance, sweet potato roots produce an antifungal compound, ipomeamerone, after infection (Uritani and Akazawa, 1955) or treatment with chemicals (Uritani, Uritani and Yamoda, 1960) which is thought to be involved in resistance to this organism. However none could be detected by Weber and Stahmann (1966) after inoculation with an isolate of C. fimbriata which caused protection from a more virulent isolate. However it was possible that the method used to detect ipomeamerone, flooding tissue with Ehrlich's reagent and examining for a specific staining reaction, was not sufficiently sensitive to rule out the possibility that ipomeamerone had been produced and was the cause of protection. Peroxidase and polyphenol oxidase activity was found by Weber, Clare and Stahmann(1967) to increase in tissue adjacent to that inoculated with an avirulent isolate and this effect rather than synthesis

Table 1 Some recent reports of acquired resistance to fungal diseases

Host plant	Fungal Pathogen	Protectant	Reference	Note
Apple	Venturia inaequalis	phenyl thiourea D. phenylalanine	Kuc, Williams and Shaey(1957)	1
Clover	Erysiphe polygoni	Bean yellow mosaic virus (systemic infection)	King, Hampton and Diachun (1964)	2
Cotton	Verticillium albo atrum	Thielaviopsis basicola	Mathre, Garber and Ravenscroft (1967)	2
	V. albo atrum (virulent strain)	V. albo atrum (avirulent strain)	Schnathorst and Mathre (1966)	
	"	"	Bell and Presley (1969)	2, 5
	"	Heat killed virulent strain	"	2, 5
	"	Heat inhibited " "	"	2
Cucumber	Cladosporium cucumerinum	Cucumber mosaic virus	Hopen and Dezeew (1962)	
Flax	Fusarium lini (virulent strain)	F. lini (avirulent strain)	Borlaug (1945)	1
	Melampsora lini (virulent race)	M. lini (avirulent race)	Littlefield (1969)	1,2,3
	"	Puccinia graminis	"	
	"	Puccinia recondita		
French bean	C. lindemuthianum (virulent race)	C. lindemuthianum (avirulent race)	Smith (1967)	1, 2
	"	"	Rahe, Kuc, Chuang and Williams (1969b)	2, 5
	"	Heat inhibited virulent race	Rahe and Kuc (1970)	2

Host plant	Fungal Pathogen	Protectant	Reference	Note
French bean	Uromyces appendiculatus	Tobacco mosaic virus	Wilson (1958)	2
Potato	Phytophthora infestans	Potato virus Y	Muller and Munro (1951)	
Prunus domestica	Cytospora cinta	C. cinta at different site	Hubert and Helton (1967)	4
	"	Prunus ringspot virus	Helton and Hubert (1968)	4
Peperomia obtusifolia	Phytophthora nicotianae (avirulent isolate)	P. nicotianae (virulent isolate)	Siradhana, Schmitthenner and Ellett (1969)	2,3,5
Rice	Pyricularia oryzae (virulent strain)	P. oryzae (avirulent strain)	Kiyosawa and Fujimaki (1967)	
	"	"	Ohata and Kosaka (1967)	
	Helminthosporium oryzae (virulent strain)	H. oryzae (avirulent strain)	Sinha and Trevidi (1969)	2,5
	"	" germination fluid	"	
	"	" heated spores	"	
Soy bean	Phytophthora megasperma (virulent race)	P. megasperma (avirulent race)	Paxton and Chamberlain (1967)	2,3,5
Squash	Fusarium solani	Squash mosaic virus Water melon mosaic virus	Diaz-Polanco, Smith and Hancock (1969)	
Sweet Potato	F. oxysporum	F. solani	McClure (1951)	
	"	"	Bega (1954)	1

Host plant	Fungal Pathogen	Protectant	Reference	Note
Sweet Potato	Ceratocystis fimbriata (Pathogenic strain)	C. fimbriata (non pathogenic strain)	Weber and Stahmann (1966)	2, 3
	"	papain, ribonuclease, acid phosphatase	"	
	"	Ethylene	Stahmann, Clare and Woodbury(1966)	2
Tomato	F. oxysporum	Cephalosporium spp.	Phillips, Leben and Allison (1967)	
	Cladosporium fulvum (avirulent race)	C. fulvum (virulent race)	Curren (1967)	
Tobacco	Peronospora tabacina	P. tabacina at another site	Cruickshank and Mandryk (1960)	4
Vine	Uncinula necator	leaf roll virus	Gohen and Schnathorst (1961)	
Water melon	Colletotrichum orbiculore (virulent race)	C. orbiculare (avirulent race)	McLean (1967)	
Wheat	Puccinia recondita	Puccinia coronata	Johnston and Huffmann (1958)	3

NOTES

1 Direct inhibitory action of protectant ruled out

2 Active host resistance mechanism suggested

3 Protection shown to be localized

4 Protection shown to be systemic

5 Fungitoxic substances known to accumulate in tissue or in inoculation drop.

of a fungitoxic compound was thought to be responsible for acquired resistance. Ethylene, which also "induced" resistance, caused an increase in the level of peroxidase activity (Stahmann, Clare and Woodbury, 1966). Chalutz and De Vay (1969) failed to repeat these findings with the ethylene concentrations they used. Ethylene is known to stimulate the production of pisatin (Chalutz and Stahmann, 1969) and isocoumarin (Chalutz, De Vay and Maxie, 1969), antifungal compounds from pea and carrot respectively, and is produced in the interaction of C. fimbriata and sweet potato roots. Therefore the relative importance of fungitoxic chemicals in sweet potato is still in doubt.

Much of the information about the operation of disease resistance mechanisms in Tobacco has come from studies of acquired resistance. Studies with fungal, bacterial and virus diseases indicated that where marked necrosis followed infection often apparently unaffected tissue in adjacent or distant parts of the plant showed less severe symptoms after infection with the same or other organisms. This suggested that the development of necrosis might be associated with a local and systemic activation of host defence mechanisms. It might also be suggested that the mechanisms of resistance to diseases caused by completely unrelated organisms are similar.

Cross protection between viruses which developed a systemic infection was thought to be due to the interference of a less virulent strain at the site of virus multiplication (Price, 1964). For this to operate, cells must contain particles of the avirulent virus. Gilpatrick and Weintraub (1952) and Weintraub and Kemp (1961) reported

that when necrotic localized lesions caused by carnation mosaic virus were present on lower leaves of Dianthus barbatus the upper leaves developed very few lesions after a subsequent inoculation. No virus was found in protected leaves, thus suggesting that a different type of protection was operating and was associated with the presence of necrosis.

Acquired systemic resistance to a virus (TMV) on the local necrotic lesion Tobacco variety "Samsun" was reported by Ross (1961a). "Pin-points" of necrosis were seen when leaves were viewed under UV light and measurements of lesion diameter suggested the effect of acquired resistance was on lesion expansion rather than infection. Tobacco necrosis virus, which also developed limited brown lesions after inoculation, also caused systemic protection but mechanical injury or infection with Pseudomonas tabaci failed to do so although both caused necrosis. Increases in peroxidase activity in uninoculated upper leaves at about the time when resistance developed (8 days after inoculating the lower leaves) were reported by Simons and Ross (1970). Ross (1961b) ~~and 1970~~) thought that the cells showing increased resistance had developed an increased potential to react to virus infection by the production of oxidized phenolic compounds. This would have tended to cause the smaller lesions found in this study. However depletion of metabolites essential for virus multiplication or products of the necrotic reaction inhibitory to this process were also considered possible causes (Ross, 1961a&b).

Loebenstein (1960) found that the application of purified TMV protein to tobacco leaves caused an increase in their resistance to

TMV in a subsequent inoculation. Systemic acquired resistance to TMV, of the type noticed by Ross (1961a), was abolished when actinomycin D solutions were injected into the leaf two days before a second inoculation (Loebenstein, Rabina and Praagh, 1968). It was suggested that actinomycin D, which did not affect virus multiplication, acted on the host to prevent the formation of an agent responsible for resistance.

Hecht and Bateman (1964) obtained evidence of localized and systemic protection against TMV in plants with restricted necrotic lesions caused by a fungal pathogen, Thielaviopsis basicola. Resistance, which was first detected in upper leaves at the time that necrosis became evident in fungus-infected lower leaves, became maximal after a further five days. Conversely acquired systemic resistance to T. basicola was demonstrated when necrotic local lesions formed in response to TMV or TNV were present. Strains of TMV which did not cause necrosis but developed systemically, failed to cause protection. These workers suggested that a sharply limited lesion was necessary for the development of acquired resistance. The production of a protective substance during the necrotic response was thought to be a possibility.

Cruickshank and Mandryk (1960) found that leaves inoculated with Peronospora tabacina developed less severe symptoms on plants with stem infections of the same organism. The growth of tobacco plants was severely affected by stem infection and it was suggested that this effect might be associated with a premature senescence. Extracts of leaves from infected plants inhibited spore germination (Shepherd and Mandryk, 1962), those from resistant plants where the least growth occurred had little activity. Shepherd and Mandryk suggested a role for fungal-produced inhibitors (substance from the spores inhibited spore germination)

but the operation of a host response could not be ruled out. Mandryk (1963) found that P. tabacina infections caused acquired systemic resistance to Tobacco mosaic virus (TMV), fewer and smaller lesions being formed on infected plants.

These reports suggested that systemic acquired resistance to fungal or virus diseases often accompanied the development of a necrotic reaction thus indicating that the whole tobacco plant, not just the immediate area of infection was affected. Important information concerning effects on cells immediately adjacent to those in which necrosis was developing was also provided by acquired resistance studies.

Ross (1961b) noticed that when a second inoculum of TMV was applied to leaves showing local necrotic lesions from a previous infection, rings of completely lesion free, non-brown tissue were found around the necrotic spot. Later electron microscope studies (Israel and Ross, 1967) indicated that the fine structure of cells adjacent to a local lesion was substantially different from that of cells of uninoculated tissue. These effects included an increase in cytoplasmic content, numbers of ribosomes attached to the endoplasmic reticulum and the numbers of other cell oganelles except sphaerosomes. These latter organelles contained crystals, possibly of peroxidase (Ross and Israel, 1970). Israel and Steward (1966) found that cells in which growth had been stimulated, e.g. by coconut milk, had similar properties, which were thought to be typical of a cell in a state of increased metabolic activity.

Heat treatment (60°C for 40 s) 2 - 14 days after inoculating with TMV, caused an area of tissue, corresponding to that which was resistant

to a second TMV infection, to collapse and become necrotic (Ross and Israel, (1970). Particles of TMV were only found at the original lesion site. During further incubation, cells adjacent to the new necrotic area developed the ultrastructural changes noticed by Israel and Ross (1967).

The effects observed in tissues adjacent to a developing local lesion and in which no virus was found (i.e. increases in cytoplasm, development of resistance to subsequent infection, and increased sensitivity to heat) were thought to be intermediate stages in a series of changes which ultimately lead to cell collapse, this being instrumental in localization of infection. Thus these would have been expected to occur in the cells which developed the necrotic reaction to TMV.

Lovrekovich and Farkas (1965) reported that they had obtained acquired resistance to a bacterial pathogen of tobacco, Pseudomonas tabaci; symptoms typical of a virulent race were reduced by prior injection of heat-killed cells, or a living avirulent race of the pathogen. A hypersensitive collapse of host cells and a cessation of bacterial multiplication was the usual response of tobacco to infection by an avirulent organism (Klement and Goodman, 1967). Thus the localized protection observed by Lovrekovich and Farkas might have been associated with a necrotic response in the host tissue. However, Lozano and Sequeira (1970) found that heat-killed cells of another bacterial pathogen of tobacco, P. solanacearum prevented the visible hypersensitive response in tissue subsequently infected by an avirulent strain. When this latter strain was applied to untreated leaves on

the same plant a few days after treatment, similar inhibition was noted suggesting a systemic effect. The number of viable cells of the avirulent race decreased after injection into the protected tissue. These results suggested that inhibition of bacterial multiplication (multiplication was thought to be necessary before a hypersensitive response can occur) was due to some factor not related to a necrotic reaction. Two factors were thought to be present in the living avirulent cells; an "inducer" of the hypersensitive response and a substance which "induces" the host to prevent the hypersensitive response, the former being heat labile. Similar factors have been reported for P. tabaci and P. glycinea (Sleesman, Perley and Hoitink, 1970). Clearly the isolation and characterization of these factors would give very valuable information about the possible cause of strain/host specificity, one of the major problems of disease resistance. Acquired resistance studies obviously provide a very useful tool for the investigation of the possible role of such factors.

Rather less information has been obtained from acquired resistance studies on French bean, the subject of this present investigation. However there are similarities between reported work on this host and that mentioned above for tobacco. Acquired resistance to bacterial pathogens of bean have been reported (Klement and Lovrekovich, 1961, Omer, 1966 and Stholasuta, 1970) when tissue was pre-inoculated with a race which caused a hypersensitive reaction. Acquired resistance to a virus causing a necrotic local lesion after pre-inoculating with the same virus was reported by Yarwood (1953) and Wilson (1958). Wilson also showed that TMV would make tissue more resistant to the rust,

Uromyces phaseoli typica. This protection was thought to be due to the production of an inhibitor of uredospore germination.

Cross protection between races of C. lindemuthianum on French bean was first shown by Smith (1967) who found that when spores of an avirulent race of C. lindemuthianum were applied to bean leaves three days before applying a virulent race at the same site, the symptoms produced by the second race were reduced. An active host response, perhaps similar to the phytoalexin response of bean pods, was a possible explanation of this effect.

The present study on acquired resistance of French bean to C. lindemuthianum (Section III, Page 208) has been carried out to:-

1) confirm the findings of Smith (1967),

2) determine whether protection is due to host response rather than the antagonism by the avirulent race,

3) determine whether protection is systemic or localized,

4) examine the ability of fungal products to cause protection and their possible role in the determination of the specificity of race/variety interactions,

5) investigate the causes of protection and thus obtain further information about the mechanisms of disease resistance in French bean.

Recently Rahe et al (1969b)demonstrated acquired resistance to C. lindemuthianum on hypocotyl tissue of P. vulgaris. Avirulent races of this fungus, and Helmithosporium carbonum, caused this effect. These results will be discussed later in the light of findings from the present research.

3. Acquired susceptibility

Salmon (1905) found that when barley leaves were treated with ether, chloroform, ethyl alcohol, heat or were mechanically wounded, tissue that was still apparently alive supported the growth of a non-pathogenic isolate of Erysiphe graminis. This suggested that the treatments had reduced the ability of the host to effect an active resistant response. Many other cases have been reported in which non-lethal heat treatment (Yarwood and Hooker, 1966) and exposure to compounds which have a "narcotic" effect on animals (ether, chloroform, ethanol; e.g. Gassner and Hassebrauk, 1937, Behr, 1949 and Muller and Behr, 1949), were found to allow infection of a normally resistant host. Yarwood (1959 and 1965) has reviewed reports of an increase in susceptibility ("predisposition") to pathogens on their natural hosts. Here an increase in symptoms followed treatment and infection by these organisms.

In other cases attempts were made to inhibit specific metabolic processes to give an indication of their role in the mechanism of resistance. Oku (1962) found that applying the reducing agents, ascorbic acid and glutathione, in drops of spore suspension, reduced the browning reaction which occurred when Cochliobolus miyabeanus penetrated epidermal cells of rice leaves, and increased infection. Noveroske, Williams and Kuc, (1962) increased the susceptibility of resistant apple tissue by applying inhibitors of an enzyme, polyphenol oxidase, thought important for resistance.

Acquired susceptibility studies have been a useful means of

testing the possible role of post-infectional antifungal compounds. Thus in cases where resistance is reduced after treatment of the host one would expect the amounts of an antifungal compound responsible for resistance also to be reduced. Chamberlain and Gerdeman (1966) treated soybean plants with heat and found that both resistance to Phytophthora spp. and the ability to produce a phytoalexin in response to these avirulent isolates, decreased. Klarman and Gerdeman (1963a) found that when substances produced by the soy bean host at the site of inoculation with an avirulent race of P. sojae, were leached away using a string wick, the host tissue became susceptible. Fungitoxic compounds were removed during this leaching (Klarman and Gerdeman 1963b).

With French bean, similar attempts have been made to demonstrate that acquired susceptibility is associated with a decreased production of antifungal compounds. Jerome and Muller (1958) heated bean pods for 2h at temperatures ranging from 44 - 50°C. The production of phytoalexin and the appearance of symptoms in pod cavities inoculated after treatment with spore suspensions of Sclerotinia fructicola was assessed. Treatment at 44°C for 2h caused pods to become susceptible to this fungus. Resistance was recovered if pods were incubated at 20°C before inoculation but did not if incubation was carried out at 2°C. The acquired susceptibility caused by treatment at 45°C for 2h was not reversible. Jerome and Muller found a very close correlation between the amounts of phytoalexin produced, and disease which finally developed. Cruickshank and Perrin (1967) inoculated bean and pea pod cavities with Monilinia fructicola and then incubated the pods in an atmosphere containing only 1% oxygen. Pod tissue became susceptible to this

organism; the concentration of phaseollin and pisatin (in pea) found in the diffusate from the cavities was less than that required to reduce germ tube growth of this organism by 50% (i.e. was not inhibitory). Therefore a relationship between phytoalexin production and resistance was suggested.

In Section IV of this study, a number of treatments were applied to resistant bean tissue before inoculation with an avirulent race of C. lindemuthianum in an attempt to increase susceptibility. A consistently effective treatment might then provide an indirect method for investigating the role of active host responses in the mechanism of resistance.

MATERIALS AND METHODS

1. Fungal Material and its preparation

a) Isolates of C. lindemuthianum

Three races of Colletotrichum lindemuthianum (Sacc. and Magn.) Bri. and Cav. were used in this study. A culture of race beta (isolate beta 1) was obtained from Dr. N. Hubbeling, I.P.O., Wageningen, Netherlands, and races gamma and delta were taken from the stock cultures used by Smith (1967). In an attempt to increase pathogenicity and to obtain consistently good sporulation, the latter races were reisolated from sub-epidermal tissue of infected plants and then freed from bacterial contamination. Single spore isolates of the three races were used to initiate stock cultures and these were stored under sterile paraffin oil at $4^{\circ}C$. The expression of virulence or avirulence by a race of C. lindemuthianum on a particular variety mentioned in the text is denoted by (V) and (AV) respectively.

b) Growth of Fungi

Cultures for experimental work were grown on the medium of Mathur, Barnett and Lilly (1950) as described by Smith (1967) and shown in Table 2.

Dense spore suspensions in sterile distilled water were spread over the surface of 50 ml of solid media in plugged 150 ml conical flasks. Excess suspension was poured off and the flasks were

Table 2

Media for the growth of fungi

A Colletotrichum growth medium (Mathur, Barnett and Lilly, 1950)

Glucose	2.8g
$MgSO_4 \cdot 7H_2O$	1.23g
KH_2PO_4	2.72g
Mycological Peptone (Oxoid)	2.0g
Agar (Davis)	20.0g
Glass distilled water to	1000 ml

B Liquid culture medium (Thomas, 1966)

A. Nutrient Solution

Sucrose	15.0g
KH_2PO_4	1.0g
$MgSO_4 \cdot 7H_2O$	0.5g
Acid Casein Hydrolysate (Oxoid)	4.6g
Trace element solution (B)	1.0ml
Glass distilled water to	1000ml

B. Trace element solution

$FeSO_4 \cdot 7H_2O$	249mg
$CuSO_4 \cdot 5H_2O$	40mg
$ZnSO_4 \cdot 7H_2O$	44mg
$MnSO_4 \cdot 4H_2O$	41mg
$Na_2MoO_4 \cdot 7H_2O$	51mg
Glass distilled water to	100ml

Sterilization of both media was effected by autoclaving at 121°C for 15 min.

incubated at 19-23°C beneath 'Philips Black Light' fluorescent tubes giving maximum emission at 360 nm. The photoperiod was 12h per day. Cultures were used for sub-culture or experiment after 5 to 11 days.

On occasions a decline in sporulation occurred but this was easily overcome by sub-culturing from the stock culture.

c) Preparation and application of inoculum

Spores were rinsed from the surface of the cultures with sterile water, filtered through four layers of muslin, washed by two centrifugations at 830g for 5 min each and adjusted to the required concentration with sterile water.

When a Pasteur pipette was used to fill the haemocytometer chamber, there was a discrepancy between the calculated spore concentration and that actually delivered by the syringe which was used for inoculation (Agla Micrometer Syringe, Burroughs Wellcome Ltd., London). This discrepancy was eliminated by filling the haemocytometer directly from the syringe. In some experiments where spore concentration was varied the concentration delivered by the syringe was checked by counting the spores in a number of drops of known volume applied to a microscope slide.

A 5µl drop of a suspension containing 5×10^5 spores/ml, (2500 spores per drop) was the normal inoculum used on detached hypocotyls. Twenty-microlitre drops were used at various concentrations for pod cavities and detached leaves. Where other volumes and concentrations were used these are specified in the text. In

pathogenicity tests both drop and spray inoculation was used, the latter being effected with a compressed air spray-gun (No. 2a from Colour Sprays, North Road, London, N.7). Before inoculation, and between subsequent inoculations with different races, the syringe or spray gun was rinsed very thoroughly in 70% ethanol and sterile distilled water.

2. Plant material and its preparation

a) Bean seed

Seed of the different varieties of French Bean (Phaseolus vulgaris L.) was obtained from: A. R. Zwaan & Zn, Voorburg, Netherlands (c.v. Kievit Koekoek); N.V. Nunhem, Haelen, Netherlands (c.v. Immuna and c.v. Dubbele Witte); Department of Agriculture, Michigan State University (c.v. Red Kidney); Sutton & Sons Ltd., Reading (c.v. Canadian Wonder and c.v. Pinto). The small stocks of Kievit (Koekoek) and Red Kidney were increased on plants grown for seed at Imperial College Field Station, Ascot, Berks. In the text varieties are described as being resistant (R), susceptible (S) or intermediate (I) in their response to a particular race of C. lindemuthianum. These classifications were given as a result of the pathogenicity tests described later.

b) Excised Hypocotyls

In many of the experiments detached hypocotyl material was used and this was prepared in the following way unless otherwise indicated.

Seeds which were not obviously damaged or diseased were washed for about 15 min in running tap water and then placed between single layers of absorbent toilet tissue, moistened with 30-35 ml distilled water, in polythene boxes (28 x 20 x 10 cm). (Excess water especially in the covering layer allowed the seeds to rot. Surface sterilization in 0.01% mercuric chloride for 5 min did not reduce this rotting). The boxes were closed and incubated at room

temperature for 4 or 5 days. Germinated seeds were then stripped of their testas and planted, 40 per box, in a depth of about 7 cm of Vermiculite (Micafil, Dupre Vermiculite Ltd.) saturated with tap water. A further 1 cm depth of Vermiculite was used to cover the seeds and the boxes were transferred to an aluminium cabinet (150 x 60 x 50 cm) fitted with an extractor fan and 3 Phillips "Cool White" fluorescent tubes, 40 cm from the Vermiculite surface. A 16h light period was applied during the normal hours of darkness, and the temperature was maintained at 23-30°C. Variation in light intensity across the breadth of the cabinet was less than 50 lumens per sq. ft.; however it varied from 580-360 between the centre and ends respectively. One to 10 boxes of beans were grown in the cabinet and when 8 or less were required they occupied the central area and received 450-550 lumens/sq.ft.

Emergence of hypocotyls from the Vermiculite and their subsequent extension occurred over a period of less than 24h, 4-7 days after planting (depending on the variety used). Hypocotyls were harvested at the end of this rapid extension period when upright, 7-10 cm long with primary leaves held between the cotyledons or just beginning to expand and fold backwards. If left uncut the hypocotyls continued to grow longer for a further 3 or 4 days. The age of hypocotyls, quoted later in the text was the time in days between sowing the seed and inoculation.

The cut hypocotyls were trimmed to a length of 6-7 cm from 0.5 cm behind the cotyledonary node and the ends smeared with "Vaseline" (white petroleum jelly) to prevent drying and to fix

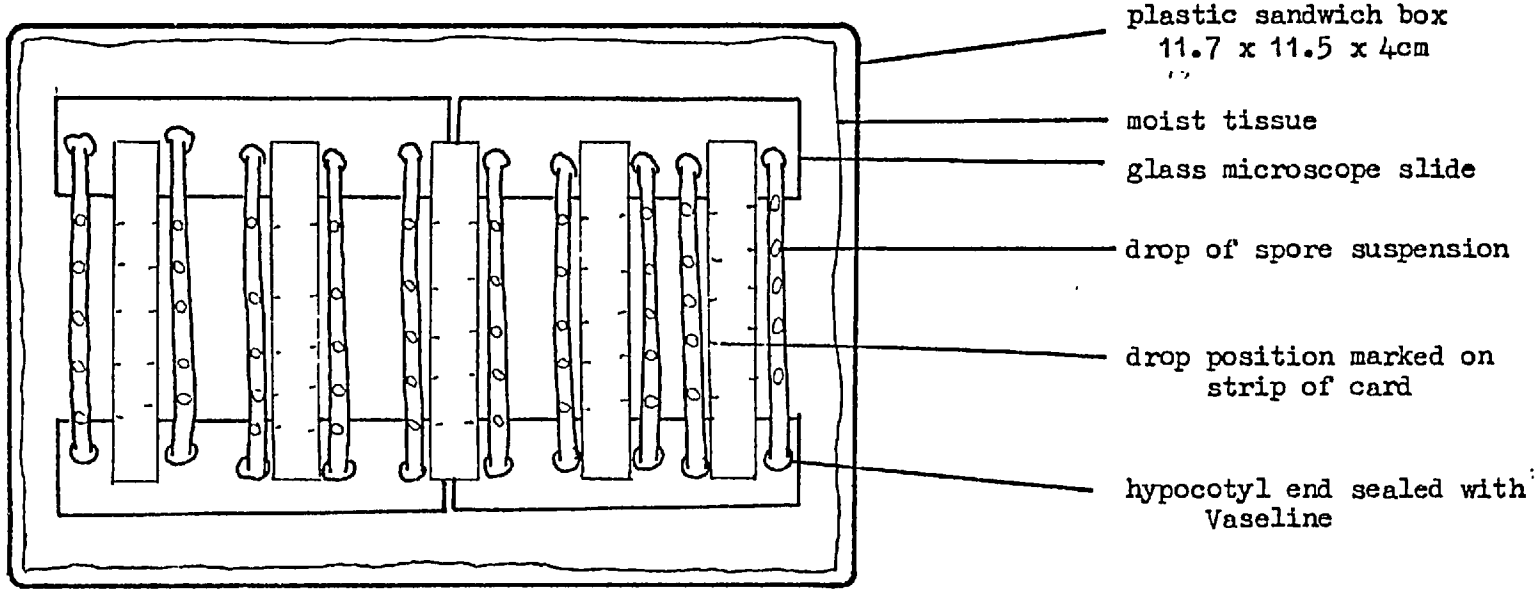
them into the incubation boxes (Fig.1). Drops of inoculum were prepared and applied, 5 per hypocotyl at 1 cm intervals, as stated above. The area of hypocotyl tissue beneath a drop is termed "inoculation site" in the text.

The box lids, lined with moist tissue to prevent formation of droplets of condensation, were sealed with Vaseline and the boxes were incubated at 15-19°C in a cooled illuminated incubator. The boxes were kept beneath white muslin for 3-4 days and in this way the drops remained and infection occurred. Non-infected hypocotyls remained turgid and apparently healthy for more than a month and adventitious roots formed at the base of the stem.

c) Stripped and abraded hypocotyls

"Stripped" hypocotyl material was prepared by removing two thin adjacent slices of tissue from along the length of a hypocotyl (epidermis and upper cortex) with a razor blade. "Abraded" hypocotyl material was prepared by rubbing hypocotyls between finger and thumb 60 times after dipping in a suspension of 2% celite (diatomaceous earth) in water. Both Stripped and Abraded hypocotyls were washed in running tap water for about 20 min, rinsed in sterile distilled water and allowed to dry before sealing and inoculating.

Fig. 1. Boxes used for incubation of inoculated hypocotyls.



d) Spray inoculation of intact hypocotyls for testing differential race reactions

Plants were grown in the manner described above in boxes containing seedlings of two or three varieties (8-10 seedlings per variety). Just before the stage of rapid expansion the contents of the boxes were sprayed with 5-10 ml of suspensions containing 5×10^5 spores per ml of the appropriate races. The boxes were incubated at $17^{\circ}C$ in the light for 10 days, being covered with transparent polythene for the first three. The scoring system of Smith (1967) was used which classified plants as resistant, if they showed no reaction, light brown flecking or a few limited lesions, and susceptible if numerous dark brown limited lesions were formed or if the whole plant collapsed.

e) Inoculation in cross protection experiments

Cross protection experiments were based on the methods used by Smith (1967) and set up in the following way:-

Drops of "protectant" (i.e. spores of an avirulent race or cell free culture fluid) and drops of water or (where noted) sterile culture medium, were applied to the tissue. After a period of incubation the drops were removed with absorbent tissue and replaced with either water, or spores of a virulent race. Thus the two treatments were; "protected" (protectant then virulent spores) and "non-protected" (water/medium then virulent spores). The controls were; "water control" (water then water) and "protectant control" (protectant then water). Where a mixed inoculum was used the spores

of virulent and avirulent races were mixed together before applying the drops. In some cases the inoculation site was "washed" before the second inoculation. This was done by repeatedly removing the drop with absorbent tissue and replacing it with sterile water.

3. Chemicals

'Analar' grade chemicals were normally used and obtained from Hopkins and Williams Ltd., British Drug Houses Ltd. Absolute ethanol was obtained from Burroughs Ltd.

4. Microscopic examinations

a) Preparation of material

Examination of the fungus/cell interaction during the development of resistant and susceptible reactions per se, and, during reactions which had been affected by certain treatments, was carried out on thin strips of tissue cut from the area under the inoculation drop with a razor blade. Before removing these "epidermal strips" from hypocotyls and pod cavities the position of the drop was marked by making vertical cuts at the drop margin. Material was normally mounted in distilled water in which medium cells maintained turgor, and protoplasmic streaming continued for several hours. Leaf material, as discs removed with a cork borer, was fixed in absolute ethanol/glacial acetic acid (2:1 for 12h) and mounted in lactophenol. Lactophenol was the mountant for strips used to measure appressorium formation.

As an indication of cell vitality and the integrity of semi-permeable membranes, strips were immersed in sucrose solution for about 20 min and examined for plasmolysis. Epidermal strips were tested in 0.5M and pod endocarp tissue in 0.8M sucrose. Tissue from hypocotyl cortex and fungal hyphae was plasmolysed in 1.0M sucrose. Sucrose isotonic with hypocotyl epidermal cells was 0.25M.

Rhodamine B was used by Muller (1958b) to detect cells showing early stages of a resistant reaction. In the present study this reagent (obtained from Hopkin and Williams Ltd.) was applied to freshly cut strips at 10ppm in distilled water or in isotonic sucrose. The Nitroso-stain method of Reeve (1951) was performed

on similar tissue by adding, in turn, separate single volumes of; 10% sodium nitrite, 20% urea and 10% acetic acid followed after 2-4 min by two volumes of 2N caustic soda; this stain gives a cherry-red colour with catechol tannins. Other stains used are mentioned in the Experimental section.

Browning and granulation obscured hyphae in cells of necrotic tissue but their presence was made visible if dilute caustic soda solution was added. Freshly cut strips were mounted in 0.1N NaOH and examined after 2-3 min.

A Richert Zetopan microscope with bright field illumination was used throughout and photographs were taken on Ilford Pan F film using a green filter. All micrographs shown in plates were of strips of epidermis mounted in water unless otherwise indicated. Measurements were made with a calibrated eyepiece micrometer.

b) Measurements of events on hypocotyls

(i) Appressorium formation

The two drops nearest the cotyledonary end of each of four hypocotyls were marked and allowed to dry. Strips cut from the tissue beneath the drops were mounted in clear lactophenol and covered with 5mm coverslips. All spores beneath the coverslips were examined and the number which had formed appressoria (defined as in Section 5) was counted. In this way spores washed from the strip during mounting were not excluded from the count but the total number of spores counted was not the same in each strip.

(ii) Use of Van Tieghem Cells

Van Tieghem cells were used when it was necessary to carry

out successive observations on the same strips for more than 1-2h.

Perspex rings (5 mm high, 17 and 10 mm outside and inside diameters) were fixed to microscope slides with Vaseline and a drop of water placed in the centre of the well which was formed. Epidermal strips (2-3 mm long and 1 per cell) were placed cuticle downwards on a clean coverslip and then covered with a piece of agar (ca. $8mm^3$ of 0.2% Oxoid Ionagar No. 2). Excess water was withdrawn from around the edge of the strips with absorbent tissue and this caused the inoculated surface to become fixed to the glass. The coverslips were then inverted and sealed on to the open end of the well with Vaseline, the epidermal cells being viewed through the coverslip using an oil immersion objective.

The position of an epidermal cell was identified for further observation by means of the vernier reading of the mechanical stage of the microscope and by a drawing showing the position of cell wall junctions and appressoria. In this way individual epidermal cells were examined at intervals although the slide was removed from the stage between observations. Protoplasts were seen to retain the capacity to stream for at least 24h in Van Tieghem cells.

(iii) Measurement of hyphae in infected cells

Hyphae less than 9-10 μm appeared spherical or crescent shaped beneath or at one side of the appressorium and thus were not wholly visible. The measurements given for hyphae of this size are of maximum dimensions, i.e. between the points of the crescent or across the sphere. Larger hyphae were usually elongate and where the base of the hypha was obscured, the edge of the appressorium

was taken as its starting point. When the hyphal tip was at a different plane of focus from its base, each was obtained in focus in turn and the distance between these two points of sharp focus recorded. The smallest unit of the eyepiece micrometer was 1.4 μm (x 1000) and measurements were made in multiples of this to the nearest whole unit. In experiments using Van Teighem cells the base and apex of the measured hyphae were indicated on the marker drawing.

c) Germination and germ tube growth tests on glass slides

The method used was that of Smith (1967). New microscope slides were scrubbed in hot detergent solution (Stergene) with a wad of cotton wool and rinsed repeatedly in hot tap water and glass distilled water. After soaking overnight in 5% acetic acid and then distilled water they were steeped in acetone for a short time before drying at 60°C. Drops of spore suspension (1 μl of 5 x 10^5 spores/ml) were applied to the centre of 20 μl drops of test solutions which had been pipetted on to the slides. Each slide held 3 test drops although the 3 replicate drops of a particular treatment were put on to different slides. After incubation for 24h at 17°C in the dark, cotton blue in lactophenol was added and 200 and 20 spores per drop were examined for germination and growth of germ tubes respectively.

The presence of an appressorium or germ tube, (defined in sub-section 5) was considered indicative of germination.

5. Definitions

Definitions of the terms used in the text to describe the macroscopic or microscopic appearance of fungi on inoculated tissues are given below:-

Brown flecking - numerous discrete, minute, red-brown spots in the inoculation site

Lesion - a continuous area of green-brown, brown or dark-brown tissue more than 1 mm in length where browning was clearly not confined only to the surface cells

Limited Lesion - a lesion which is confined to part of the area of the inoculation site

Non-limited lesion - a lesion which is the same size as or larger than the area of the inoculation site

Germ tube - a hyaline structure arising from the spore without an intervening septum and being longer than the spore width

Appressorium - a darkly pigmented, spherical or pear shaped structure arising from the spore or germ tube and being separated from it by a septum

Germinated spore - a spore with either an appressorium or a germ tube attached

Penetration - the presence in a cell of a clearly visible hypha directly beneath and at a lower plane of focus than the appressorium

6. Preparation of culture filtrates and mycelial extracts

Culture filtrates were prepared from cultures of C. lindemuthianum grown on the sucrose/casein-hydrolysate medium shown in Table 2 (Thomas, 1966). Eight oz. medical flats containing 40 ml of sterile medium were inoculated with two drops of sterile spore suspension and incubated horizontally for 7 days at 25°C in the light. The culture fluid was then poured out and filtered through a sterile Oxoid membrane under reduced pressure. This bulked sterile filtrate was stored at -20°C. The pH of filtrates from beta, gamma and delta cultures was 5.87, 5.82 and 4.69 respectively when measured with a Beckman Zeromatic pH meter. The pH of the medium was 6.1.

Extracts of mycelium were prepared as follows:-

Mycelium from the above liquid cultures was bulked and then rinsed five times in distilled water. Excess water was squeezed from the mass and the fresh weights obtained. A volume of 80% ethanol equivalent to 10 times the fresh weight was added and the mycelium blended for 10 s in a Sorvall Omnimixer. The fragments were collected by centrifugation, re-suspended in a similar volume of ethanol and the extraction repeated twice. The combined supernatants from three extractions were passed through an Oxoid membrane filter.

The ethanol was replaced by water at 40°C under reduced pressure, water being added on three successive occasions when the volume had been reduced to one third of the original. A pink precipitate formed in the ethanolic extract when its volume had been reduced by one half. The final volume of aqueous suspension of this precipitate was equivalent to the fresh weight of extracted mycelium.

7. Treatments used to induce susceptibility

a) Volatile compounds

Small known volumes of chloroform or diethyl ether were applied to absorbent tissue at the bottom of large medical flats (584-599 ml). These were sealed and maintained upright at 25°C for 1h so that evaporation and diffusion of the vapour could occur. Excised hypocotyls were introduced and the sealed bottles, which were inverted to allow the vapour to reach the tissue, were incubated for various lengths of time.

b) Heat

Beakers of sterile distilled water were maintained at the required temperature in a thermostatically controlled water-bath. Detached hypocotyls, in groups of 3 or 4, were immersed in the distilled water for appropriate lengths of time, the temperature of the water being checked, after stirring, at the beginning and end of the immersion period. This temperature, which did not change more than 0.5°C during treatment, was measured with a total immersion thermometer (Range 40-70°C, B.S. 593, G. H. Zeal Ltd., London) held under water to just below the first expansion bulb. After treatment the hypocotyls were transferred immediately to water at room temperature, rinsed and allowed to dry before sealing and inoculating in the normal way.

EXPERIMENTAL

Section I Studies on fungal development and host response

This section is concerned with the events which lead to the visible expression of the resistance or the susceptibility (described in the first subsection below) of an infected bean.

1. Differential reactions of Bean varieties to races of C. lindemuthianum

The ability of the isolates of races beta, gamma and delta to form lesions on the varieties listed in Tables 3, 4 and 5 was tested as described below:-

Excised hypocotyls from 11-day plants were inoculated with 5 drops of spore suspension and incubated at 17°C. Twelve hypocotyls, 2 for microscopic observation and 10 for lesion formation, were used for each combination. Four days after inoculation 2 epidermal strips were taken from hypocotyls of each race/variety combination and examined for the presence or absence of browning in cells to which appressoria were attached. The other 10 hypocotyls were examined at intervals for the appearance of symptoms of infection (i.e. lesions and flecking as defined earlier). The results are shown in Tables 3, 4 and 5.

After incubation for 8-11 days the resistant and susceptible reactions of varieties to the three races of C. lindemuthianum were clearly differentiated by the presence or absence of lesions on

Table 3

Differential reaction of varieties of P. vulgaris to a single spore isolate of C. lindemuthianum - race beta

Variety	Cellular reaction at 4 days	% of 50 inoculation sites with lesions after (days)				Classification
		5	6	8	11	
Canadian Wonder	C	-	62	70	70	S
Red Kidney	C	-	-	98	100	S
Pinto	C + B	-	F	F	4	I
Immuna	B	-	F	F	F	R
Kievit	B	F	F	F	F	R
Dubbele Witte	B	-	-	-	-	R

Abbreviations:

C Penetrated cells colourless

B Browning

- No visible reaction

F Brown flecking only

S Susceptible

R Resistant

I Intermediate

Table 4

Differential reaction of varieties of P. vulgaris to a single spore isolate of C. lindemuthianum - race gamma

Variety	Cellular reaction at 4 days	% of 50 inoculation sites with lesions after (days)				Classification
		5	6	8	11	
Canadian Wonder	C	-	-	98	100	S
Red Kidney	C	-	-	90	100	S
Pinto	B	-	-	F	F	R
Immuna	B	-	F	F	F	R
Kievit	C	-	42	92	96	S
Dubbele Witte	B	-	-	-	-	R

Abbreviations as in Table 3

Table 5

Differential reaction of varieties of P. vulgaris to a single spore isolate of C. lindemuthianum - race delta

Variety	Cellular reaction at 4 days	% of 50 inoculation sites with lesions after (days)				Classification
		5	6	8	11	
Canadian Wonder	C + B	12	40	48	60	S
Red Kidney	B	F	F	F	F	R
Pinto	C	26	86	98	100	S
Immuna	C	32	40	40	40	S
Kievit	B	-	F	F	F	R
Dubbele Witte	C	F	F	34	44	S

Abbreviations as in Table 3

hypocotyls. With race beta on c.v. Pinto, however, although most sites showed brown flecking a few superficial limited lesions were also formed. When the reaction of this variety was checked by the spray-inoculation test of Smith (1967), the results indicated that the response of c.v. Pinto to race beta was "intermediate" and not definitely resistant or susceptible. Reactions identical with those listed above for excised hypocotyls were obtained with the other varieties and races in the spray inoculation test. Further studies on the intermediate Pinto/beta response are reported on Page 134.

Limited lesions were produced on Immuna, Dubbele Witte and Canadian Wonder inoculated with race delta while all other susceptible reactions gave rise to non-limited lesions.

With the exception of the reaction of c.v. Kievit Kockoek to race beta, the classifications given above agree with those of Smith (1967) and English and Albersheim (1969). Both Smith (1967) and Hubbeling (1957) reported that Kievit was susceptible to race beta but in the present study a resistant response was obtained although on other varieties the isolate consistently behaved as race beta and not as either of the other races used. One possible explanation was that the single spore isolate was atypical. To test this, hypocotyls of c.v. Kievit and Red Kidney (S) were inoculated with spores from the single-spored and the original isolate obtained from Hubbeling. No lesions had developed after 10 days on Kievit hypocotyls inoculated with either isolate although those on Red Kidney were typical of a normal susceptible reaction.

Thus the single spore isolate of race beta behaved like the

original culture but appeared to differ from that of Smith (1967) in its interaction with c.v. Kievit. However it was also possible that differences between the batches of bean seed used by Smith and the author could account for this discrepancy.

The microscopic examination of infected cells showed that browning had occurred in those of resistant varieties after 4 days while in susceptible hypocotyls cells had been colonized without any change in their colour. This was in agreement with the suggestions of other workers (see Review of Literature) that the "browning reaction" was closely associated with resistance. A more detailed study of the microscopic events preceding browning and the possible role of this response in resistance is described later.

Conclusion

Races beta, gamma and delta used in the present study caused similar responses in differential varieties to those found by other workers with these races. The isolate of race beta behaved differently on c.v. Kievit to that of Smith (1967).

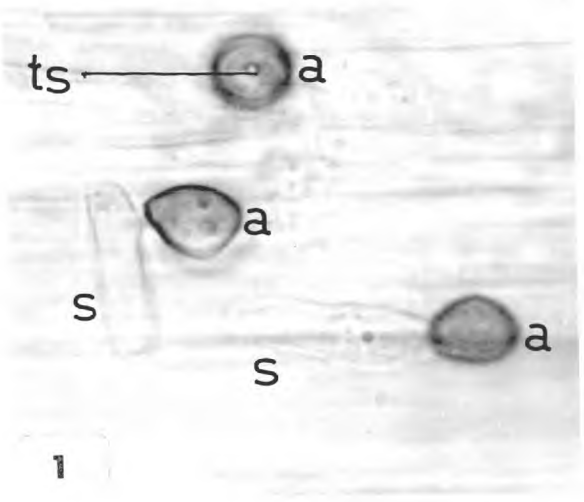
2. Pre-penetration events

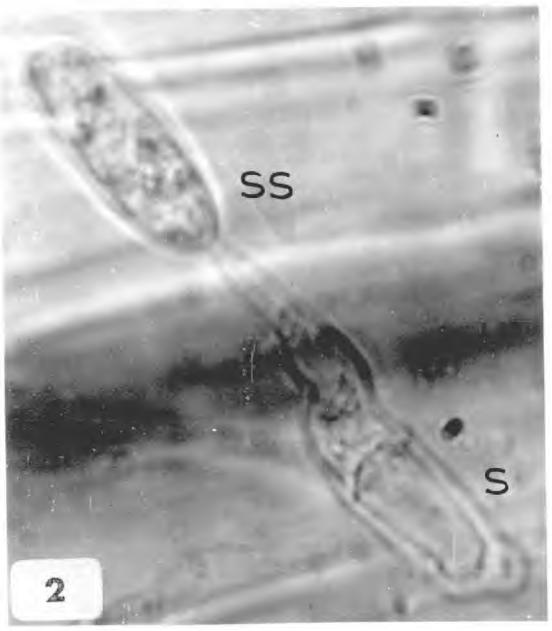
Spores of C. lindemuthianum germinated to produce either an appressorium (Plate 1) or a germ tube. Occasionally a second spore was formed (Plate 2) but normally germ tubes either developed appressoria after a limited growth or continued to grow as superficial mycelium (Plate 3). The appressorium was the only structure ever found to be involved in penetration of the cuticle of the hypocotyl. Although germination has been studied qualitatively by many workers little data is available regarding the quantitative aspects of this process. Quantitative differences in the formation of appressoria on resistant and susceptible hosts might be able to account, to some degree, for the presence or absence of lesions in these varieties. A variety might appear "resistant" if: a) spores germinated to form structures other than appressoria or b) germination was inhibited. These possibilities were examined in the following experiments:-

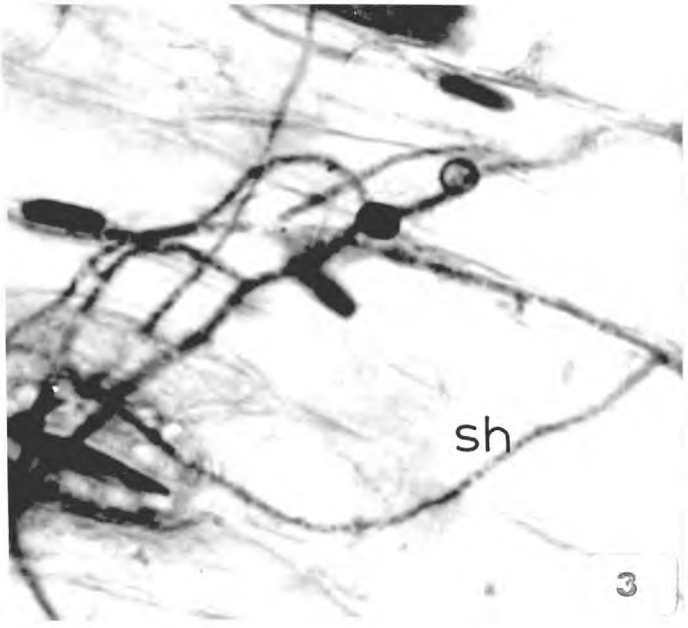
a) To determine the relative development of appressoria and germ tubes of virulent and avirulent races on c.v. Immuna, hypocotyls were inoculated with spores of races gamma (AV) and delta (V). After incubation for 48h, strips (2 on each of 4 replicate hypocotyls) were removed and 50 germinated spores examined on each. The results are shown in Table 6.

Although spores of both races germinated to form appressoria and germ tubes, only race delta produced lesions after a further

Plates 1, 2 and 3 Products of spore germination on excised hypocotyls







1) Appressoria (a) formed by spores (s) in an inoculation drop on the hypocotyl surface. Note transparent spot (ts) at base of appressorium (Immuna(R)/beta* 3 days+ x 1600)

2) Second spore (ss) formed during germination in an inoculation drop (Immuna(R)/beta* 3 days+ x 2500)

3) Superficial hyphae (sh) in an inoculation drop (Immuna(R)/beta* 5 days+ x 750)

Note: * Variety/race used + after inoculation

Table 6

The relative proportions of appressoria and germ tubes produced by germinated spores of races gamma and delta on Immuna hypocotyls

	% spores germinating as:-			
	Appressoria		Germ Tubes	
Race	Mean[1]	Range[2]	Mean[1]	Range[2]
gamma (AV)	69	58 - 75	31	25 - 42
delta (V)	74	67 - 81	26	19 - 33

1 Mean of individual percentage values for 4 replicate hypocotyls

2 Highest and lowest percentage values of single replicates

period of incubation. The two races did not differ much in their ability to form appressoria rather than germ tubes.

b) To determine whether fewer appressoria were formed on resistant than susceptible hypocotyls for a given spore inoculum and to determine the time of maximum appressorium formation, counts of these structures were made at intervals after inoculation. Two epidermal strips were removed from each of 4 replicate hypocotyls of c.v. Immuna 24, 48 and 72h after inoculating with races gamma (AV) and delta (V). The strips were prepared as described in Materials and Methods and all the spores (500 - 2800) on 2 strips were examined for each replicate. Spores with germ tubes but no appressoria were counted as ungerminated. The results are shown in Table 7.

It would appear from Table 7 that the numbers of mature appressoria increase for 48-72h and that the maximum percentage production was similar for both races.

The time course of germination of race beta on c.v. Immuna hypocotyls was studied in a separate experiment (Table 8) using the above methods. A total of 450-750 spores were examined for each replicate.

In this experiment, appressorium production reached 90% in 48h but was close to this maximum value after 24h.

The results of both these experiments show that resistance is not caused by any marked inhibition of appressorium formation.

Table 7

The production of appressoria on bean hypocotyls (c.v. Immuna) inoculated with races gamma (AV) and delta (V)

Time after inoculation (h)	% appressorium production by spores of race :- gamma (AV) % [1]	gamma (AV) Range [2]	delta (V) % [1]	delta (V) Range [2]
24	21	16 - 35	12	7 - 14
48	45	32 - 54	45	19 - 66
72	53	41 - 63	43	32 - 74

1 Total number of appressoria on 4 replicate hypocotyls as a percentage of the total number of spores examined

2 Highest and lowest values for the percentage of appressoria produced by spores on individual hypocotyls

Table 8

The production of appressoria on bean hypocotyls (c.v. Immuna) inoculated with race beta (AV)

Time after inoculation (h)	% appressorium production	
	% [1]	Range [2]
24	70	61 - 82
48	90	85 - 92
72	88	81 - 96

1 Total number of appressoria on 4 replicate hypocotyls as a percentage of the number of spores examined

2 Highest and lowest values for the percentage of appressoria produced by spores in individual hypocotyls

Conclusion

Differences in the growth of virulent and avirulent races before penetration could not account for their ultimate ability to cause or fail to cause lesions.

3. System used for quantitative study of infection and cellular changes

The data given earlier and the observations of other workers suggest that the interactions which take place during or after penetration of the host might be decisive in determining whether the fungus grows to produce a lesion or not. It was clear that a microscopic examination of host cells infected and/or affected by the fungus would be of value, especially if this were made quantitatively. Information could be provided on quantitative changes in cellular events with time or resulting from different treatments. The uniformity of cellular reactions could be assessed in tissues which might be used for biochemical studies.

A system of grading cells (Table 9) and hyphae (Table10) was established from observations of different race/variety combinations. This was used later to give a semiquantitative account of the appearance of cells and hyphae in tissue giving susceptible and resistant responses.

The grading system was used in the following way:-

Epidermal strips (2 per replicate hypocotyl) were cut from the inoculation site and mounted in sterile distilled water on clean slides. Using a magnification of x 1000, the strips were scanned and cells with a single appressorium attached were examined. On each strip 50 of such cells were graded for cell appearance (Table 9) and hyphal development (Table 10).

The host/fungus interaction of each cell is described by 2 grade numbers, the first referring to the cell and the second to the hypha. Thus the cell shown in Plate 13 was graded as 2.1. It was colourless but contained larger particles than adjacent cells and those of

uninoculated tissue. Reaction material was found beneath the appressorium.

The results obtained were expressed as the percentage of all cells examined at a single time that were assigned to individual grades or groups of grades. These were then tabulated. Changes taking place in individual cells during the course of incubation would be reflected by increases or decreases in these percentages at different times after inoculation (i.e. across the table of results). The response of tissues at any one time would be represented by the proportion of cells in different grades at that time (i.e. the columns of the table).

When calculating the percentage of penetrated or affected cells those containing "reaction material" were excluded.

Table 9

Grades used for describing the appearance of epidermal cells of bean hypocotyl tissue inoculated with C. lindemuthianum

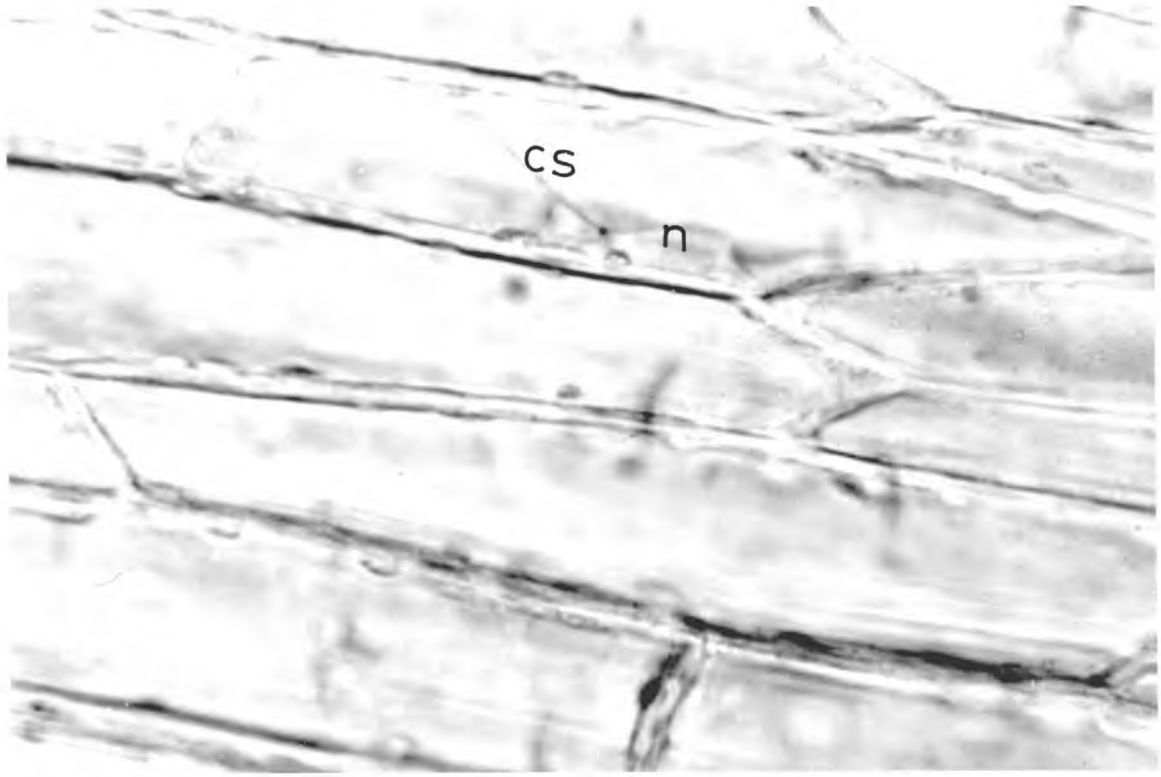
Grade	Description	Plate
	Unaffected cell	
1	Particles in cytoplasm (not plastids): just visible at a magnification of x 1000; streaming within cell; not aggregated near appressorium Cytoplasm: not granular; not aggregated near appressorium; colourless	4
	Affected cell - colourless	
	Cells different from above in following respects:-	
2	Particles in cytoplasm obvious (x 1000 magnification)	5
3	As 2 - Particles in cytoplasm: aggregated near appressorium	6
4	Particles in cytoplasm: slowly moving or motionless; aggregated near appressorium with or without other cytoplasmic material	7 & 8
5	Particles in cytoplasm: motionless Cytoplasm: showing fine granulation throughout cell (protoplast may be pulled away from cell wall; nucleus may be rounded and shrunken)	9
6	Particles in cytoplasm: showing Brownian motion	
	Affected cell - brown	
7	Particles in cytoplasm: motionless Cytoplasm: granular; very pale brown throughout cell	
8	As 7, Cytoplasm: light brown throughout cell	10
9	As 7, Cytoplasm: brown to dark brown throughout cell	11
10	As 7, Cytoplasm: pale to dark brown in region of appressorium	12
11	Particles in cytoplasm: As 2 Cytoplasm colourless - browning in cell wall near appressorium	

Table 10

Grades used for describing structures in epidermal cells of bean hypocotyl tissue beneath appressoria of C. lindemuthianum

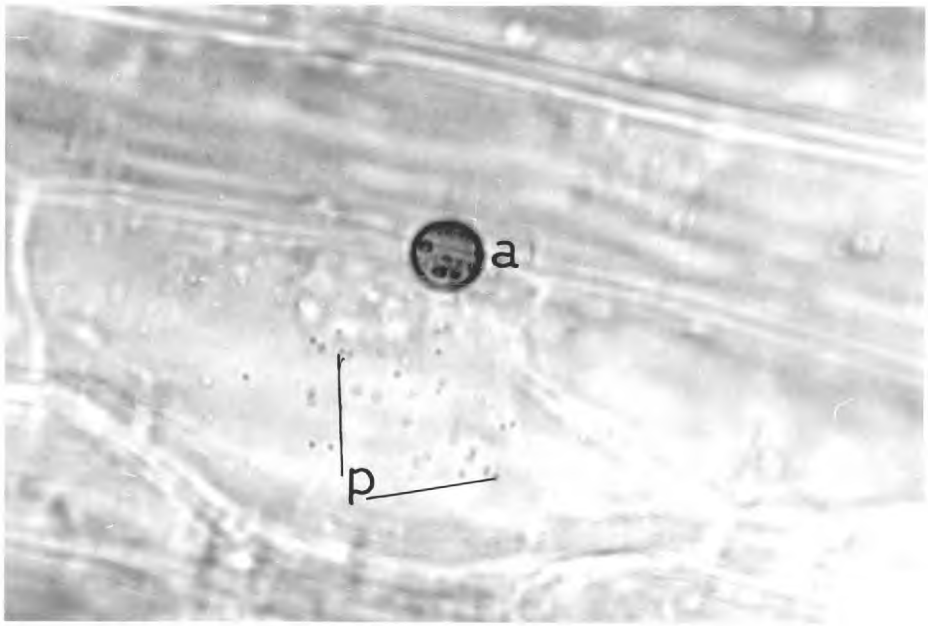
Grade	Description	Term used	Plate
1	Dense, opaque material at base of appressorium; spherical or elongate; colourless, yellow or brown	Reaction material	13
2	Thin walled, transparent, colourless hypha	Primary hypha	14
3	As 2 but has grown into adjacent cell	" "	
4	No structure visible		

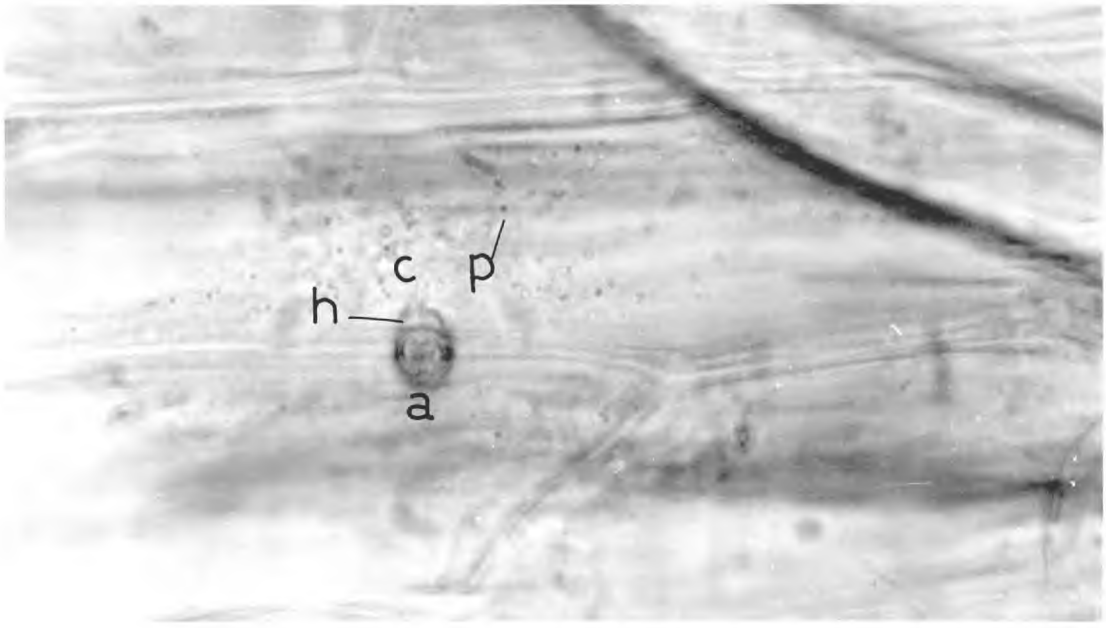
Plate 4 Grades for description of hypocotyl epidermal cells



4) Cell grade 1 - unaffected. Note nucleus (n) and cytoplasmic strands (cs). No large particles or granulation of cytoplasm (c.v. Immuna x 1600)

Plates 5 & 6 Grades for description of hypocotyl epidermal cells (contd.)

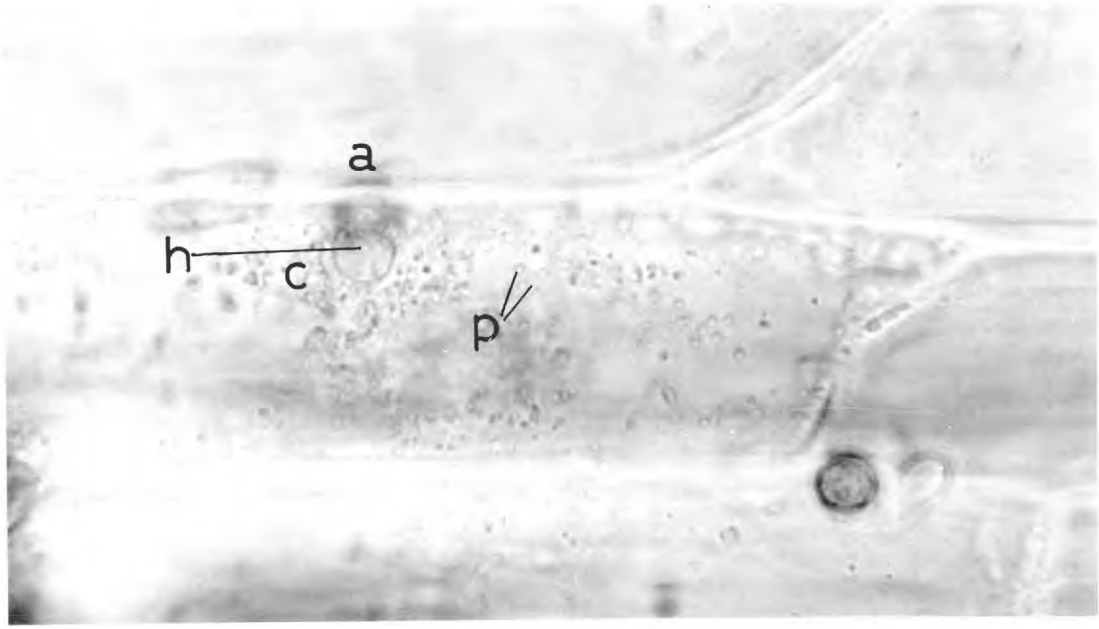


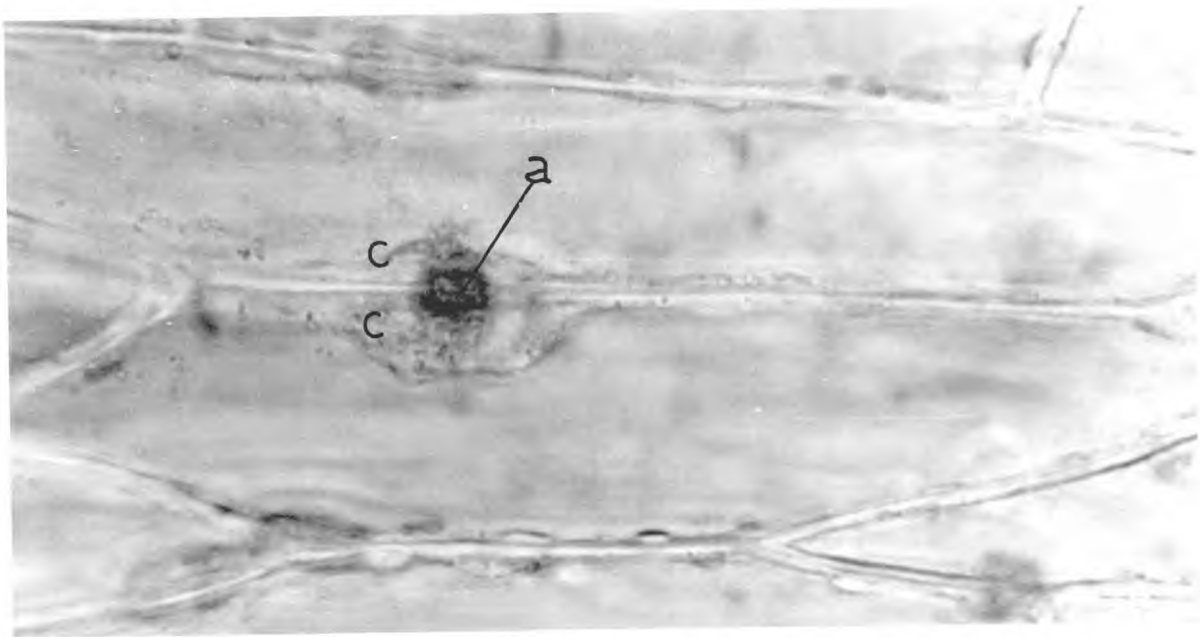


5) Cell grade 2 - affected. Note obvious particles (p) throughout cytoplasm not aggregated near appressorium (a) (Kievit(R)/delta 70h x 1600)

6) Cell grade 3 - affected. Note obvious particles (p) mainly in the vicinity of the appressorium (a). Cytoplasm (c) was accumulated beneath the appressorium around a hypha (h). (Kievit(R)/delta 60h x 1600)

Plates 7 & 8 Grades for description of hypocotyl epidermal cells (contd.)

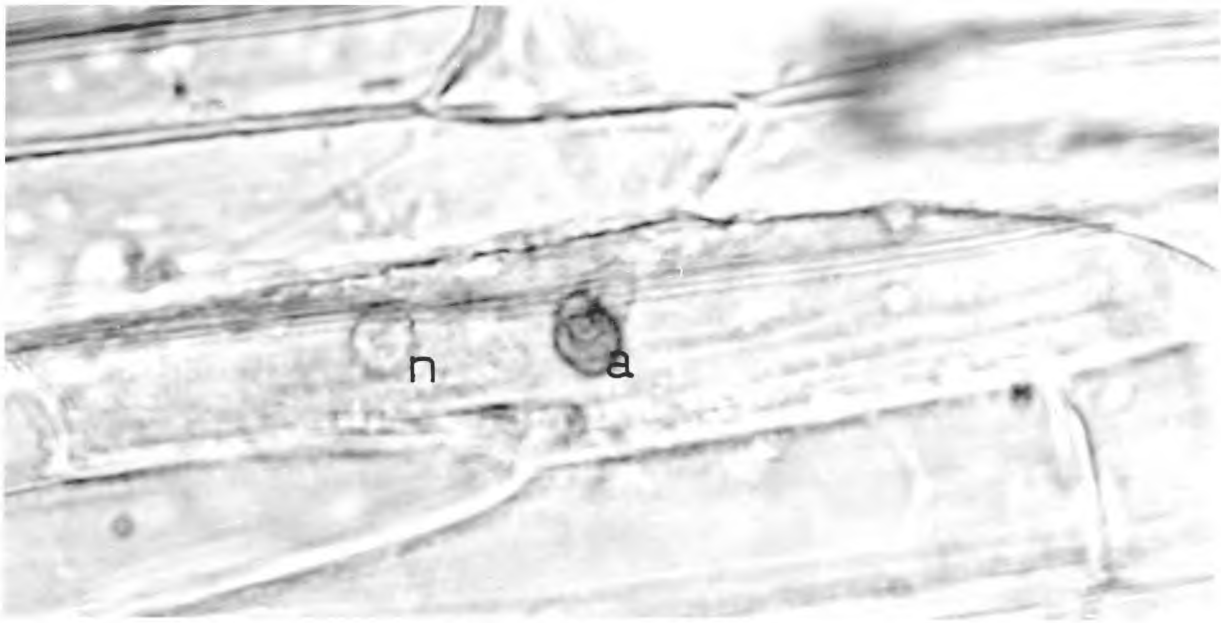




7) Cell grade 4 - affected. Note marked accumulation of obvious particles (p) around a thin walled hypha (h) beneath the appressorium (a) seen in outline. Particles were motionless. (Kievit(R)/delta 70h x 1600)

8) Cell grade 4 - affected. Note accumulation of cytoplasm (c) in 2 cells beneath an appressorium (a) seen in outline. No motion was observed in cell. (Kievit(R)/delta 72h x 1600)

Plates 9 & 10 Grades for description of hypocotyl epidermal cells (contd)

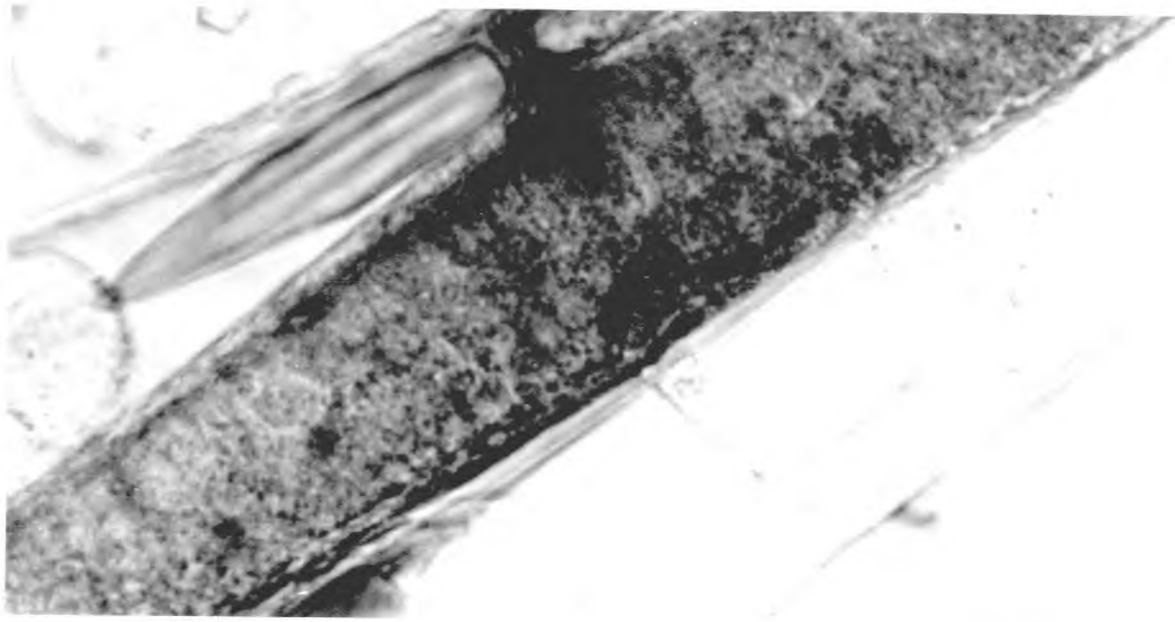


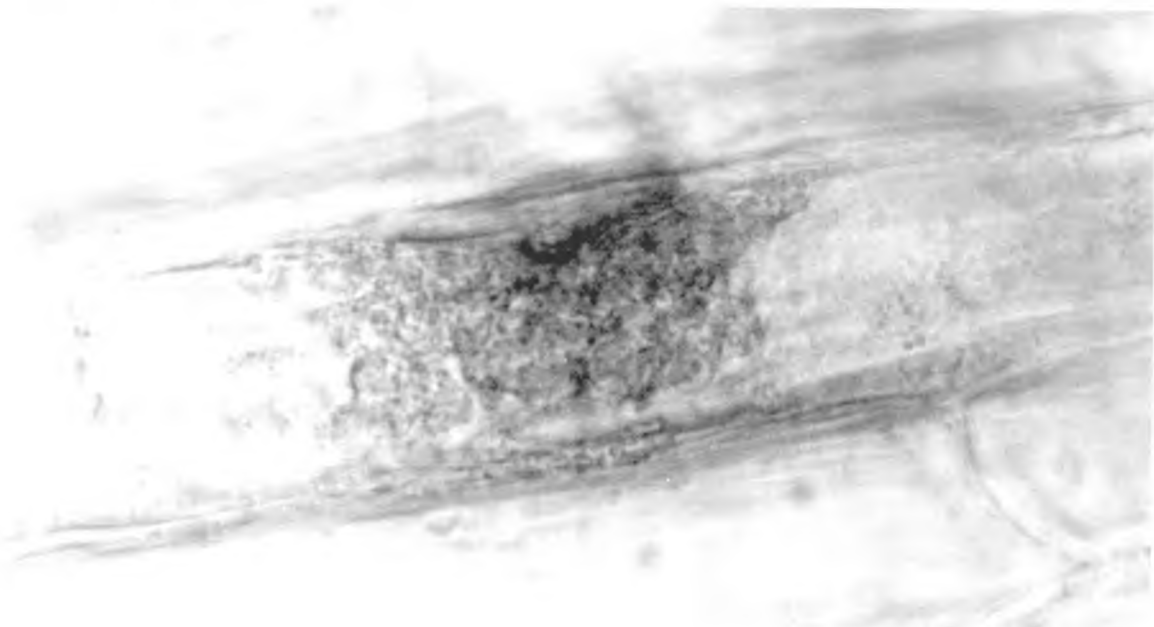


9) Cell grade 5 - affected. Note very fine granulation throughout cytoplasm and rounded nucleus (n). (Immuna(R)/beta 3 days x 1250)

10) Cell grade 8 - affected, light brown. Note homogeneous granulation and darkening of whole of cytoplasm; adjacent cells plasmolysed in 0.5M sucrose (Immuna(R)/beta 4 days x 1600)

Plates 11 & 12 Grades for description of hypocotyl epidermal cells (contd)

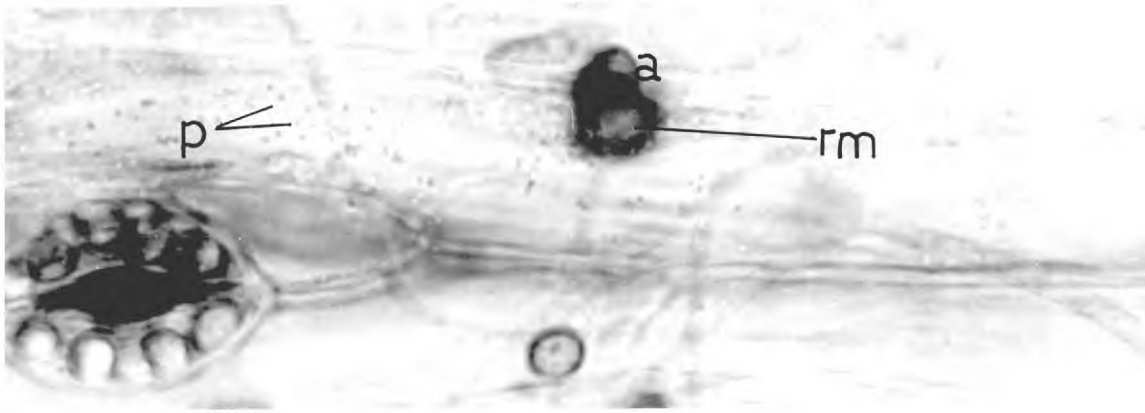


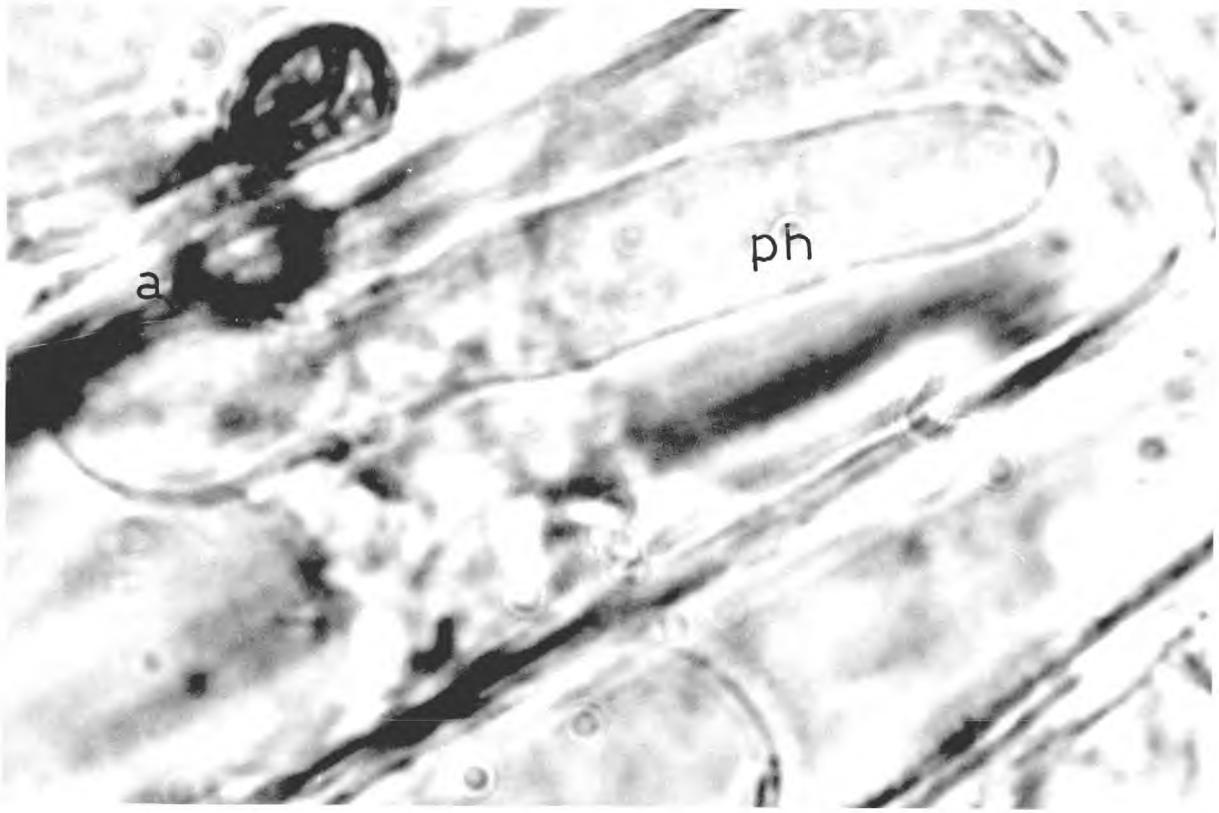


11) Cell grade 9 affected, dark brown. Note coarse granulation of cytoplasm; adjacent cells plasmolysed in 0.5M sucrose. (Kievit(R)/delta 5 days x 1600)

12) Cell grade 10 - affected, brown. Note coarse granulation and browning in part of cell. (Kievit(R)/delta 4 days x 1600)

Plates 13 & 14 Grades for description of structures in bean hypocotyl epidermal cells beneath appressoria





13) Grade 1 A dark deposit reaction material (rm) is visible beneath the appressorium (a) in a cell of grade 2 showing streaming of obvious particles (p) (Immuna(R)/beta 5 days x 1250)

14) Grade 2 A thin walled primary hypha (ph) is visible; the appressorium (a) seen in outline was visible at a higher plane of focus. (Kievit(S)/gamma 3 days x 3100)

4. Development of a susceptible response

Descriptions of the microscopic events leading to the formation of lesions in susceptible hypocotyls have been given by other workers (see Review of Literature). However it seemed well worthwhile to attempt to obtain a complete picture of this process as it was taking place in the varieties used in the present study. This is summarized on Page 96 and in Fig. 2 (Page 97) but is described in more detail below where special attention was paid to quantitative aspects of the time course of the infection process and changes in the appearance and vitality of infected cells.

a) Course of infection

Ten day old hypocotyls (c.v. Kievit) were inoculated with spores of race gamma (V). Epidermal strips were removed from 2 inoculation sites on each of 3 replicate hypocotyls at the times shown in Table 11 and these were examined using the cell grading method. The results of these observations are summarized in Table 11.

Intracellular hyphae were first found after 50h and a maximum penetration of about 25% of cells was observed after a further 16-24h. Growth into a second cell was not detected until 74h and reached a maximum at 114h or later. Penetrated cells remained colourless and showed no other effects of reaction to the fungus. Reaction material was found in cells after the first observations of penetration and its frequency increased throughout. Brown, water-soaked, non-limited lesions were visible under 98% of the drops after 164h.

It is clear that the percentages of penetrated cells varied

Table 11 Hyphal development and reaction of epidermal cells of susceptible bean hypocotyls (c.v. Kievit) at various times after inoculation with race gamma

Grades	Description	% of cells examined[1] assigned to different grades after (h):-							
		42	50	58	66	74	82	90	114
	Cell unaffected								
1.2	Hypha in penetrated cell	0.0	1.3	4.0	24.0	24.3	10.0	30.3	11.0
1.3	Hypha in second cell	0.0	0.0	0.0	0.0	0.7	0.3	8.0	11.0
	Reaction material produced in cell								
2.1	Obvious particles streaming throughout cell	0.0	0.0	0.0	0.3	1.0	1.0	2.0	5.0
2.4, 3.2, 4.2, 4.4, 5.4, 11.4	Other grades	0.0	0.3	2.0	0.7	0.3	0.7	0.7	1.0
	Total penetrated cells[2] (%)	0.0	1.3	4.3	24.3	25.3	10.3	38.7	22.3

1 Three replicate hypocotyls, giving a total of 300 cells, examined at each time

2 Cells containing reaction material excluded

considerably during the period of study although the trends outlined above were quite obvious. As with the data for germination given in Tables 7 and 8 there was much variation between results for individual replicate hypocotyls. The problem of variation and the significance of results obtained by this cell grading method are discussed on Page 283.

b) Observations on the development of intracellular hyphae and their effects on infected and adjacent cells

Excised hypocotyls of c.v. Kievit and c.v. Immuna (where noted) were inoculated with spores of races gamma and delta respectively; under the conditions of study these combinations gave non-limited lesions after 5-7 days. Strips were removed at intervals and examined using an oil immersion objective. The results of these observations are described below.

(i) Prior to the development of macroscopic symptoms

Appressoria of C. lindemuthianum (Plate 1) were dark brown, thick walled and 4-7 µm in diameter. They were attached to the cuticle, often over the cell-wall junction between epidermal cells, and were not removed by the manipulations of stripping and mounting; ungerminated spores were often seen in the mounting medium around a strip. Occasionally a circular area at the base appeared to be more transparent than the rest of the body (Plate 1). The intracellular hypha resulting from penetration was first seen as a hyaline ring around, or a crescent at one side of the opaque appressorium and this body expanded to form an elongate "Primary hypha" (as described by Leach 1923) (Plate 14).

Primary hyphae were 5-8μm in diameter when examined soon after penetration. They developed between the plasmalemma and the cell wall (Plates 14, 15 and 16). Growth was most commonly observed parallel to the longitudinal axis of the cell but centripetal growth towards the cortex and growth across the cell also occurred. A characteristic swelling (Plate 17) was produced at either side of a cell wall penetrated by a primary hypha and there was no obvious gelatinization of cell wall material between these swellings. Hyphae of this type colonized epidermis and cortex intracellularly.

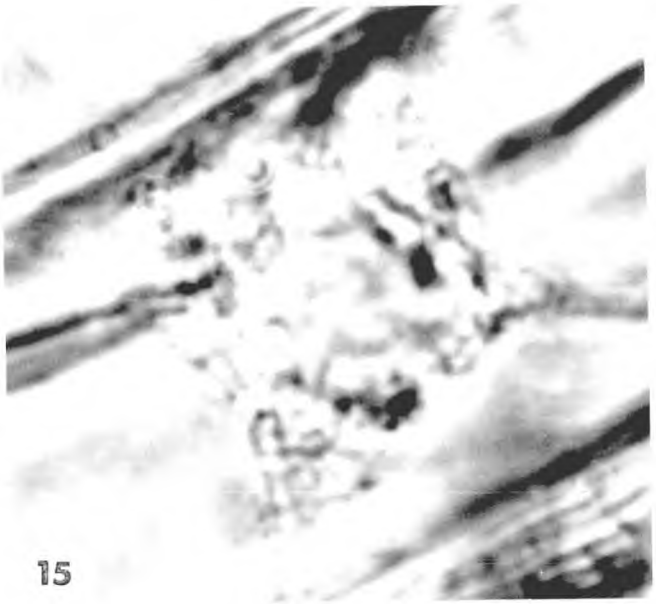
Throughout the period shown in Table 11 and later, when extensive colonization of tissue under the inoculation drop had taken place, infected cells could be plasmolysed and de-plasmolysed when immersed in turn in 0.5M sucrose and distilled water (Plates 15 and 16). Streaming was visible in infected cells and although this sometimes appeared to be more active than in adjacent uninfected, or than in uninoculated tissue, the particles were no larger. No coagulation or browning of cytoplasm was observed and this remained colourless when cells were treated in Rhodamine B (10ppm in 0.3M sucrose for 2h). A faint pink colour was detected in the vacuole of all cells after this treatment. These vital properties were displayed by infected cells until just before symptoms became visible.

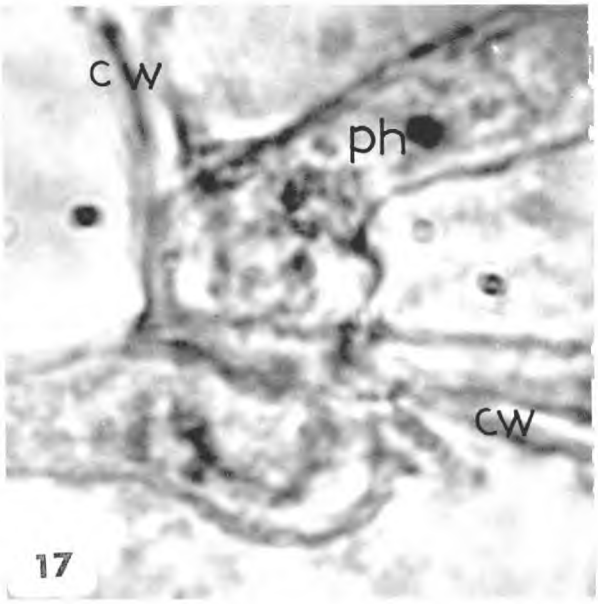
Details of the development of reaction material in combinations showing resistant and susceptible responses are given later (Page142).

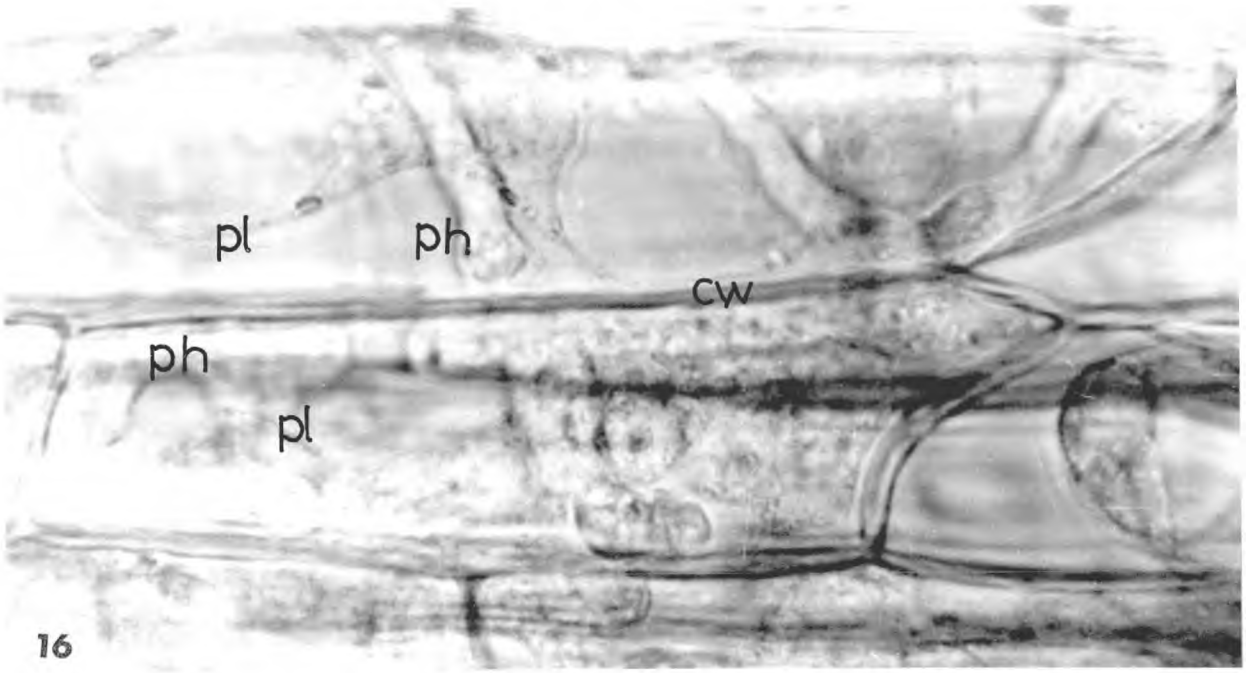
(ii) At the time of appearance of macroscopic symptoms

Water-soaking, which was the first visible sign of infection,

Plates 15, 16 & 17 Epidermal cells containing primary hyphae from susceptible hypocotyls







15) A portion of the cell in Plate 14 at lower focus to show protoplast plasmolysed in 0.5M sucrose. (Plates 14 and 15 indicate that hypha was above protoplast) x 3100

16) Plasmolysed epidermal cells containing primary hyphae. Note primary hyphae (ph) lie between plasmalemma (pl) and cell wall (cw) (Kievit (S)/gamma 5 days x 1600)

17) Primary hypha (ph) swelling at point of passage through a cell wall (cw) (Kievit(S)/gamma 5 days x 3100)

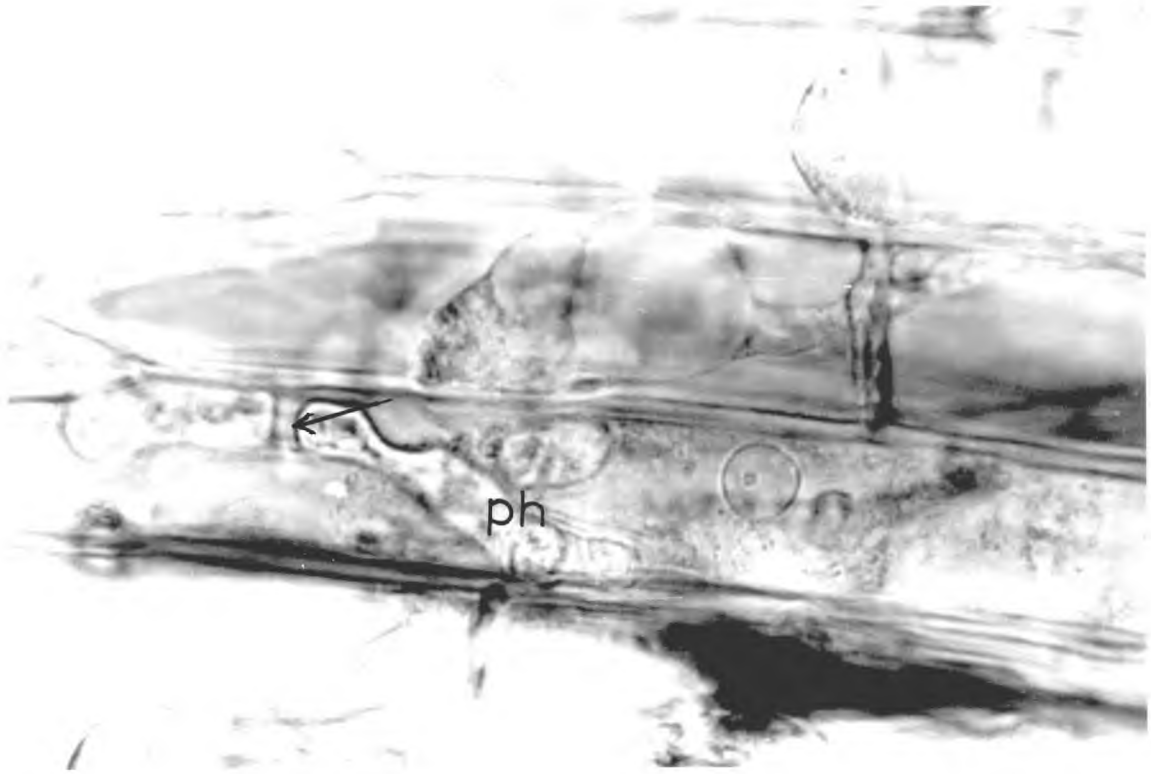
was often noticed initially at sites nearest the apex of the hypocotyl, the other sites appearing unaffected. Observations were made on strips from infected water-soaked and symptomless tissue.

In symptomless tissue, groups of epidermal cells were found, which had been colonized by primary hyphae and which failed to plasmolyse in 0.5M sucrose. A typical cell is shown in Plate 18. The cytoplasm was finely granular throughout and the nucleus often rounded with an obvious nucleolus.

"Secondary hyphae" (as described by Leach 1923) were seen occasionally in this symptomless tissue but were much more common in the water-soaked and brown lesions which appeared later. They were abundant in pith and in cortical tissue around the inoculation site. Secondary hyphae could be distinguished from primary hyphae in the same tissue by their smaller diameter (3-4μm compared with 5-10μm) and by the lack of any marked swelling or constriction at the site of passage through a cell wall (Plate 19 compared with Plate 17). In the epidermis they were found to grow mainly at right angles to the longitudinal cell axis and were seen to originate from established parts of a primary hypha. Branches arising from a primary hypha in a cortical cell are shown in Plate 20.

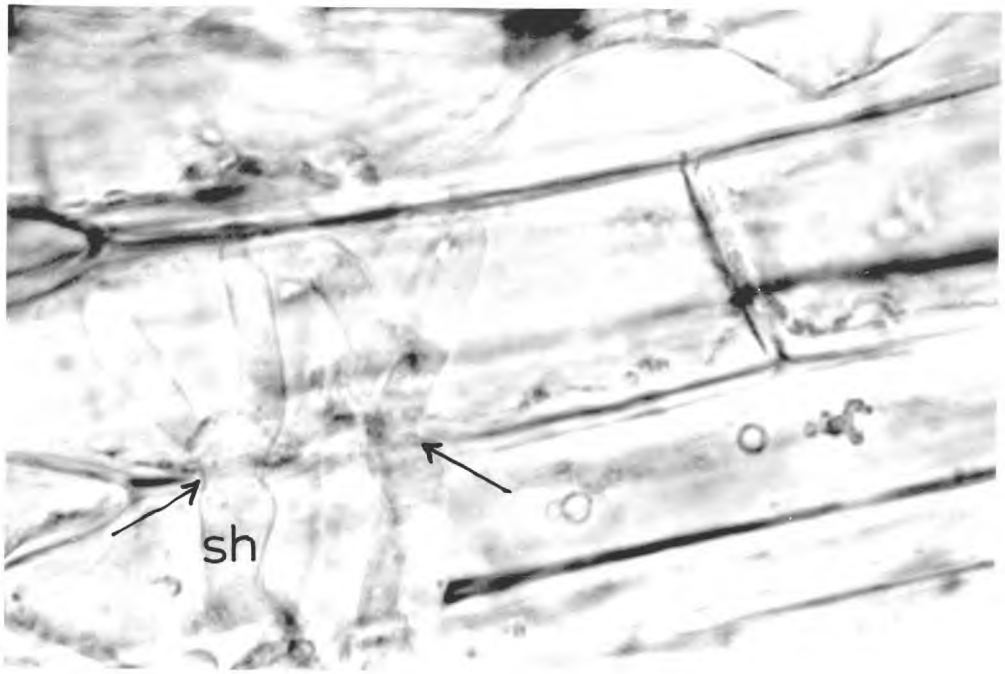
Epidermal cells, penetrated by secondary hyphae, were unable to plasmolyse in hypertonic sucrose solutions although adjacent uninfected cells did so on occasions (Plate 19). In more advanced lesions, cells at some distance from those containing hyphae failed to plasmolyse. The plasmalemma of

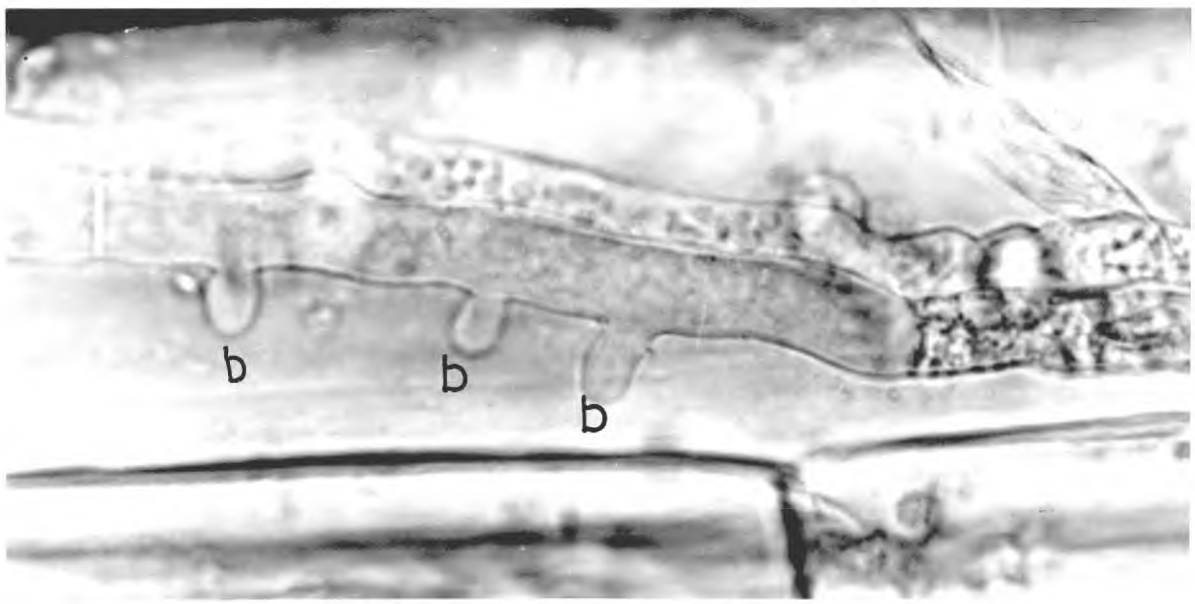
Plate 18 Death of hypocotyl epidermal cell containing primary hyphae of a virulent race



18) Granulation and lack of plasmolysis in molar sucrose compared with neighbouring cells is visible in a cell containing primary hyphae (ph). Brownian movement was apparent within the cell. Plasmolysis of the fungal protoplast is evident (arrowed). (Kievit(S)/gamma 5 days x 1600)

Plates 19 & 20 Secondary hyphae in susceptible hypocotyls





19) Branching secondary hyphae (sh) in hypocotyl epidermal cells. Note little constriction at point of passage through cell walls (arrowed) and failure of infected not adjacent cell to plasmolyse in 0.5M sucrose. (Kievit(S)/gamma 5½ days x 1600)

20) Branches (presumed to be secondary hyphae) arising from a hypha in a cell of the cortex. (Kievit(S)/gamma 5½ days x 1600)

Plate 21 Secondary hyphae in epidermal cells of susceptible hypocotyls



21) A secondary hypha (sh) appeared to have caused a marked localized effect on the plasmalemma of an infected cell at the point of penetration through the cell wall (arrowed)
(Kievit(S)/gamma 6 days x 1600)

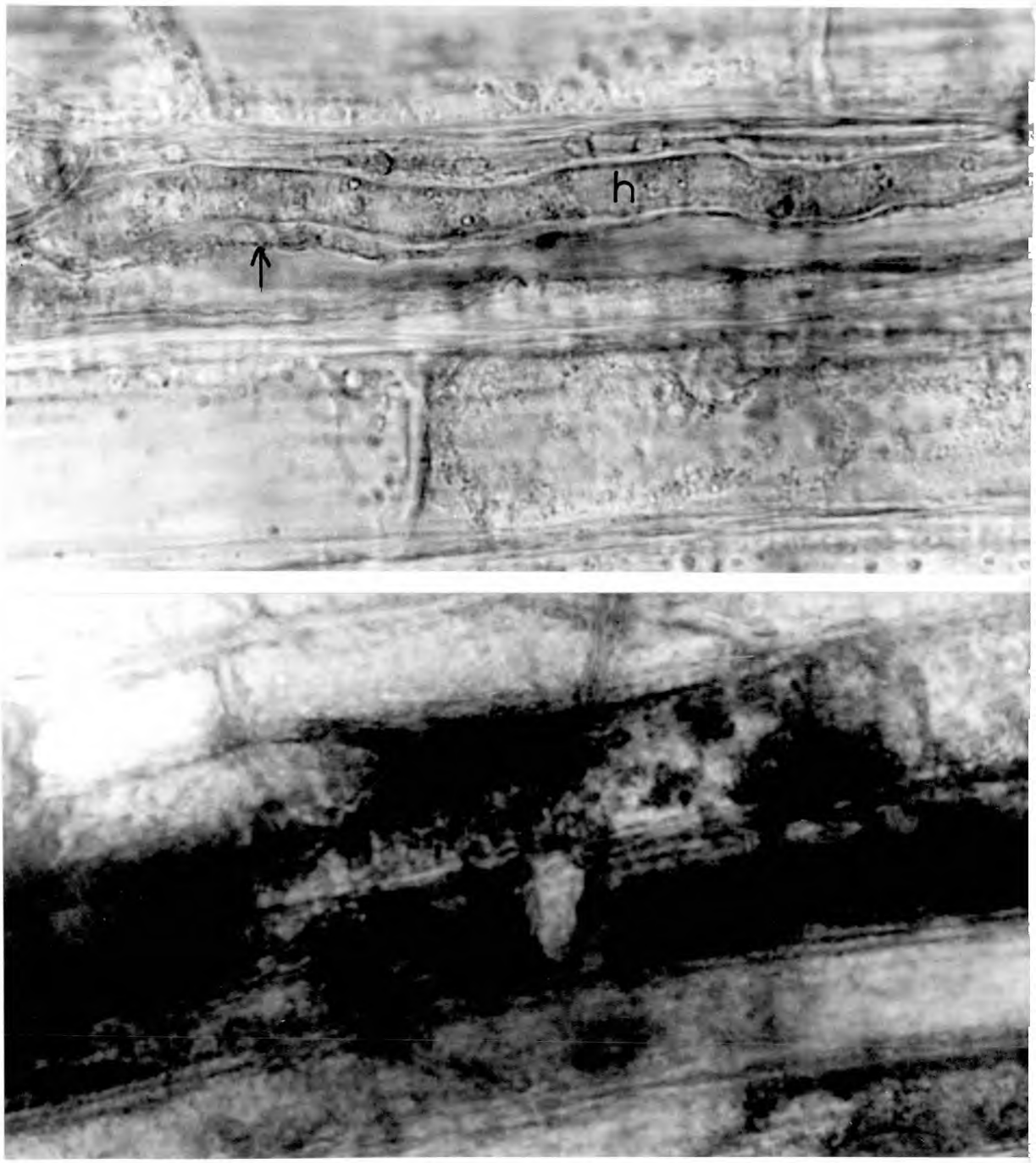
the cell shown in Plate 21 appears to be affected at the point where the secondary hypha has contacted it after passing through the wall.

Brownian movement of particles in some cells and a diffuse granulation with the absence of movement in the majority (Plate 22 c.v. Immuna) was typical of infected and adjacent cells in strips in which browning had begun to occur. Cellular browning was seen to occur first in cells of the cortex while often epidermal cells above such tissue remained colourless and plasmolysable. Progressive browning and granulation occurred and that typical of later stages in lesion development is shown in Plate 23 (c.v. Immuna). In tissue where a large proportion of the epidermal cells was dead, the stomata were open. Hyphae present in dead, pale brown cells could often be plasmolysed in 1.0M sucrose (Plate 18).

(iii) Development and spread of non-limited lesions

Symptoms of infection of c.v. Kievit by race gamma appeared $5\frac{1}{2}$ or more days after inoculation, depending on the age of the hypocotyl. A slight depression which was grey green at first but which later became a light chocolate brown, appeared at the inoculation site. This colour change was followed by the appearance of a drop of brown liquid on the hypocotyl surface, exudation occurring whether the inoculation drop was still present or had dried previously. When examined under "Anoptral" phase contrast, these brown drops were often found to contain large numbers of bacteria.

Plates 22 & 23 Granulation and browning in epidermal cells of infected susceptible hypocotyls



22) Infected and uninfected cells showing fine granulation of cytoplasm and effects on the plasmalemma (arrowed) especially near the hypha (h). (Immuna(S)/delta 6 days x 1600)

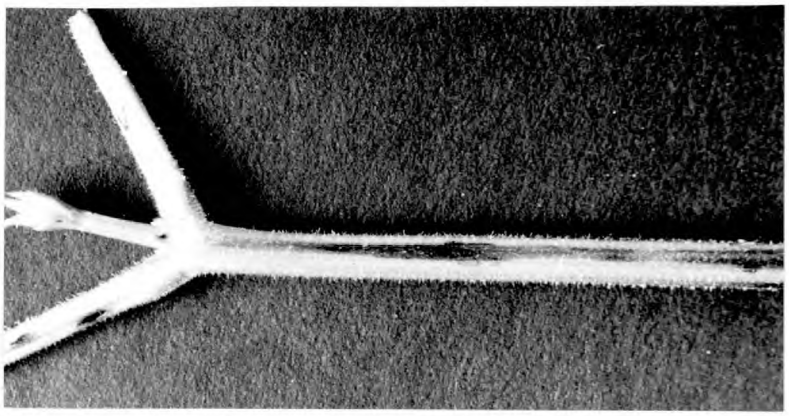
23) Coarse granulation in dark brown epidermal cells of a lesion (Immuna(S)/delta 7 days x 1600)

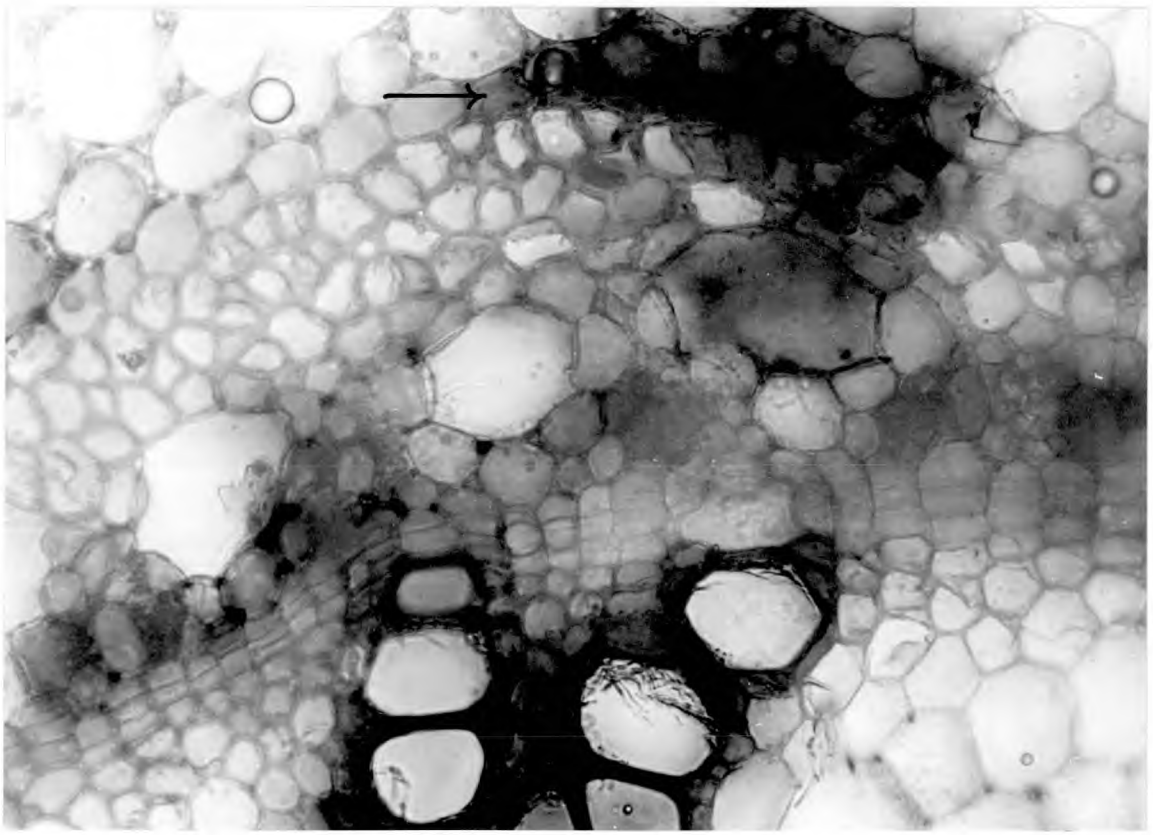
By the time drop exudation had occurred, tissue beyond the original inoculation site showed signs of being affected and often brown streaks were seen in the tissue between lesions (Plate 24 - from a spray inoculated hypocotyl). Transverse and longitudinal sections taken from this region were mounted in sterile water and examined. Neither fungal hyphae nor bacterial contamination (seen as exudation out of the tissue) could be detected although both were clearly visible in sections from the lesion area. Browning was not seen in epidermal cells but was confined to cells of the lower cortex and to vascular elements and parenchyma (Plate 25). The cell contents were granular and brown and moved as a 'gel' when the coverslip was pressed down on the tissue.

The exuded drops normally disappeared after a further 2 or 3 days incubation and the lesions then appeared to be sunken with a slightly raised area in the centre. They were a dark chocolate brown in colour which decreased in intensity along the stem. Acervuli were seen as minute "bumps" on the lesion surface. Complete collapse of hypocotyl tissue occurred later and was associated with fungal colonization and bacterial contamination.

Development of non-limited lesions in Pinto/delta, Red Kidney/beta and Canadian Wonder/beta combinations were similar to this except that in Pinto/delta exudation preceded browning in some hypocotyls.

Plates 24 & 25 Browning in susceptible hypocotyls at a distance from the site of inoculation





24) A hypocotyl inoculated with spore suspension from a spray gun showing a few black sunken lesions and long streaks of dark tissue in between (Kievit(S)/gamma 7 days x 1.5)

25) T.S. bean hypocotyl from a region between lesions showing browning like that in Plate 24. Note granulation and darkening of vascular parenchyma and cortex (arrowed) (Kievit(S)/gamma 7 days x 240)

c) Summary of observations on the susceptible reaction

A diagram showing the approximate time course of events which occurred during the interaction of Kievit (S) and race gamma (V) is given in Fig 2. The main features of the susceptible reaction were as follows:-

1. Penetration of epidermal cells after about 2 days.
2. Intracellular growth of primary hyphae, without any apparent effect on host cells, for a further 3-4 days.
3. Death (inability to plasmolyse in hypertonic sucrose, collapse of cell membranes, loss of streaming etc.) of some infected cells just before symptoms appeared on hypocotyls. Later adjacent uninfected cells also died.
4. Development of narrower secondary hyphae from primary hyphae at the time symptoms appeared and the continued colonization of tissue.
5. Progressive granulation and browning of dead cells.
6. Exudation of a drop of fluid from the brown water-soaked lesion followed by the collapse and drying of lesion tissue.
7. The appearance of brown streaks in uninfected parts of the hypocotyl.

Fig. 2 Diagram to illustrate the course of resistant and susceptible responses of epidermal cells of French bean hypocotyls to races of C. lindemuthianum

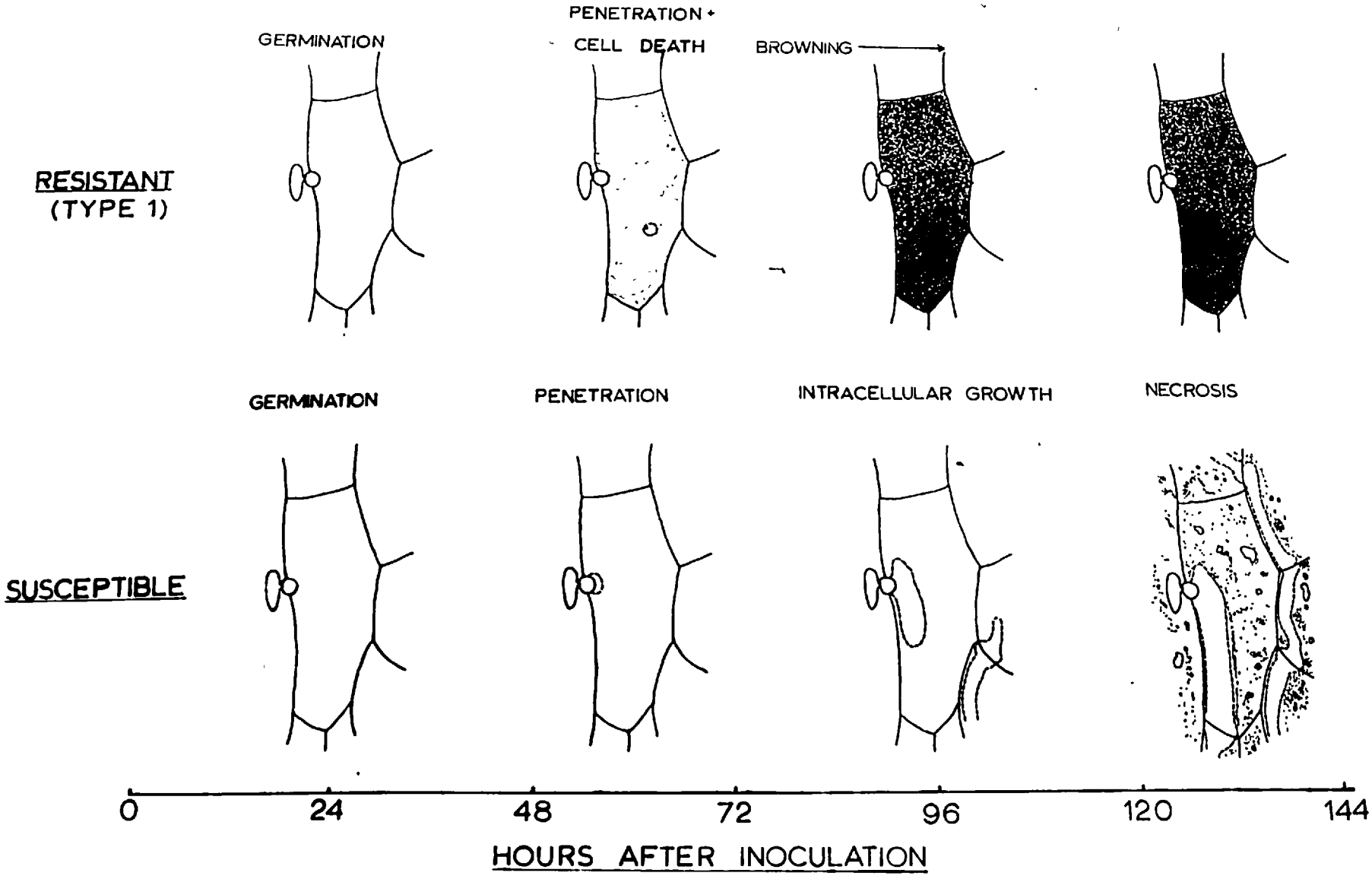
Resistant Type 1 (Immuna(R)/beta)

An appressorium was formed by the spore but no penetration took place until 48h or more after inoculation. Soon after penetration the infected cell became finely granular and the nucleus became rounded and shrunken (cell grade 5 - Table 9). Browning then occurred and increased in intensity for a further 24-48h.

Susceptible (Kievit(S)/gamma)

An appressorium was formed by the spore but no penetration took place until 48h or more after inoculation. After reaching the cell lumen, the infection hypha enlarged to form a primary hypha (dashed line). Primary hyphae colonized apparently unaffected epidermal cells for a further 48-72h. These cells then underwent necrosis and became granular and brown.

Fig. 2


GERMINATION
PENETRATION + CELL DEATH
BROWNING
RESISTANT (TYPE 1)
GERMINATION
PENETRATION
INTRACELLULAR GROWTH
NECROSIS
SUSCEPTIBLE
0
24
48
72
96
120
144
HOURS AFTER INOCULATION

5. Development of resistant responses

A fundamental difference between a susceptible and a resistant response (Tables 3, 4 and 5) was that in the former, infected cells remained colourless for some time while hyphal growth took place within them, but in the latter, cells became brown and extensive colonization of host tissue did not occur. A number of important questions present themselves :-

Is the cell reaction always associated with the presence of a hypha or might it occur before penetration?

Could resistance be determined before penetration, cell response being merely a secondary phenomenon?

Is there any evidence of an increase in cell activity before death and browning?

How are these changes related to the inhibition of hyphal growth?

Do all varieties respond in the same way to all races?

Thus it was of some interest to know what changes took place in the cell protoplasts of different varieties before the completion of the "browning reaction".

It was suggested earlier that a single chemical compound, possibly phaseollin, could be responsible for hyphal inhibition and that a critical test of its role would be to determine whether phaseollin reached inhibitory concentrations at the time when hyphal growth was affected. Bailey (1970 - see Review of Literature) has studied

the increase in levels of phaseollin that occurs in resistant hypocotyls after inoculation with an avirulent race and has suggested a method whereby the average phaseollin content of infected cells might be estimated. In the present study (see Page120 et seq) the relationship between hyphal growth rate in vivo and the degree of cell response has been investigated in order to determine the time of growth inhibition. If these two types of investigation, one on gross changes in infected tissue and the other on the response of individual cells, could be related then the role of phaseollin in varietal resistance could be examined more critically. The cell grading system seems to be of some use here as a means of defining the range and degree of cell reaction of tissues at different times after inoculation (i.e. the sort of material that would be used in biochemical studies).

In the following studies the variety/race combinations of Immuna(R)/beta(AV) and Kievit(R)/delta(AV) were examined by this system in an attempt to detect early stages of the resistant reaction and to see whether this method might be applied to biochemical studies. These combinations were those used by Bailey (1970) and by the author in the cross protection and acquired susceptibility studies of Sections 3 and 4. The detailed studies given below are summarized in Fig. 2 (Page 97), Fig. 5 (Page112) and on Page 132.

a) Race beta on c.v. Immuna

(i) Course of infection and cell response

Nine day Immuna hypocotyls were inoculated with spores of race beta and at intervals epidermal strips taken from the inoculation

sites, were examined using the cell grading method. The results are shown in Table 12 and Fig. 3.

Some penetration was detected at 38 and 46h (Table 12) but it was seen more frequently at 54h and later. Only once was a hypha observed to have grown from the penetrated cell into an adjacent cell (110h).

The course of the reaction was suggested by changes in the proportions of cells assigned to the different "affected" grades (5, 7, 8 and 9) during the period of incubation. Many affected cells were found to be granular and colourless (grade 5, Plate 9) at 54 and 62h but the proportion of this type of cell decreased rapidly as browning (indicated by increases in % of cells of grades 7, 8 and 9) took place. Browning was homogeneous throughout the cell and not confined to the region of the appressorium. As there was no evidence that any other clearly recognisable stage preceded that described by grade 5 it is suggested that the cellular response of Immuna to race beta occurred in the manner shown diagramatically on Page 97 (Fig. 2, Resistant Type 1) i.e. from grade 5 to 7 to 8 to 9.

Cells containing reaction material were observed some time after the first observations of granulation and browning although some cells containing this material were later seen to be affected in this way.

The results shown in Fig. 3 indicated that about 50% of the cells examined became affected but only a few were seen to contain clearly recognisable hyphae. Three explanations seemed possible:-

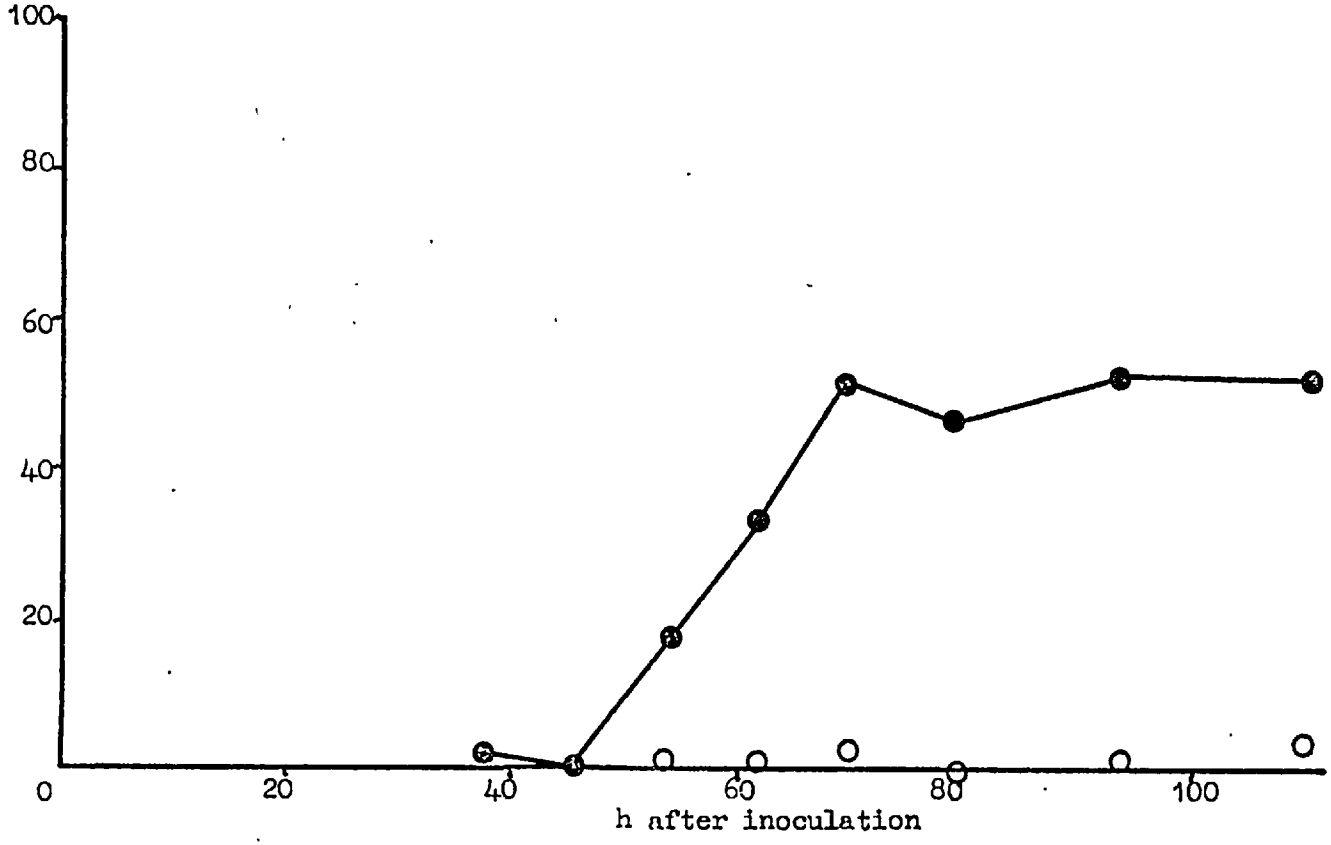
1) That hyphae were obscured by browning and granulation

Table 12. Hyphal development and reaction of epidermal cells of resistant bean hypocotyls (c.v. Immuna), at various times after inoculating with race beta

Grades	Description	% of cells examined[1] assigned to different grades after(h):- 38	46	54	62	70	78	94	110
	Cells unaffected								
1.2	Hypha in penetrated cell	0.3	1.0	4.5	4.8	5.0	2.5	0.8	1.0
1.3	Hypha in second cell	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3
	Cells affected								
5.2, 5.4	Cytoplasm granular, colourless	0.0	0.0	5.3	4.0	3.8	0.0	0.0	1.3
7.2, 7.4	" " , v.pale yellow brown	0.0	0.0	1.5	1.5	2.8	1.3	0.3	0.8
8.2, 8.4	" " , light brown	0.0	0.0	4.8	10.3	18.3	11.8	10.3	11.3
9.2, 9.4	" " , brown/dark brown	0.5	0.0	4.0	16.8	26.5	31.8	41.3	36.5
10.2,10.4	Browning of part of cell only	0.3	0.0	0.8	0.5	0.5	1.3	0.8	1.5
	Reaction material produced in cells								
2.1	Obvious particles streaming throughout cell	0.0	0.0	0.0	4.8	4.0	7.5	7.3	11.0
6.1,8.1,9.1, 10.1, 11.1	Cell affected, colourless or partly or completely brown	0.3	0.0	0.0	0.8	1.0	1.0	1.0	3.3
2.4,3.4,6.2	Other grades	0.5	0.0	0.5	0.3	0.5	0.3	1.3	1.5

1 Four replicate hypocotyls, giving a total of 400 cells, examined at each time

Fig. 3. Increases in the proportion of penetrated and affected epidermal cells in c.v. Immuna hypocotyls after inoculation with race beta



2) That c.v. Immuna was resistant to penetration by race beta and that the observed cell response was not associated with the mechanism of resistance, response occurring in the absence of penetration

3) That growth was inhibited before the infection hypha had expanded and emerged from beneath the opaque appressorium.

As the difference between the proportions of affected and penetrated cells was evident as early as 54h when many affected cells, although faintly granular, were colourless the first explanation seemed unlikely. When Immuna hypocotyls grown from a second batch of bean seed were examined 60h after inoculation with race beta, many cells were found to contain hyphae. Observations were continued at intervals in an attempt to resolve the problem of the relationship between cell response and the presence of intracellular hyphae. The results are shown in Table 13.

In this second experiment many more cells were found to contain hyphae (more than 35% at 60 and 80h) than in the previous experiment (max. 7%) but again of all those examined, only one hypha had grown into a second cell during the period of incubation. The proportion of penetrated cells that had become granular and brown increased throughout so that at the later times very few of these cells were unaffected. This decline in the proportion of penetrated cells of grade 1 preceded a similar decline in cells of grade 5 and an increase in cells of grade 8 and 9. Thus it was concluded that after penetration the response of infected cells took place in the manner reported earlier (Fig. 2, Page 97). It was clear resisistance to penetration was not the prime cause of the failure of race beta to

Table 13 Hyphal development and reaction of epidermal cells of resistant bean hypocotyl (c.v. Immuna - different seed batch from that of Table 11) at various times after inoculating with race beta

Grades	Description	% cells examined* assigned to different grades after(h):- 60	84	108	360
	Cell unaffected				
1.2	Hypha in penetrated cell	14.5	2.5	1.0	0.0
	" " second cell	0.0	0.3	0.0	0.0
	Cell affected - hypha in penetrated cell				
5.2	Cytoplasm granular, colourless	18.8	18.5	1.5	0.0
7.2	" " , v.pale brown	2.8	6.0	5.5	1.0
8.2	" " , light brown	0.8	7.8	8.5	5.0
9.2	" " , brown/dark brown	0.0	3.3	9.5	9.8
	Total	22.4	35.6	25.3	15.8
	Cell affected - no hypha visible				
5.4	Cytoplasm granular, colourless	3.8	7.0	0.5	0.0
7.4	" " , v.pale brown	1.8	2.5	2.5	1.8
8.4	" " , light brown	0.8	5.5	10.0	10.8
9.4	" " , brown/dark brown	0.5	5.3	27.8	37.0
	Total	6.9	20.3	40.8	49.6
10.2, 10.4	Browning of part of cell only	0.0	0.8	0.3	0.5
	Reaction material produced in cells				
2.1	Obvious particles streaming throughout cell	0.0	3.8	6.8	6.5
5.1,6.1,7.1 8.1,9.1,10.1	Other grades	1.3	1.3	2.0	0.8

* Four replicate hypocotyls, giving a total of 400 cells, examined at each time

cause lesions on c.v. Immuna and that hyphal growth ceased in cells which showed very marked changes in appearance.

As cells became more granular and brown, intracellular hyphae became more difficult to detect. This was reflected in the decrease in the proportion of penetrated affected cells at a time when the total percentage of affected cells remained constant (108 and 360h). To check that such cells actually contained hyphae and to determine whether, after a long period of incubation, all affected cells were clearly penetrated, the epidermal strips (108 and 360h) were immersed in 0.1N NaOH and then re-examined. At 108h, 26.3 and 51.5% of cells were found to be penetrated in uncleared and cleared tissue respectively, 65.1% of cells were affected. Similarly at 360h the respective values were 15.8%, 47.0% and 65.9%. In this second experiment therefore, although clearing the granular and brown cells allowed the detection of many more intracellular hyphae, some affected cells could not be proved to have been penetrated and thus the cellular response might have been initiated without the hypha coming into direct contact with the cell protoplast.

(ii) Qualitative observations

As suggested earlier, the effects typical of cell grade 5 appeared to mark the first stage of the Immuna/beta resistant response. Cells of this type had the following properties:-

1) No movement was apparent within them compared with active streaming around the margin and in transvacuolar strands of healthy cells

2) No plasmolysis or deplasmolysis occurred when strips were immersed in 0.5M sucrose then distilled water although both were observed in adjacent cells

3) Fine granulation, just visible at a magnification of x 1000, occurred evenly throughout the cell

4) Nuclei were rounded and shrunken (4-8μm compared with 11-21 x 4-7μm in unaffected cells), nucleoli were obvious (Plate 26)

5) A deep pink/red colour developed in the cytoplasm after immersing in 10ppm Rhodamine B for 1h, unaffected epidermal cells only showing a faint pink coloration of the vacuole.

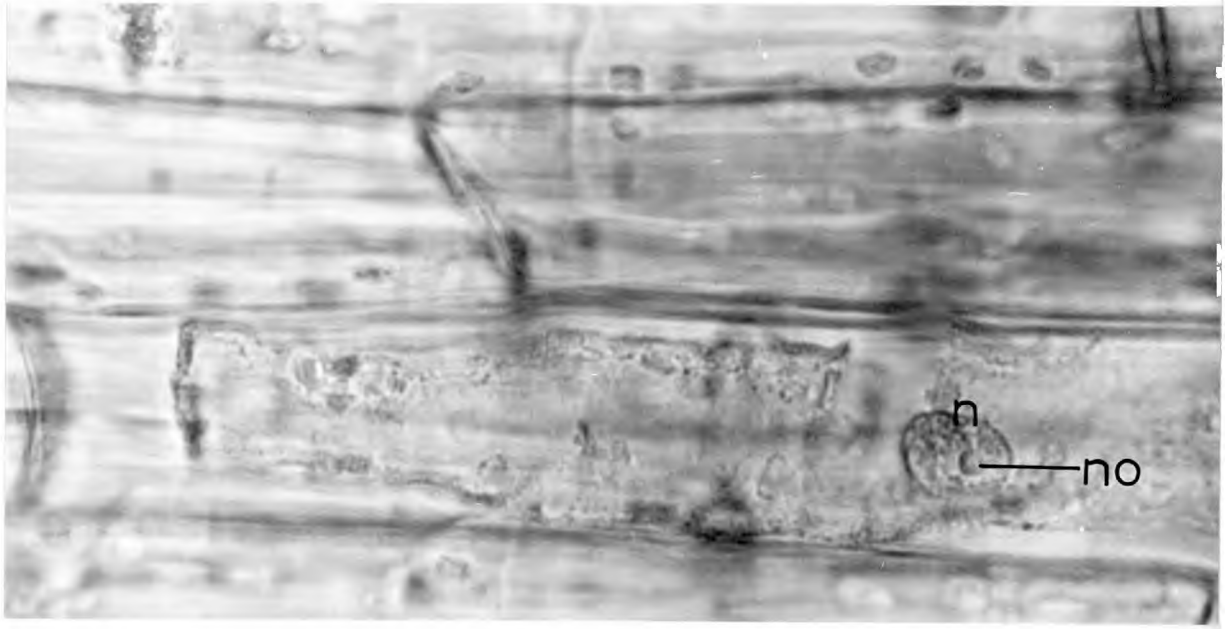
6) The protoplast was sometimes found to be shrunken (Plate 27)

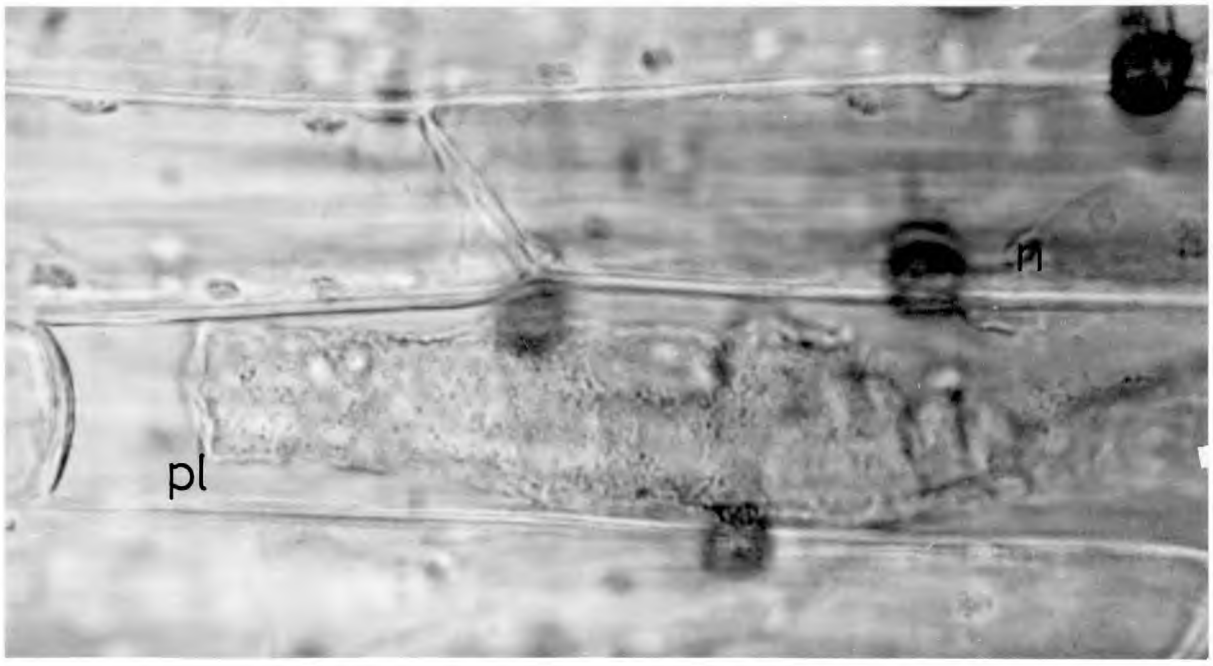
When tested for the presence of catechol tannins by the "Nitroso-method" cells failed to show a positive reaction although the cell walls at the base of hairs of inoculated tissue stained red in this stain and Rhodamine B.

After observing a large number of cells at 40-65h no conclusive evidence was obtained of a visible increase in cell activity or any other change that might be considered to precede that which was responsible for the loss of vital properties shown by cell grade 5. However in some cells a more active streaming of particles was observed.

Cells which were adjacent to affected cells were normally similar in appearance to those of uninoculated tissue. However after prolonged incubation (14 days) the streaming particles often appeared to be larger than those of control cells. Granulation and browning was never seen in a cell adjacent to an affected cell when no appressorium was present on the cell. Hyphae were not observed in adjacent cells in epidermis and cortex (except as noted in Tables 12 and 13).

Plates 26 & 27 Appearance of epidermal cells of c.v. Immuna hypocotyls showing the first visible signs of resistant response to race beta





26) Cell of grade 5. Note shrunken nucleus (n) with obvious nucleolus (no). (Immuna(R)/beta 72h x 1600)

27) As Plate 26 higher plane of focus. Note shrinkage of plasmalemma (pl) and overall fine granulation of cytoplasm. Nucleus (n) of adjacent cell was not rounded.

b) Race delta on c.v. Kievit

A second variety/race combination was studied by the cell grading method to determine whether the course of cell response outlined in Fig. 2 only occurred with Immuna/beta or could be regarded as typical of resistant reactions of bean to C. lindemuthianum. The detailed "time-course" information would also serve as a basis for the interpretation of the cross protection studies reported later.

(i) Course of infection and response

Excised hypocotyls of c.v. Kievit (R), which were grown at the same time as those used for a study of the susceptible reaction (Table 11), were inoculated with spores of race delta (AV). The results of observations made on three replicate hypocotyls at each of the times given are summarized in Table 14 and Fig. 4.

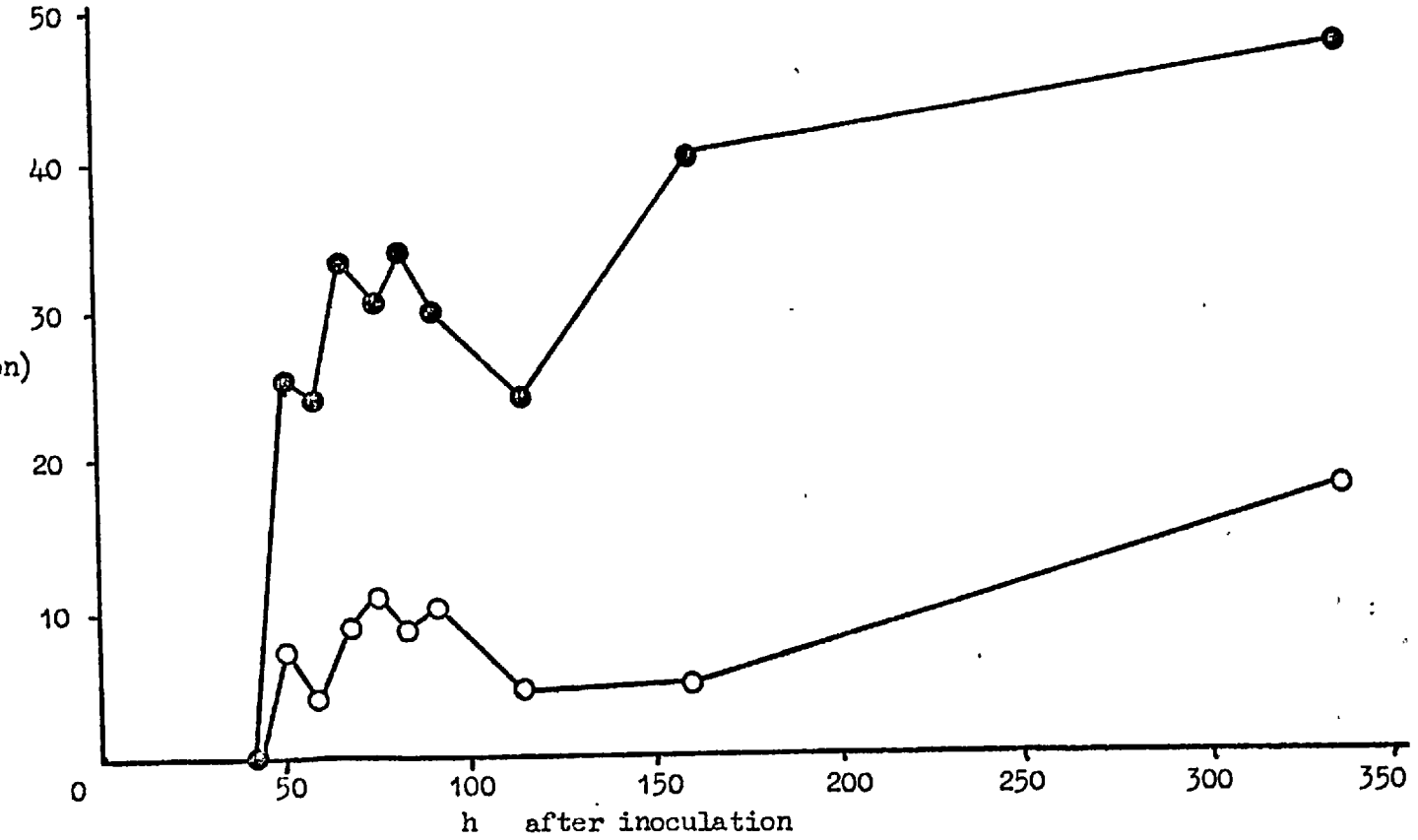
Little penetration had taken place by 42h but the proportions of affected and penetrated cells approached their maximum value during the following 24h. As in the previous experiments hyphae failed to grow from the penetrated cells (except at 336h), all of which cells had shown some reaction by 114h. However the types of cell response found during the period 50 to 114h were quite different from those observed with Immuna/beta. Very few cells of grade 5 were seen, instead many cells contained obvious particles which streamed throughout the cell (grade 2, Plate 5), or in the region of the appressorium (grade 3, Plate 6). In others little streaming was observed but cytoplasm and particles had accumulated beneath and around the appressorium (grade 4, Plates 7 and 8). Later the

Table 14 Hyphal development and reaction of epidermal cells of resistant bean hypocotyls (c.v.Kievit) at various times after inoculating with race delta

Grades	Description	% cells examined[1] assigned to different grades after(h):- 42	50	58	66	74	82	90	114	162	336
	Cells unaffected										
1.2	Hypha in penetrated cell	1.7	3.0	3.7	0.7	3.0	1.7	0.3	0.0	0.0	0.0
1.3	Hypha in second cell	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Cells affected - no browning										
2.2, 2.4	Obvious particles streaming throughout cell	0.0	3.7	1.7	5.0	0.7	0.3	0.3	2.7	3.3	3.0
3.2, 3.4	" " predominently near appressorium	0.0	2.3	1.3	4.0	0.0	0.3	0.3	0.0	0.0	0.0
4.2, 4.4	Aggregation of cytoplasmic material near appressorium	0.0	16.0	9.7	9.3	8.3	6.7	7.0	4.0	4.3	0.7
5.2, 5.4	Cytoplasm granular throughout	0.0	0.3	0.7	0.0	0.3	0.7	0.0	0.3	0.3	0.0
	Cells affected - browning										
10.2,10.4	Cytoplasm granular, pale to dark brown near appressorium	0.0	1.3	7.7	11.3	14.0	9.3	10.0	6.0	5.3	1.7
7.2, 7.4	" " , v.pale yellow brown	0.0	0.3	0.0	0.0	1.7	0.7	3.0	0.7	2.7	0.7
8.2, 8.4	" " , light brown	0.0	0.3	0.3	0.3	3.0	3.7	3.7	3.3	5.3	6.0
9.2, 9.4	" " , brown/dark brown	0.0	0.7	2.7	3.0	2.0	12.0	4.3	7.0	18.3	25.0
7.3,8.3,9.3	Cell brown hypha in second cell	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.7
	Reaction material produced in cells										
2.1	Obvious particles streaming throughout cell	0.3	3.3	3.7	1.7	3.7	4.7	7.0	7.0	4.7	5.7
4.1,5.1,7.1, 8.1,9.1,10.1)	Cell affected, colourless or partly or completely brown	0.0	2.3	2.3	1.7	1.0	3.3	3.3	3.0	1.0	1.7
11.4, 6.2	Other grades	0.0	0.0	0.0	0.0	0.7	0.3	0.0	0.3	0.3	0.0

1 Three replicate hypocotyls, giving a total of 300 cells, examined at each time

Fig. 4. Increases in the proportion of penetrated and affected epidermal cells in c.v. Kievit hypocotyls after inoculation with race delta



proportion of these cells decreased as cells similar to grade 4 but with granulation and browning of the accumulated cytoplasm (grade 10, Plate 12) were seen more frequently. These trends and further evidence given later suggested that the course of response of epidermal cells of c.v. Kievit to race delta corresponded to changes from grade 1 to 2 to 3 to 4 to 10 to 7, 8 and 9. This is shown diagrammatically in Fig. 5.

Figure 4 shows the progress of penetration and cell response. As with Immuna/beta (first batch of beans), the proportion of affected cells greatly exceeded that in which hyphae were visible even at times when there was little granulation and browning to obscure them.

Variation in the proportion of affected cells at different examination times was apparent (Fig. 4) and its significance is discussed on Page 283. The effect of between-hypocotyl variation is also shown in Table 14 where at 336h penetration of adjacent cells (grades 7.3, 8.3 and 9.3) had occurred. This was almost completely confined to one of the replicate hypocotyls and the high level of penetration observed with this replicate could account for the increase shown between 160 and 336h in Fig 4.

(ii) Qualitative observations

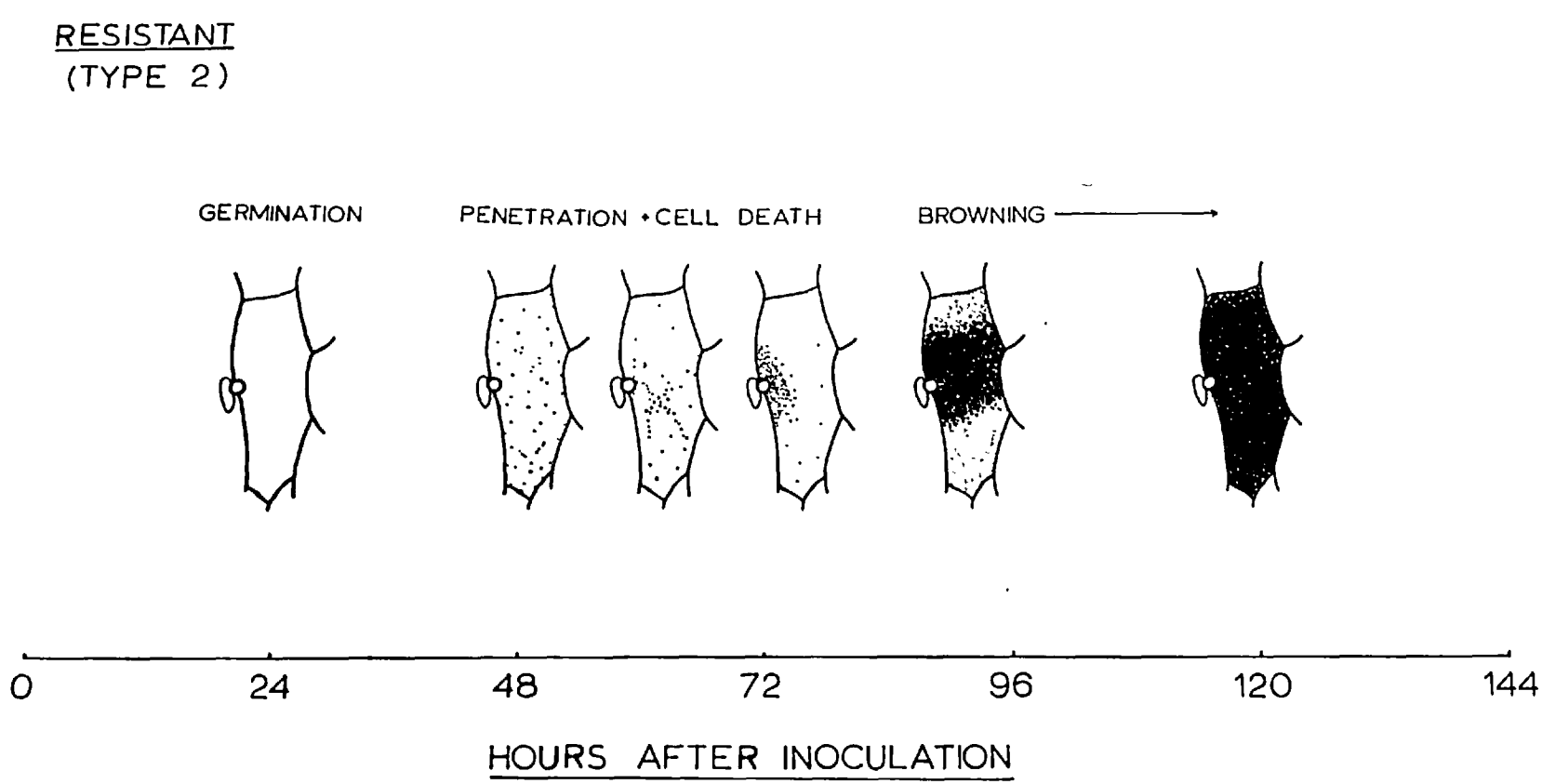
As mentioned above it appeared that events characterized by cell grades 2 - 4 probably preceded granulation and browning in the response of c.v. Kievit to race delta. However no evidence was obtained to indicate that events definitely took place in the sequence 2, 3, 4. In the following experiment Van Tieghem cells were used so that individual cells could be observed at intervals. These

Fig. 5 Diagram to illustrate the course of the resistant response of an epidermal cell of a French bean hypocotyl to a race of C. lindemuthianum

Resistant Type 2 (Kievit(R)/delta)

An appressorium was formed by the spore but no penetration took place until 48h or more after inoculation. Soon after penetration obvious particles became visible in the cell (cell grade 2 Table 9). Particles and cytoplasm aggregated in the cell at the site of infection (cell grades 3 and 4); browning then occurred and spread from this site throughout the cell (cell grades 10 to 7, 8 and 9).

Fig. 5


RESISTANT
(TYPE 2)
GERMINATION
PENETRATION + CELL DEATH
BROWNING
0
24
48
72
96
120
144
HOURS AFTER INOCULATION

were set up as in Materials and Methods. A number of cells of the above grades were examined, "marked" and then re-examined after 12h incubation at room temperature.

At the first examination, most streaming in the 6 cells of grade 3 was towards the appressorium although more randomly orientated movement was seen in parts of the cell furthest from this body. The movement of particles aggregated beneath the appressorium was random in direction and restricted in extent. Where very marked aggregation had taken place, particle movement sometimes resembled Brownian motion but was less rapid than that shown by bodies in the cell vacuoles. This effect was seen in 2 out of 3 cells of grade 4 while in the third no motion was evident and some granulation of cytoplasm had taken place. After 12h incubation this latter cell had not changed in appearance but cells of grades 3 and 4 in which moving particles had previously been aggregated were classed as grade 2. Thus the particles had become randomly distributed. No change was observed in cells previously classed as grade 2 and no coagulation or browning occurred during the re-incubation period.

These results suggested that when strips of inoculated material were removed from hypocotyls and incubated in Van Tieghem cells the process of cytoplasmic accumulation which preceded granulation and browning (as shown in Table 14)was reversed and a return to an "earlier" stage (grade 2), where particles streamed randomly throughout the cell, took place. Thus the sequence of events suggested in Fig. 5 is confirmed by this experiment.

To determine the stage at which cell vitality was first affected,

observations were made on cells of strips taken 40-70h after inoculation and which were examined soon after removal from the hypocotyls.

Cells of grades 2 and 3 exhibited the vital properties of: cytoplasmic streaming, ability to plasmolyse in hypertonic sucrose solution then deplasmolyse in water and to show no reaction with Rhodamine B (10ppm for 2h) apart from a slight pink coloration of the vacuole. Many cells of grade 4 however, failed to plasmolyse or plasmolysed incompletely, the protoplast being withdrawn only at a point farthest from the appressorium. A pink colour developed after Rhodamine B treatment and was of greatest intensity in the mass of accumulated, slightly granular cytoplasm. Cytoplasmic streaming was absent in these dead cells but in those of grade 4 where some particle movement was observed the other vital properties could also be demonstrated. Thus cell death occurred at a time when the accumulated cytoplasm became granular and was indicated by the loss of streaming activity, semipermeable properties of the cell membrane and an increased affinity for Rhodamine B. In contrast to the Immuna/beta combination, where cell death was the first sign of a response, this process occurred after an increase in cell activity (streaming) and a movement of cytoplasm, had taken place.

The obvious particles typical of grades 2 and 3 were not found to stain with Rhodamine B, neutral red or the Nitroso reagent. When strips of inoculated tissue were floated on a 10ppm solution of Janus Green (G.T.Gurr Ltd.) in 0.3M sucrose for 2h (according to the method of Sorokin, 1955, for the detection of mitochondria) particles in healthy or affected cells gave a negative reaction. Thus no

evidence was obtained concerning the identity of these particles.

Effects on adjacent cells were more commonly observed with this combination than with that of Immuna/beta. Larger particles were often found in cells and after 5-6 days these were sometimes seen 2-3 cell widths from a brown granular cell. The cells around large groups of brown cells were affected for an even greater distance and particles or vesicles were often motionless and up to 1·5 μm in size. On prolonged incubation (1-2 weeks), granulation was seen in some adjacent cells.

c) Cellular response in other combinations of race and resistant variety

As the early stages of the two resistant reactions described above were found to be different, it was decided to investigate others in an attempt to discover whether the reaction type is determined by either the variety of bean used or the race of fungus.

Hypocotyls of c.v. Kievit and Dubbele Witte were inoculated with spores of race beta (AV), those of Red Kidney with race delta (AV) and Immuna with race gamma (AV). Epidermal strips were removed after 72h incubation and the results of observations made on these are shown in Table 15.

It can be seen from Table 15 that the response of Kievit and Dubbele Witte to race beta was very like that reported earlier for the reaction of Immuna to this race (Table 12) in that a large proportion of affected cells were classed as grade 5 while few were of grades 2, 3 and 4. The nuclei of grade 5 cells in both these combinations

Table 15. Response of epidermal cells of different bean varieties 72h after inoculation with races beta, gamma and delta

Grade	Description	% cells examined[1] assigned to grades for the following variety race combinations			
		Kievit/ beta	Dubbele Witte/ beta	Red Kidney/ delta	Immuna/ gamma
	Cell unaffected				
1.2	Hypha in penetrated cell only	0.0	0.3	2.8	0.0
	Cell affected - colourless				
2.2, 2.4	Obvious particles streaming throughout cell	0.0	0.0	1.0	0.0
3.2, 3.4	" " predominantly near appressorium	0.0	0.0	0.3	0.0
4.2, 4.4	Aggregation of cytoplasic material nr.appressorium	0.0	0.3	3.8	0.0
5.2, 5.4	Cytoplasm granular throughout	26.3	8.5	0.8	2.8
	Cell affected - brown				
7.2, 7.4	Cytoplasm granular, v.pale brown	4.0[2]	2.3[2]	5.0[2]	1.3[2]
8.2, 8.4	" " light brown	0.0	1.5[2]	5.8	0.8
9.2, 9.4	" " brown/dark brown	0.0	0.3	4.0	0.0
10.2,10.4	" " pale to dark brown near appressorium	0.0	0.3	1.8	0.0
	Reaction Material				
2.1	Obvious particles streaming throughout cell	0.0	16.5	6.5	0.8
3.1	" " near appressorium	0.0	0.0	0.8	0.5
4.1	Aggregation of cytoplasmic material nr.appressorium	0.0	4.8	5.8	4.3
5.1	Cytoplasm granular throughout	0.0	1.3	0.8	0.0
7.1, 8.1, 9.1	Cell brown	0.0	0.8	6.5	0.0

1 Four replicate hypocotyls, giving a total of 400 cells, examined at each time

2 Nuclei rounded and shrunken

were rounded and shrunken, the fine granulation in the cytoplasm being homogeneous throughout the cell. These results suggest that the response observed in these two combinations was of Resistant Type 1 (Fig.2, Page 97).

A number of cells of grades 2, 3 and 4 were found in the Red Kidney/delta combination. However shrunken nuclei were seen in pale brown cells and in relatively few cells was browning restricted to the region of the appressorium. Thus the response of this variety had features in common with both Resistant Type 1 and Type 2 (Fig.5 Page 112) although it was more like Type 2. It is noted that in cells containing reaction material, characteristics of grades 2, 3 and 4 were found.

On this and several other occasions when the Immuna/gamma combination was studied, very few cells were affected. Of the cells without reaction material, most affected cells showed characteristics of Resistant Type 1 i.e. fine overall granulation and shrunken nuclei.

These results, taken together with those obtained earlier, suggest that the type of reaction is more closely related to fungal race than bean variety. Races beta and gamma appeared to initiate Resistant Type 1 and race delta, Resistant Type 2.

Conclusion (sub-section 5)

Epidermal cells of a resistant variety died rapidly after being penetrated by an avirulent race and the contents became granular and brown. Penetration of a cell showing such a response could not always be proved but those hyphae which were present did not normally grow from the cell. Based on the changes preceding cell death, 2 types of reaction were distinguished; Resistant Type 1 (Fig. 2 , Page97), where cell death and granulation of cytoplasm were the first recognizable signs of response, and Resistant Type 2 (Fig. 5 Page 112), where increased streaming and the accumulation of cytoplasm at the site of penetration preceded cell death. The type of response shown appeared to depend on the fungal race used rather than on the variety of bean.

6. Stage of inhibition of hyphal growth in cells showing a resistant response

The close association between the death and browning of infected cells and the failure of an avirulent race to colonize more than one or two cells of a resistant variety was suggested by the data given in Tables 12,13 & 14. Further experimentation appeared to be necessary to answer two questions:-

a) At what stage in the response of an infected cell does the growth of an intracellular hypha become inhibited?

b) Do hyphae continue to grow in cells after the resistant reaction has been completed?

The significance of the first question in relation to biochemical studies has been stressed earlier. Growth of a fungal hypha after the completion of a resistant response would indicate that this response had had an inhibitory rather than a fungicidal effect on the hypha.

In the following experiments the qualitative demonstration of hyphal growth and evidence of marked changes in growth rate were sought by measuring hyphal length at intervals after inoculation, the stage of cell response at these times also being determined. Race beta (AV) and c.v. Immuna (second batch of beans) were used here because penetration of 10-39% of cells had been obtained consistently with this combination. Hyphae were rarely found in cells of other resistant varieties inoculated with avirulent races.

a) Relationship between degree of cell reaction and hyphal growth

This relationship might be demonstrated in two ways:

a) by the presence of longer hyphae in unaffected than in granular and brown cells

b) by a decrease in hyphal growth rate, as suggested by a reduced increase in mean hyphal length, at a time when a particular cell grade was predominant.

In the following experiment, strips were taken from Immuna hypocotyls at 40h, then at 12h intervals, after inoculation with race beta until browning had obscured the hyphae. The cell grade and length of any intracellular hypha was determined for each of 75 cells on 2 strips for 4 replicate hypocotyls, the results of these observations being presented in Table 16.

The results shown in Table 16 indicate that the longest hyphae were found in unaffected cells. At 52h the maximum hyphal length in a living cell was 39μm compared with 10μm in dead and brown cells. Although growth appeared to be very limited in affected cells some did take place. This was suggested by the fact that while the proportions of cells containing hyphae of less than 10μm remained constant, an increasing proportion of hyphae in affected cells exceeded 5μm in successive observations. Little increase occurred in mean hyphal length after 52h when almost half the penetrated cells were affected, the majority of these affected cells being of grade 5.

It was concluded that there was some evidence of hyphal inhibition in cells which were at the earliest recognisable stage of the resistant reaction (grade 5) and thus browning did not necessarily precede inhibition.

Table 16. Size of hyphae of C. lindemuthianum race beta and reaction of epidermal cells of resistant hypocotyls (c.v. Immuna) at intervals after inoculation

	% of cells examined[1] assigned to categories after (h) :-											
	40			52			64			76		
	Alive[2]	Dead[3]	Brown[4]	Alive	Dead	Brown	Alive	Dead	Brown	Alive	Dead	Brown
1.4 - 4.2 µm	3.0	2.0		1.5	1.3	0.7	0.5	0.3	1.0	0.0	1.7	1.5
5.6 - 8.4 µm	0.7	0.5		1.2	1.7		0.7	3.0	3.2	0.2	2.2	2.0
9.8 - 12.6 µm	0.2			0.5			0.0	0.5		0.0	1.2	0.7
14.0 - 16.8 µm				0.5			0.3			0.0	0.5	0.7
18.2 - 21.0 µm				0.2			0.2			0.2		
22.4 + µm				0.2			0.3					
Mean hyphal length, all grades		3.9 µm			6.9 µm			7.1 µm			7.4 µm	
% penetrated cells in different states	61.0	39.0	0.0	52.5	38.5	9.0	16.0	39.8	44.2	3.8	51.2	45.0
% cells penetrated		6.4			7.8			10.0			10.7	

NOTES

1 Four replicate hypocotyls, giving a total of 600 cells, were examined at each time

2 Cell grade 1 - No granulation or browning

3 Cell grade 5 - Granulation no browning

4 Cell grade 7, 8 and 9 - Granulation and browning

A number of difficulties in interpreting this data are apparent:-

1) The reaction of individual cells was a continuous process and even those penetrated cells which remained unaffected for some time became granular and brown eventually. As hyphal growth and cell reaction were not examined in the same cells at each observation, it was impossible to say when the response of individual cells had taken place. The hyphal length recorded for an affected cell may have been attained during a time when the cell was unaffected and thus the effect of inhibition would have been underestimated. Between 64 and 72h there was an apparent increase in the length of hyphae in affected cells. This increase could have been due to just such an effect.

2) The proportion of penetrated cells increased throughout. This meant that the numbers of hyphae in the smallest length grade would have been increased and thus inhibition overestimated.

In any event it was clear that other methods were necessary to confirm that some inhibition of hyphal growth occurred in granular but non-brown cells. A means of measuring hyphal growth rates in individual cells which were at different stages of reaction appeared necessary to overcome the shortcomings of the first method and thus a Van Tieghem Cell seemed to be applicable. As mentioned earlier, changes in cell appearance are retarded when strips of inoculated tissue are removed from hypocotyls, so it seemed possible that some penetrated cells at least would remain in the same grade during a period of incubation and thus overcome the first problem. The difficulties caused by continuous penetration would be overcome by measuring only established hyphae. A major assumption was made; that the chemical

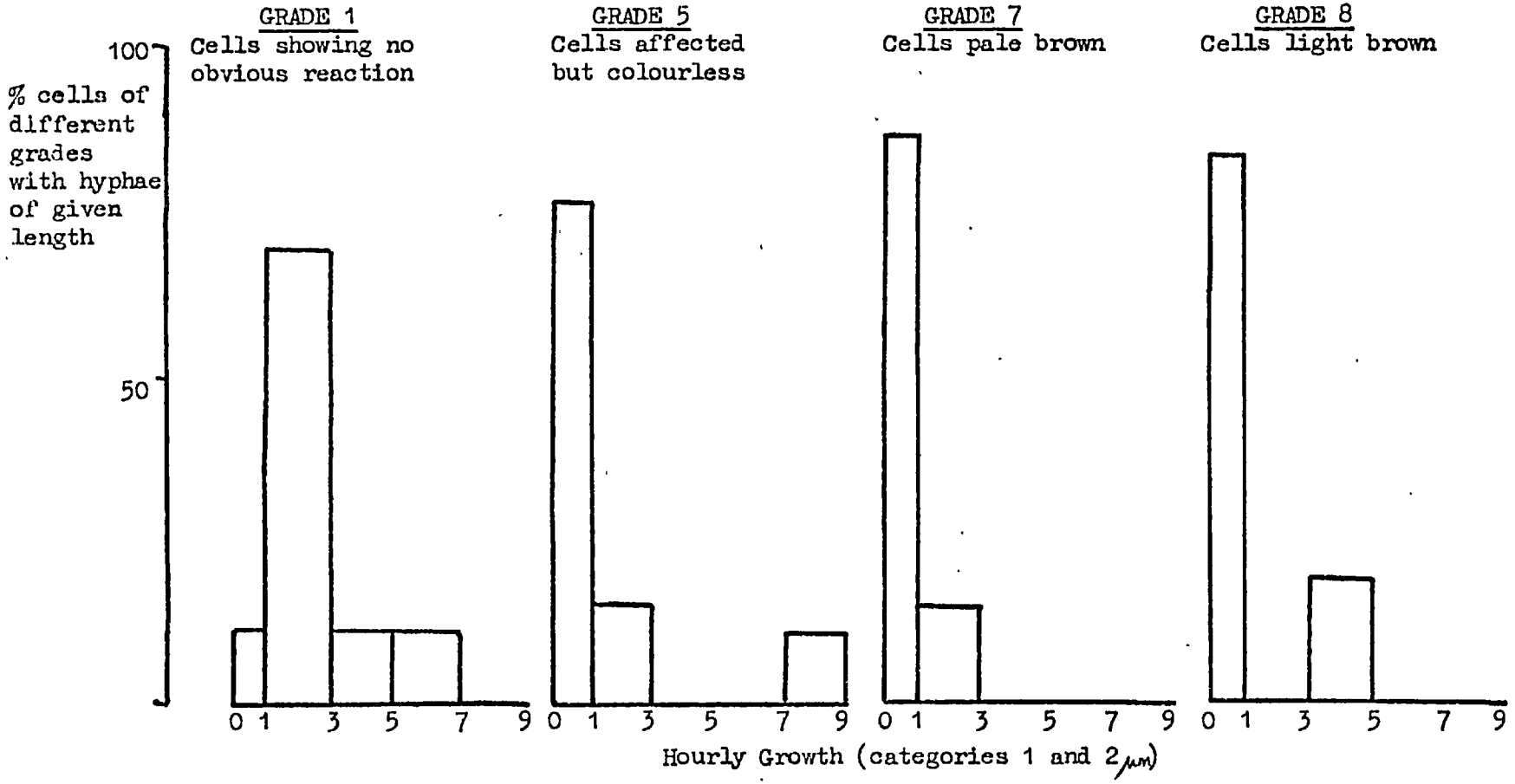
content and physiological activity of the host cells were typical of those at the same reaction stage in intact hypocotyls.

As a preliminary test to determine whether hyphae grew freely in cells of strips detached from hypocotyls, c.v. Immuna was inoculated with spores of race delta (V) and strips removed after 72h. These were mounted in Van Tieghem Cells and the lengths of hyphae in marked (vernier reading and drawing) epidermal cells were measured. The times of the individual measurements were noted and after incubation at room temperature for 9h the hyphae were re-measured. The mean growth per h of 29 hyphae was $3.32 \pm 2.03 \mu m$

This method was then applied to race beta in the resistant variety Immuna and two sets of observations were made on 9 and 10 day hypocotyls respectively. Strips taken from hypocotyls about 60h after inoculation with race beta were placed in the Van Tieghem cells. The cell grade and length of intracellular hypha were recorded for a number of "marked" epidermal cells, about 6h elapsing between removal of strips from the hypocotyl and completion of the first measurements. The second measurements were made after 16-18h incubation at room temperature ($20^{\circ}C$), the exact interval between the two measurements for each cell being recorded. In Figs. 6 and 7 the results of these observations are shown as the percentage of cells at different stages of reaction which contained hyphae possessing different hourly growth rates. The grades of the individual cells examined in this study did not change during incubation.

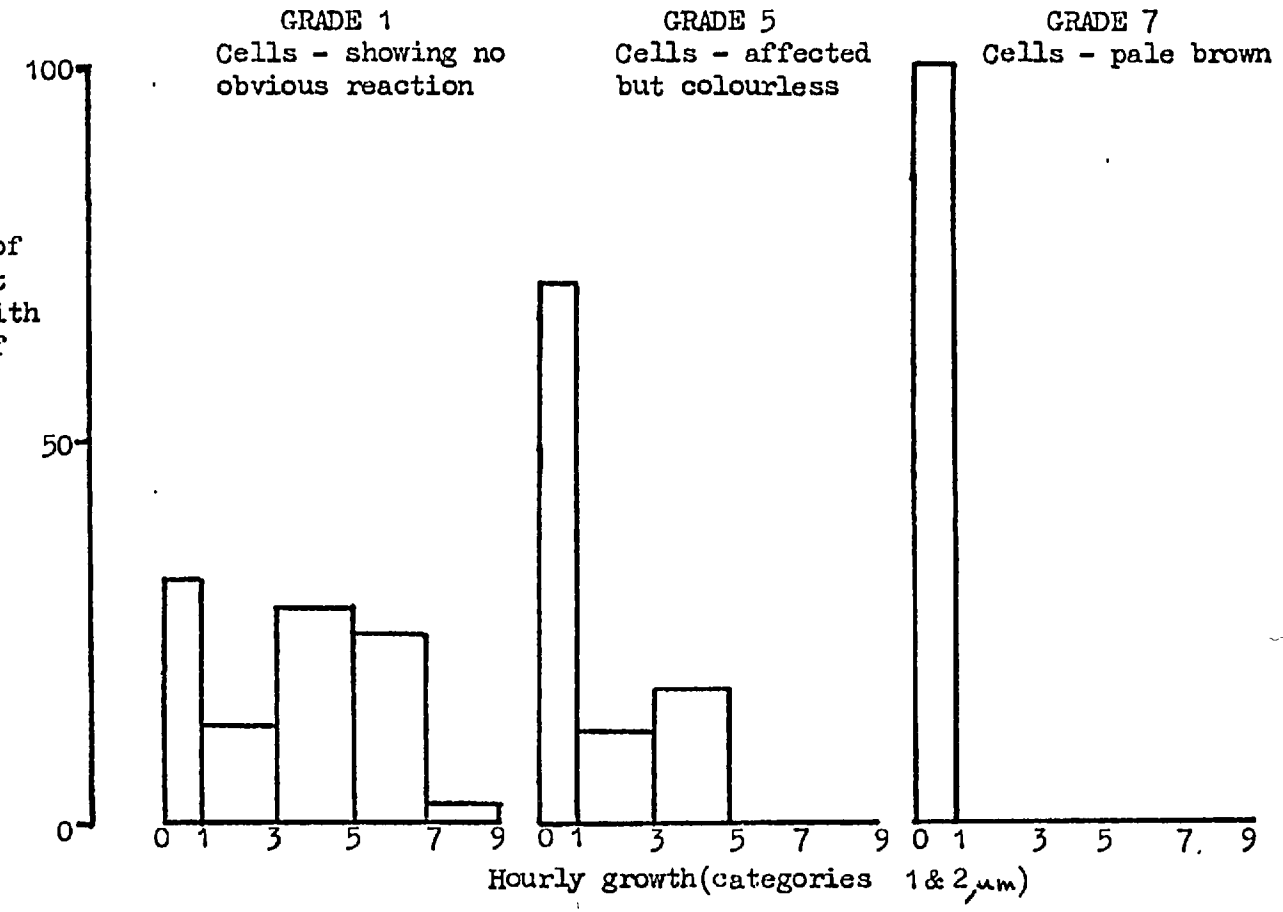
It is clear from the results shown in Figs. 6 and 7 that, as with the virulent race (delta) used in the preliminary experiment,

Fig. 6. Growth of hyphae of race beta (AV) in epidermal cells of c.v. Immuna (R - 10 day hypocotyls) exhibiting different grades of cell response



No. hyphae measured	10	19	16	6
Mean increase	1.69 μm	0.93 μm	0.29 μm	0.47 μm
Max. increase	5.60 μm	10.50 μm	2.80 μm	3.80 μm

Fig. 7. Growth of hyphae of race beta(AV) in epidermal cells of c.v. Immuna (R - 9 day hypocotyls) exhibiting different grades of cell response



No. hyphae measured	24	34	6
Mean increase /h	1.99 µm	0.75 µm	0.19 µm
Max. increase /h	8.00 µm	4.20 µm	0.97 µm

unaffected cells (grade 1) supported the growth of most hyphae of race beta. Growth of the majority of hyphae in affected cells, i.e. those of other grades, was less than 1 μm per h indicating that the growth of intracellular hyphae had been inhibited in comparison with those in unaffected cells. Some affected cells however did support considerable growth, notably colourless granular cells (grade 5, Fig. 6 and 7) and light brown cells (grade 8 Fig. 7).

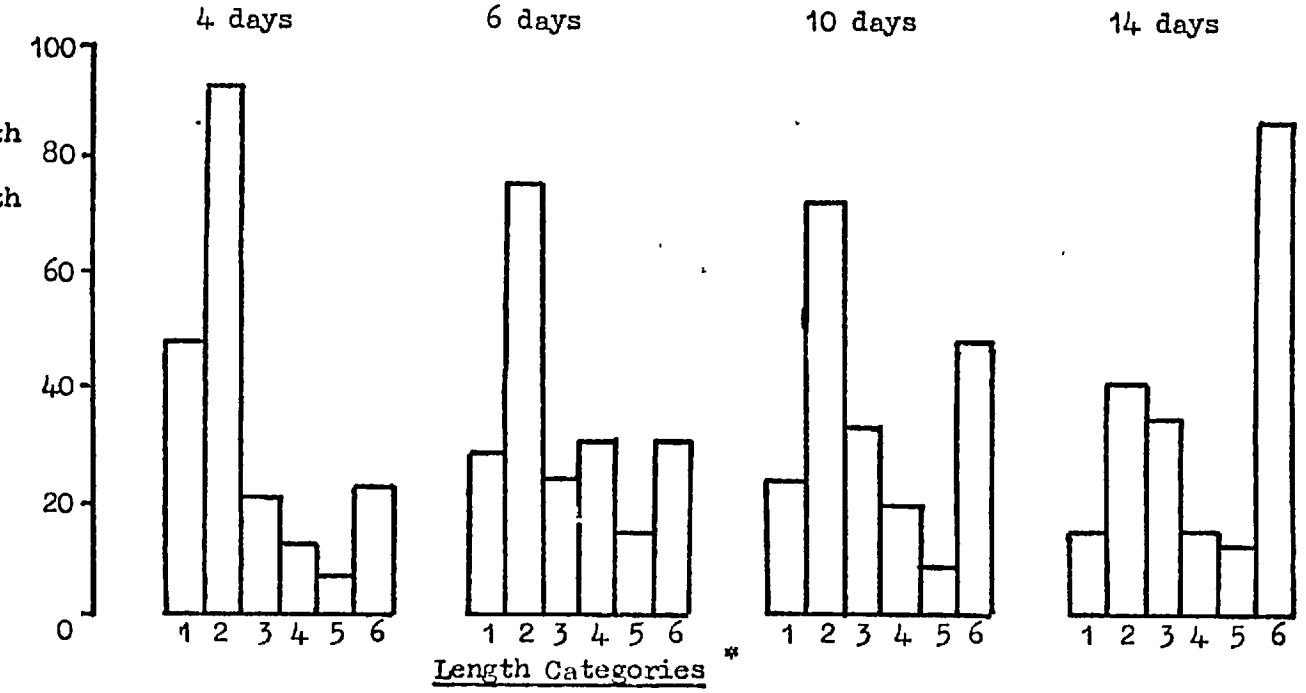
Applying these findings to the situation of intact Immuna hypocotyls, where cell appearance changed continuously, it seems likely that the growth of hyphae of race beta would have become increasingly inhibited from the time when the first visible signs of cell response appeared. This conclusion agreed with that made after the experiment on intact Immuna hypocotyls described above.

b) Hyphal growth in brown cells

The fact that hyphal growth occurred in brown cells (Fig. 7) suggested that if hyphae were killed, their death did not take place until after that of the infected cell. To determine whether hyphae continued to grow after the resistant response had been completed, the following experiment was carried out.

Young (8 day) Immuna hypocotyls were inoculated with spores of race beta and incubated at 17°C. Strips were taken from each of four replicate hypocotyls 4, 6, 10 and 14 days after inoculation and then mounted in 0.1 NaOH, all infected cells being brown and granular at these times. The results of measurements of hyphae found within the strips of cleared tissue are shown in Fig. 8. These results are

Fig. 8 Length of hyphae of race beta in epidermal cells of resistant hypocotyls (c.v. Immuna) at intervals after inoculation



	4 days	6 days	10 days	14 days
No. hyphae measured:	130	138	95	150
Mean length	11.5 µm	14.0 µm	17.1 µm	25.4 µm
Max. length	56 µm	101 µm	146 µm	158 µm

* 1 1.4 - 4.2 µm
2 5.6 - 8.4 µm
3 9.8 - 12.6 µm
4 14.0 - 16.8 µm
5 18.2 - 21.0 µm
6 22.4 + µm

expressed as the percentage of cells examined whose maximum length or diameter fell within a given category.

It can be seen that throughout the incubation period the number of cells containing hyphae of the "smallest length" grade decreased while those of the largest increased as did the mean and maximum values. Thus growth of some hyphae continued for at least 14 days after inoculation although all penetrated cells had become brown before the fifth day. The fact that no hypha was ever observed to have entered a second cell, indicated that this growth was extremely slow.

To determine the effect of caustic soda on hyphal length, spores of race beta were allowed to germinate for 24h in liquid culture medium (Table 1B) on a glass slide. Forty-five germ tubes were measured and then dilute caustic soda added to give a concentration in the drop of 0.1N. After leaving the slide for 2h, the same number of germ tubes was measured again. The mean germ tube lengths before and after treatment were 14.8 μm and 13.6 μm respectively. Thus no evidence of an increase or decrease in hyphal length following treatment with 0.1N NaOH was obtained.

Conclusion

The general conclusion for the combination Immuna (R)/beta (AV) is that hyphal growth began to slow markedly at the time when cells showed the first visible signs of response but that some hyphae remained alive and grew, although at a much reduced rate in brown apparently dead cells. Cell browning did not necessarily precede inhibition.

7. Relationship between cell response and phaseollin production

The following experiment, to relate the phaseollin content of tissue to the degree of host cell reaction was undertaken jointly with Dr. J.A.Bailey who carried out the tissue extractions, silica-gel thin layer chromatography, UV spectrophotometry and gas-liquid chromatography used in the quantitative determination of phaseollin in inoculated tissue. Host cell reactions were studied by the author using the cell grading system.

Hypocotyls of c.v. Kievit inoculated with races gamma (V) and delta (AV) were incubated at 17°C and samples removed at intervals for extraction. Epidermal strips were examined at the same time from four replicate hypocotyls. The results are summarized in Table 17.

In the susceptible reaction (race gamma), no phaseollin could be detected in tissue extracts 0, 48 and 96h after inoculation. At 96h (Table 17) penetrated cells were unaffected by hyphae apart from the production of reaction material. A small amount of phaseollin was detected by gas liquid chromatography later at the time when lesions became visible (0.145μg /site at 142h when 72% of sites bore lesions). A considerable amount was found still later (164h) when 0.830μg /site was detected. At this time lesions were a dark chocolate brown.

No phaseollin was detected in delta inoculated tissue (AV) at 0 or at 45h (Table 17) although a small proportion of cells were affected and brown at this latter time. About twice as many cells were obviously undergoing a reaction to infection at 66h, when phaseollin was first detected. Phaseollin content increased rapidly during the period 66-96h after inoculation and although no more cells had reacted there

Table 17 Cell response and phaseollin content of excised Kievit hypocotyl after inoculation with C. lindemuthianum races gamma and delta

Grade	Description	% cells examined[1] assigned to different grades after(h):- Race gamma(V)	Race delta (AV)			
		96	45	66	96	168
1.2, 1.3	Hyphae in unaffected cells	17.3	0.5	0.0	0.0	0.0
	Cells affected but not brown					
2.2, 2.4	Larger particles than uninoculated	0.4	2.8	3.3	0.2	0.0
3.2, 3.4	" " near appressorium		0.0	1.3	0.0	0.0
4.2, 4.4	Cessation of movement + coagulation near appressorium		5.3	1.8	0.2	0.0
	Total cells affected	0.4	8.1	6.4	0.4	0.0
	Cells affected - brown					
10.2,10.4(7) (8+9)	Part of cell v. pale brown	0.0	1.0	3.0	2.3	0.3
	" " light brown or brown		0.3	5.5	5.7	1.5
7.2, 7.4	Whole cell v. pale brown		1.3	2.5	0.7	0.0
8.2, 8.4	" " light brown		0.8	5.8	5.3	3.5
9.2, 9.4	Whole cell brown/dark brown		0.0	4.0	7.3	18.0
	Total cells affected and brown	0.0	3.4	20.8	21.3	23.3
2.1,8.1,10.1	Reaction material	6.5	0.5	1.8	6.6	8.3
	Total affected cells (%)[2]	0.0	11.5	27.2	21.7	23.3
	Phaseollin content (per inoculated site)					
	a) Determined by gas liquid chromatography	- *	- *	0.045	0.335	
	b) Determined by UV spectrophotometry	- *	- *	0.13	0.44	0.44

1 Four replicate hypocotyls, giving a total of 400 cells, examined at each time
2 Reaction material containing cells excluded
* No phaseollin detected by analytical method used

was some indication that browning had intensified (a greater proportion of affected cells being of the light-brown/brown grades and fewer being colourless at 96h) This intensification continued at least up to 168h after inoculation although no further increase in phaseollin content occurred after 96h.

Conclusion

This data indicates that in both susceptible and resistant reactions phaseollin formation followed cellular damage, major increases in phaseollin content occurring during a period when cell browning became intensified.

Summary of observations on resistant reactions

The following conclusions were reached after the studies on resistant reactions reported in sub-sections 2, 5, 6 and 7 above:-

1 As on susceptible varieties appressoria were produced during spore germination

2 Spore germination was not inhibited on resistant varieties

3 Penetration occurs after about 48h as in susceptible varieties

4 Hyphae were not always, sometimes rarely, detected in responding cells; indicating that cell response might occur before penetration

5 With Immuna(R)/beta (second batch of beans), where hyphae were commonly found in epidermal cells:-

a) penetration preceded cell response

b) hyphal growth was inhibited to some extent soon after cell death and before any browning had occurred

c) hyphal growth continued, although very slowly, in dead, brown host cells, indicating that hyphae were alive

6 Two main types of cell response were found:-

Reaction type 1 (Fig. 2 Page 97, e.g.Immuna/beta). Cell death occurred soon after penetration, was not associated with any visible increase in cellular activity and preceded cell browning which took place to the same extent throughout the cell.

Reaction type 2 (Fig. 5 Page 112e.g.Kievit/delta). Cell death was preceded by the appearance and rapid motion of large particles which accumulated, together with cytoplasmic material, near the appressorium. Browning spread outwards from the point of accumulation.

7 The type of reaction which took place was not related to the variety of bean but may have been related to the fungal race used

8 An antifungal compound, Phaseollin, was detected at the time of cell damage in a resistant (as well as a susceptible) reaction, major increases occurring at the time when cell browning became intensified.

8. Development of an intermediate response

The variety Pinto was studied to determine whether differences between the initial responses of cells of this variety to race beta and those observed in the susceptible and resistant reactions described above could explain the intermediate reaction reported in Table 3.

Course of infection and response

Hypocotyls of c.v. Pinto were inoculated with 5×10^5 spores/ml of race beta and strips examined at 2, 3 and 4 days. The results of these observations and of hypocotyls inoculated with race delta(V) at the same concentration and at the same time are given in Table 18

Penetration by race beta into 25% of epidermal cells had taken place by 2 days. During the following 2 days, infection resembled that of a susceptible variety (Kievit by race gamma - Table 11 and Pinto by race delta) in that hyphae grew from infected cells, many of which appeared to be unaffected by the presence of the fungus. However effects on cells were evident at 4 days with race beta, although not with race delta (V). Effects similar to those described above for the resistant response of Kievit to race delta were seen (i.e. cell grades 2, 3 and 4) and the particles involved were large (about 1μm). In cells of grade 5, shrinkage of the nucleus and fine granulation (typical of cells of this grade in the Immuna(R)/beta(AV) combination) were not often observed, instead the large particles were motionless and spread evenly throughout the

Table 18. Hyphal development and reaction of epidermal cells of bean hypocotyls (c.v. Pinto) at various times after inoculation with races beta and delta

Grades	Description	% cells examined assigned to different grades after (days):- Race beta[1] 2	Race beta[1] 3	Race beta[1] 4	Race delta[2] 4
	Cells unaffected				
1.2	Hypha in penetrated cell	23.3	25.5	12.0	14.1
1.3	Hypha in second cell	0.0	1.7	6.3	21.4
	Cells affected - no browning				
2.2, 3.2, 4.2	Streaming of particles - hypha in penetrated cell	1.3	1.0	2.3	0.0
5.2	Cytoplasm granular throughout cell - hypha in penetrated cell	0.0	0.7	1.3	0.0
4.3, 5.3	Hypha in second cell	0.0	0.0	1.5	0.0
2.4, 3.4, 4.4, 5.4	Hypha not visible	0.7	0.0	1.7	0.0
	Cells affected - brown				
7.2, 8.2, 9.2	Hypha in penetrated cell	0.0	0.0	3.7	1.2
8.3, 9.3	Hypha in second cell	0.0	0.0	1.7	0.0
7.4, 8.4, 9.4	Hypha not visible	0.0	0.7	1.7	1.2
	Reaction material produced in cells				
1.1, 2.1, 5.1, 10.1		3.3	5.2	14.7	9.2
	Other grades				
10.2, 10.4, 11.2, 11.4		0.0	0.3	0.7	0.0
	Total penetrated cells[3]	24.6	28.9	28.7	36.7
	Total reacting and/or penetrated cells[3]	25.3	29.9	32.8	37.9

1 Three replicate hypocotyls, giving a total of 300 cells, examined at each time

2 Total of 163 counted as fewer than 50 appressoria/strip

3 Reaction material containing cells excluded

cell. Cell browning was also observed after 4 days.

Seven days after inoculation, lesions had formed at 25 and 78% respectively of sites inoculated with races beta and delta. Chocolate brown flecking was seen at the beta-inoculated sites where no lesions had developed. To determine the extent to which hyphae of race beta had grown in this tissue, epidermal strips were removed from beneath five randomly chosen drops and cleared in 0.1N NaOH. Twelve penetrated hyphae were examined on each. In the four strips taken from non-lesion tissue, colonization of five cells or less had taken place in most cases (96%). Greater growth was observed in a strip taken from a superficial lesion, some hyphae (6 out of 12) having invaded more than 10 cells but in this tissue as with that showing flecking many hyphae had colonized only a few cells.

It is evident that the processes of infection and cell response described in Table 18 are intermediate between those of the susceptible and resistant reactions described earlier in two respects, namely; the time of cell response relative to penetration and the extent to which hyphae colonized the tissue.

Conclusion

If, as seems likely, inhibition of hyphal growth results from some process which occurs at about the time of changes in cell appearance, then the development of obvious but limited symptoms on c.v. Pinto with race beta could be explained by the fact that cell response occurs after some intracellular hyphal growth has taken place but before the time when effects normally appear in cells of a susceptible variety.

9. Failure of isolates of races gamma and delta to elicit marked resistant or susceptible responses in c.v. Immuna hypocotyls

In some preliminary experients, the original isolates of gamma and delta of Smith (1967) were used as inoculum for c.v. Immuna. When young plants (8 to 9 days old) were inoculated with race delta (V) only a few limited lesions developed after prolonged incubation. Race gamma (AV) caused only a small number of brown cells to appear at inoculated sites. These combinations were examined further in an attempt to ascertain the cause of this "poor response".

The results of an experiment where 2 strips from each of 4 replicate hypocotyls were examined from each race/variety combination are shown in Tables 19 and 20.

Race delta (V - Table 19), penetrated very few cells and almost no growth into a second cell was observed. Some cells became brown although at very late stages (6 and 9 days) when scattered minute areas of necrosis appeared at the inoculation sites. Necrosis was not sufficiently extensive to give rise to "lesions" but was confined to groups of 2 - 70 epidermal cells and some cortical cells. Few cells of tissue inoculated with race gamma (AV - Table 20) contained hyphae at any stage and only 2-3% became granular and brown. This cell response occurred early (3-4 days) in cells without reaction material. Visible flecking was found at very few inoculation sites even after prolonged incubation.

With both races, reaction material was commonly observed in cells and was particularly abundant after 6 and 9 days. Browning of the cytoplasm (cell grade 10) or cell wall (cell grade 11)

Table 19. Hyphal development and reaction of epidermal cells of susceptible bean hypocotyls (c.v.Immuna) inoculated with race delta (V)

Grade	Description	% of cells examined[1] assigned to grades after (days):- 2	3	4	6	9
	Cells unaffected					
1.2	Hypha in penetrated cell	0.0	0.0	0.3	0.0	0.0
1.3	Hypha in second cell	0.0	0.0	0.0	0.3	0.3
	Cells affected					
7.4, 8.4, 9.4	Cell brown hypha not visible	0.0	0.0	1.0	2.3	7.0
6.3, 8.3, 9.3	" " hypha in second cell	0.0	0.0	0.0	0.8	0.5
	Reaction Material produced in cells					
2.1	Obvious particles streaming throughout cell	0.0	5.3	4.5	16.3	18.3
10.1	Cytoplasm brown in region of appressorium	0.0	0.3	0.3	5.3	3.0
11.1	Cell wall brown near appressorium	0.0	0.0	0.0	3.8	3.5
	Total cells with reaction material	0.0	5.6	4.8	25.4	24.8
	Total cells showing any response	0.0	5.6	6.1	28.8	32.6

1 Four replicate hypocotyls, giving a total of 400 cells, examined at each time

Table 20. Hyphal development and reaction of epidermal cells of resistant bean hypocotyls (c.v.Immuna) inoculated with race gamma (AV)

Grade	Description	% of cells examined* assigned to grades after (days):- 2	3	4	6	9
	Cells unaffected					
1.2	Hypha in 1 cell	0.0	0.3	0.0	0.0	0.3
1.3	Hypha in second cell	0.0	0.0	0.0	0.0	0.0
	Cells affected					
7.4, 8.4, 9.4	Cell brown, no hypha visible	0.0	2.5	2.8	2.3	0.5
	Reaction Material					
2.1	Obvious particles streaming throughout cell	0.0	3.5	1.3	4.5	9.5
10.1	Cytoplasm brown near appressorium	0.0	0.8	0.8	3.8	2.5
11.1	Cell wall brown near appressorium	0.0	0.0	0.5	1.5	1.0
	Total cells with reaction material	0.0	4.3	2.6	9.8	13.0
	Total cells showing any response	0.0	7.1	5.4	12.1	13.8

* Four replicate hypocotyls, giving a total of 400 cells, examined at each time

accompanied the formation of reaction material in a number of cases in both race/variety combinations.

It is clear from Table 21 that a major factor in the resistance of c.v. Immuna to race gamma (AV) and "apparent resistance" to race delta (V) was the failure of these races to penetrate the cuticle and epidermal cell wall. Reaction material may have been important in preventing the development of hyphae which had been able to reach the cell lumen.

Table 21. Fate of spores in inoculation drops of races delta and gamma at different stages of infection of Immuna hypocotyls

	Race delta(V)	Race gamma(AV)
Mean No. spores/drop[1]	2550	2200
Mean No. germinated spores[2]	1096 (57%)*	1166 (47%)
Mean No. spores to any structure[3] in cell	56 (95%)	82 (93%)
Mean No. to primary hyphae (race delta)[3] or cell response (race gamma)	3 (95%)	32 (61%)
Mean No. infections associated with reaction material	53	50

* % decrease from previous total

NOTES

1 Estimated from number of spores in 3 drops

2 From Table 3 days same inoculation

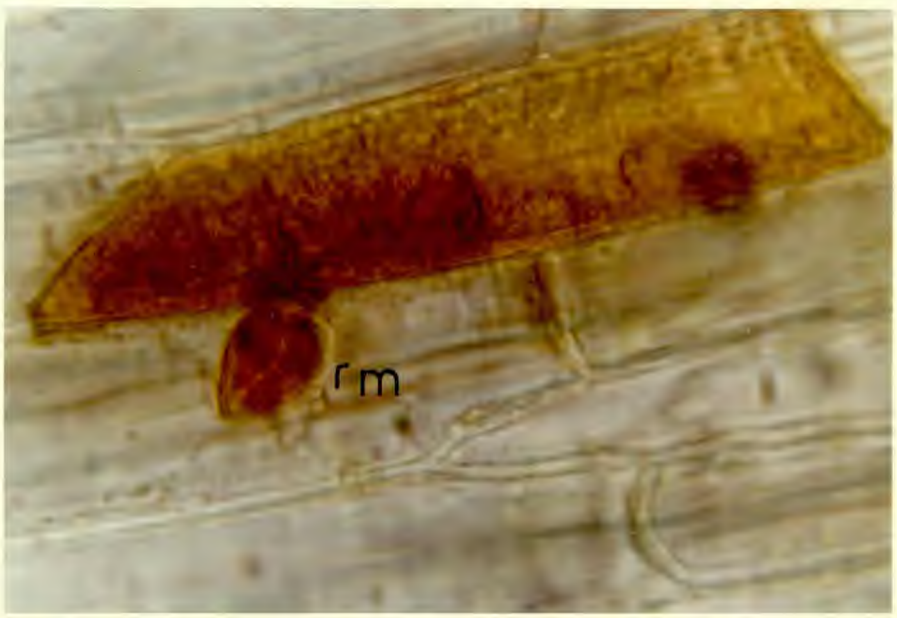
3 From Tables 4 days

10. Reaction material - its appearance, properties and associated phenomena

A dense deposit, which was colourless, creamy yellow, yellow brown or dark brown (Plate 28), was found in some cells of nearly all varieties used and after inoculation with all three races. Its production therefore, was not specifically confined to a resistant or susceptible response. This deposit was given the non-committal term "reaction material". It appeared at the same time as, or later than the onset of penetration or the granulation/browning type of cell reaction and, as suggested in sub-section 9, might have a role in preventing initial hyphal development. The results of observations on hypocotyl epidermal cells and their associated reaction material in a number of varieties is described below.

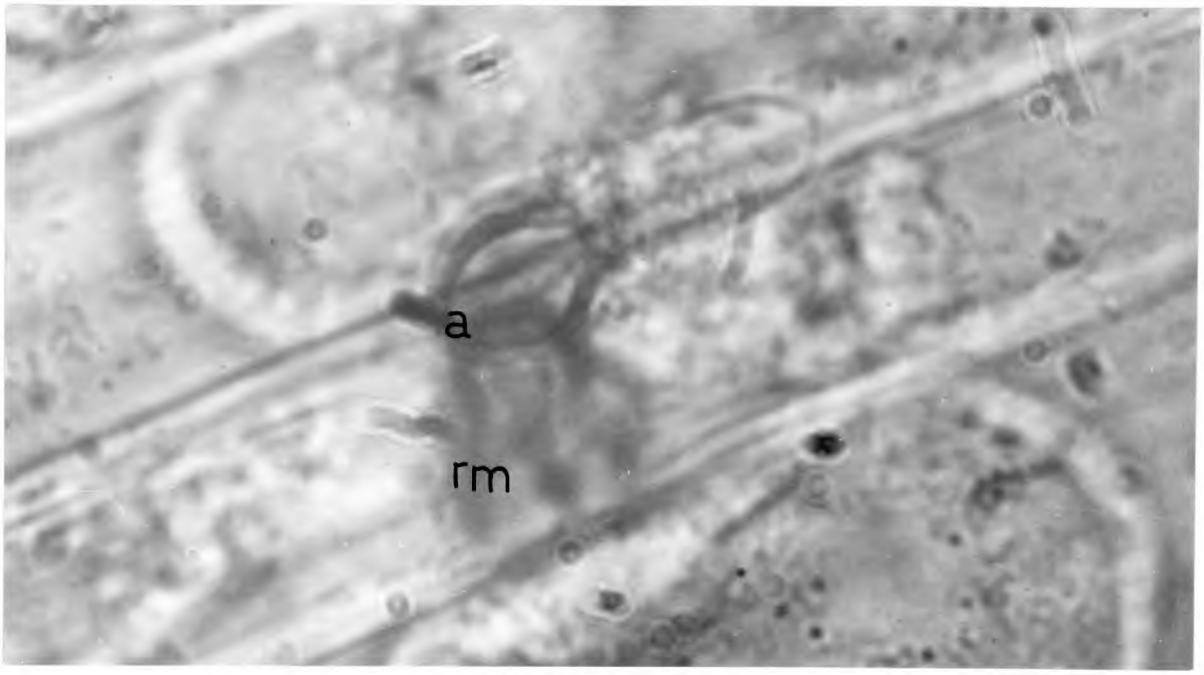
Reaction material was found beneath the appressorium, between the cuticularized wall and the plasmalemma of epidermal cells. On three occasions no appressorium was present. Plasmolysis of the cell protoplast drew away the plasmalemma leaving a spherical or elongate body inside the cell wall (Plates 29 and 30). Sometimes the brown deposit was restricted to an area beneath only part of the appressorium. A clearly recognisable hypha was rarely detected within or beneath reaction material, however a less dense area was commonly seen at the centre of the body of dark material (Plates 28 and 30). This was often thin and elongate. Thickening and browning around the walls of some short hyphae was sometimes observed and brown material was seen on one occasion to form a narrow ring around the base of a primary hypha, the hypha was constricted at this point. A thickening similar to this but which extended along part of the

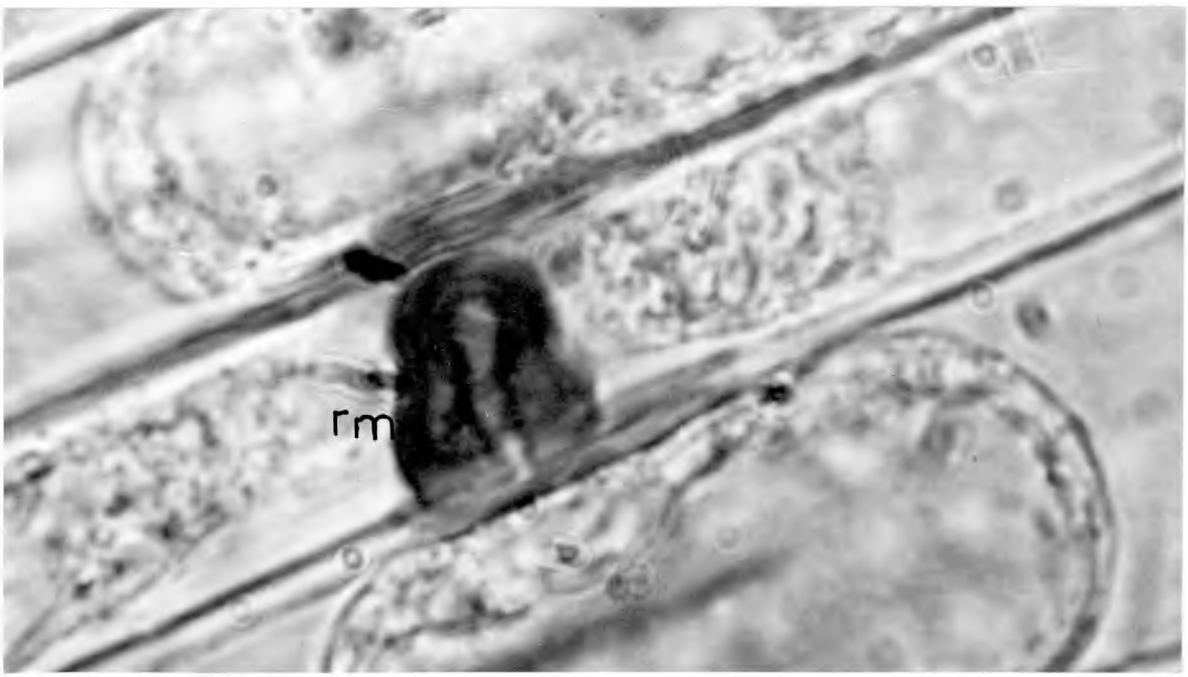
Plate 28 Reaction Material



28) Hypocotyl epidermal cell containing brown reaction material (rm). Note less dense area at centre of deposit. Adjacent cell shows granulation and browning typical of a resistant response. (Kievit(R)/delta 5 days ca. x 1000)

Plates 29 & 30 Reaction Material





29) Hypocotyl epidermal cell containing reaction material (rm) beneath an appressorium (a) (Immuna(R)/gamma 4 days stained in Rhodamine B x 3100)

30) As Plate 29 but at lower plane of focus. Note transparent area at centre of reaction material (rm) and plasmolysis of cell protoplast indicating that the material was formed outside the plasmalemma.

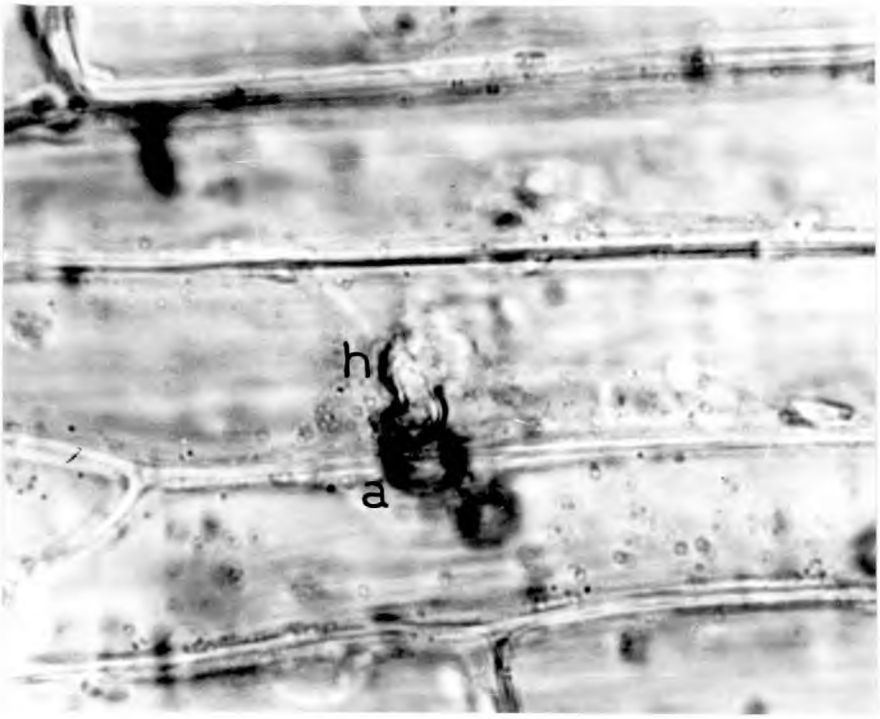
hypha was found once in c.v. Pinto (I) inoculated with race beta (Plates 31 and 32).

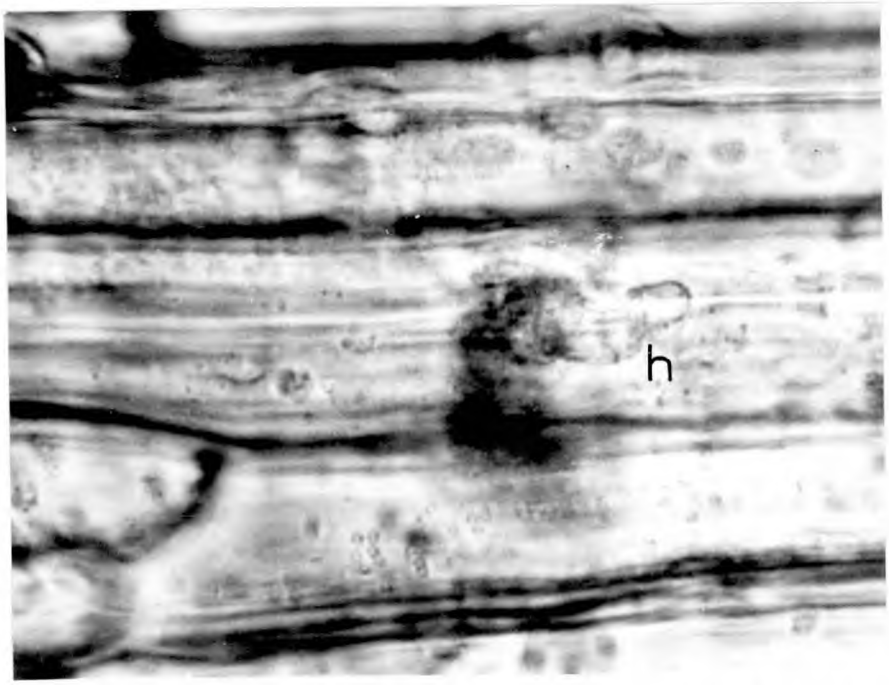
Few cells containing reaction material were unaffected. Larger particles, randomly distributed throughout the cell or accumulated near the appressorium, were found in the majority of cells with reaction material present (Plate 13). These cells plasmolysed in 0.5M sucrose and deplasmolysed in water.

The accumulation of cytoplasmic material near the appressorium and the subsequent appearance of browning, in the response of c.v. Kievit (R) to race delta, was described earlier. In this region of accumulation, the plasmalemma was sometimes seen to be invaginated by an unthickened, pear-shaped infection hypha but more commonly, colourless material was found around the pear-shaped body and between the cell wall and plasmalemma (Plate 33 and 34 - brown cell). The production of reaction material was associated with cytoplasmic accumulation in the response of c.v. Immuna to races gamma (AV) and beta (AV). With race gamma (AV) and the virulent race delta, browning and coagulation of cytoplasm was seen (Plate 35). Changes typical of a "normal" resistant reaction often took place in cells containing reaction material, however with Immuna(R)/gamma and Dubbele Witte/delta, its production was the most commonly observed response.

Browning of the cell wall (cell grade 11) was often associated with the presence of reaction material. In c.v. Immuna (inoculated with races gamma-AV, and delta-V) and Kievit (inoculated with delta-AV) this occasionally took the form of a brown halo (Plate 36). Areas of wall

Plates 31 & 32 Reaction Material

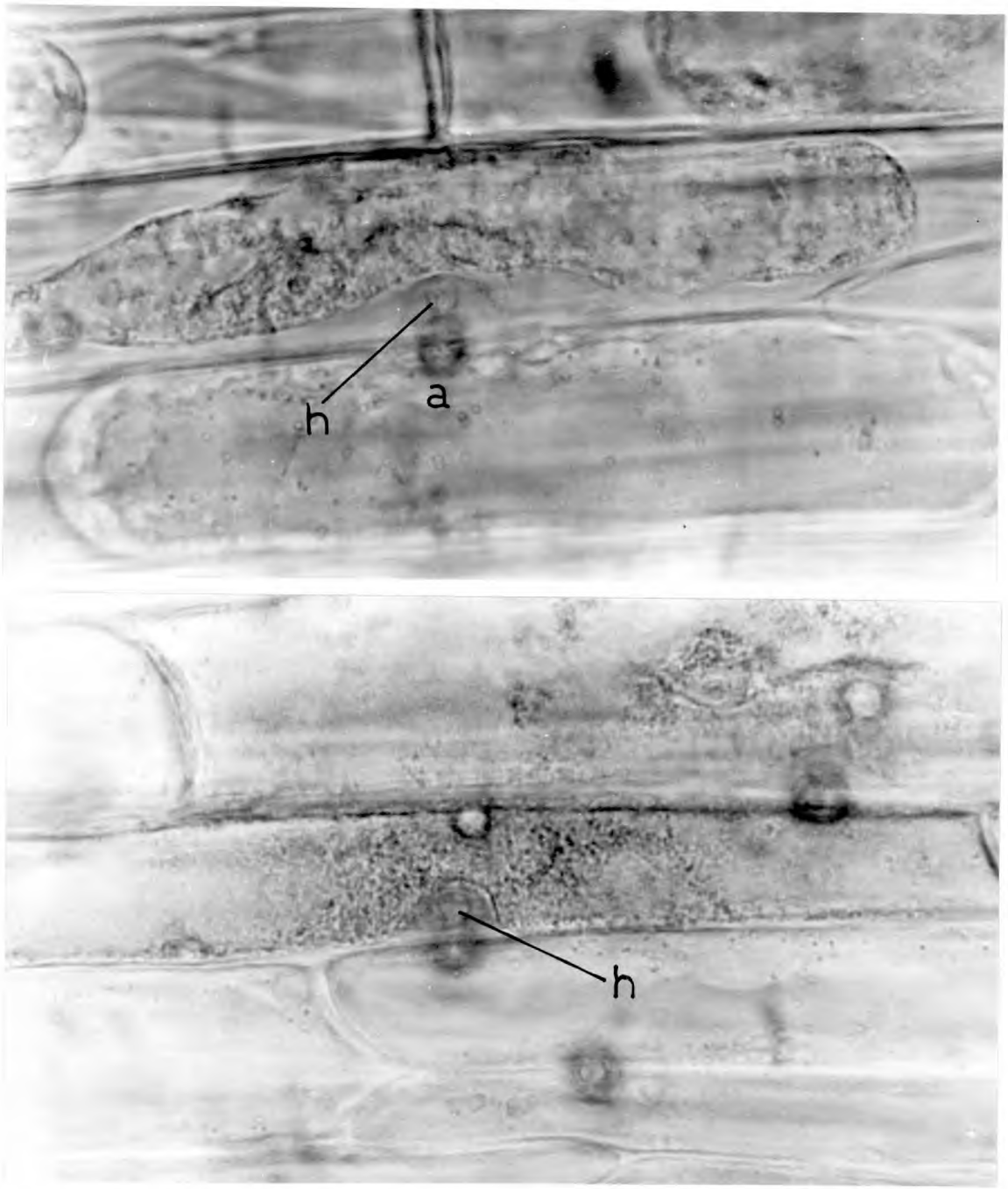




31) A hypocotyl epidermal cell containing a hypha (h) which appeared to be thickened at the base. Appressorium (a) in outline. (Pinto(I)/beta 4 days x 1600)

32) As Plate 31 but at a lower plane of focus showing hypha (h) thin walled at apex.

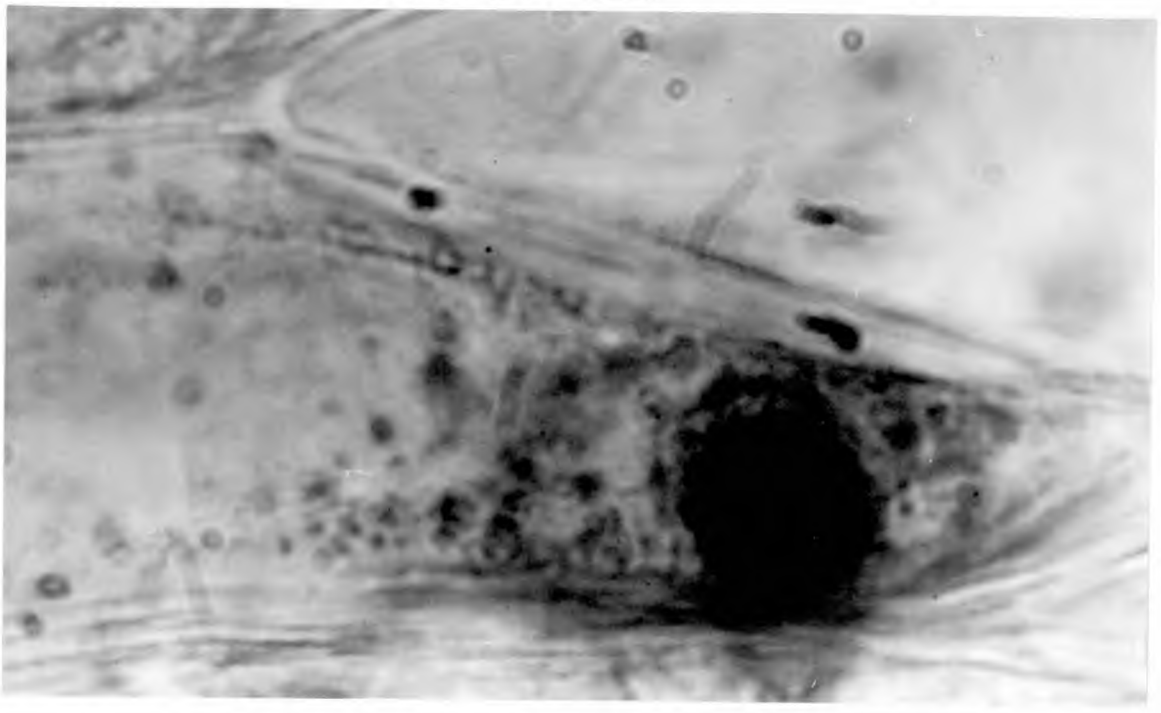
Plates 33 & 34 Reaction Material

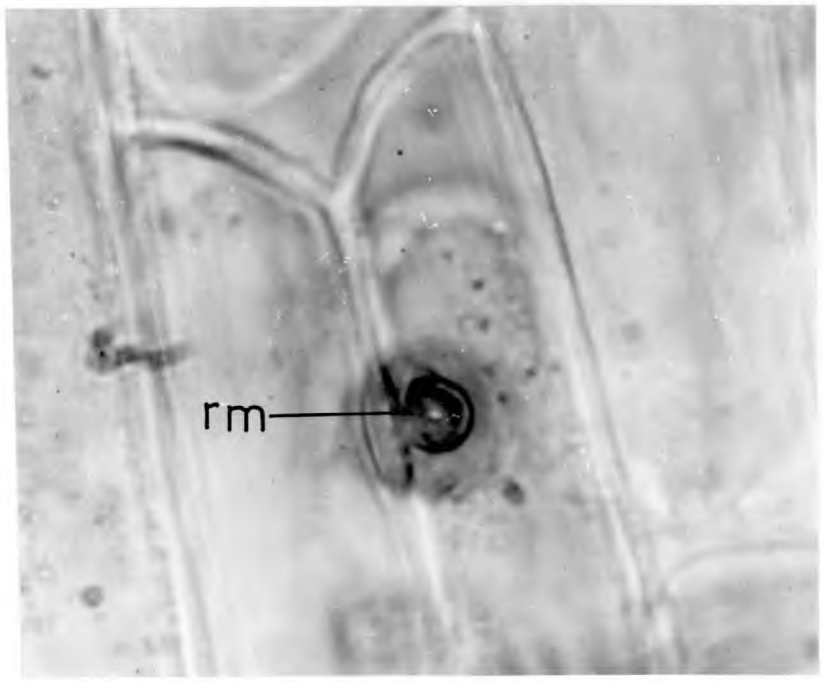


33) Protoplast of a hypocotyl epidermal cell plasmolysed to reveal a pear shaped hypha (h) surrounded by a deposit (possibly reaction material) beneath an appressorium (a) in outline. Note accumulation of cytoplasm in protoplast. (Kievit(R)/delta 84h x 1600)

34) Brown - granular hypocotyl epidermal cell showing hypha and deposit similar to that in Plate 34. (Kievit(R)/delta 84h x 1600)

Plates 35 & 36 Reaction Material





35) Affinity for Rhodamine B (darkening) and coagulation of cytoplasm around reaction material in a hypocotyl epidermal cell (Immuna(R)/ gamma 5 days x 3100)

36) Dark halo in cell wall above reaction material (rm) in a hypocotyl epidermal cell. An appressorium was visible above the material at a higher plane of focus. (Immuna(R)/gamma 4 days x 2500)

adjacent to reaction material (Immuna/gamma and Kievit/delta) often appeared intensely pink after strips had been immersed in 10ppm Rhodamine B (30 min). Such a reaction was shown by the cell in Plate 30 in a region of wall 10-20μm on either side of the broadest part of the deposit. Reaction material itself stained red with Rhodamine B. Unlike cytoplasmic browning, the brown colour of unstained reaction material did not disperse after treatment with 0.1N NaOH.

Reaction material was never seen to be produced during penetration of cell walls by primary hyphae but only during attempted penetration from appressoria.

Conclusion

Dense material which later became brown was often produced beneath appressoria between the cell wall and protoplast of living epidermal cells. Hyphae were sometimes clearly seen to be encapsulated by reaction material whose production might represent a host resistance response resulting in the limitation of an infection hypha to the penetrated cell.

11. Infection of stripped hypocotyl tissue

Experiments to detect whether certain treatments reduced the amount of necrosis caused by a virulent race of C. lindemuthianum are described in the acquired resistance studies of Section III. A reduction in symptoms produced by race delta on c.v. Immuna was difficult to detect because often only a few limited lesions developed in untreated tissue. However, following the earlier discovery that race delta was rarely able to penetrate epidermal cells of this variety, it was thought that hypocotyls from which the epidermis had been removed would probably provide a better experimental model for such studies. The following experiment was carried out to determine; a) whether stripped hypocotyls remained resistant to an avirulent race while lesion production by a virulent race was increased and b) whether the course of infection and response in stripped and unstripped hypocotyls differed.

Washed, stripped and unstripped hypocotyls were allowed to dry and then inoculated with spores of the two races, or were used as uninoculated controls (sterile distilled water). Twenty hypocotyls were used for each race/treatment combination and five for either control. These were incubated and examined for the appearance of symptoms while others, prepared in the same way, were used for microscopic examination. The results are summarized in Tables 23 & 24.

Race beta(AV) (Table 23) failed to produce lesions on either stripped or unstripped hypocotyls while race delta produced many more on stripped than on the latter. Typical symptoms produced by the two races on stripped tissue after 14 days are shown in Plate 37

Table 23. The development of lesions on stripped and unstripped Immuna hypocotyls inoculated with races beta and delta

Race	Treatment	% of inoculated sites* with lesions after (days):- $1\frac{1}{2}$	$3\frac{1}{2}$	$5\frac{1}{2}$	14
beta (AV)	Stripped	0	0	0	0
	Unstripped	0	0	0	0
delta (V)	Stripped	0	96	100	100
	Unstripped	0	0	3	4

* 100 drops, 5 on each of 20 hypocotyls were applied for each treatment and race

Table 24. The products of spore germination of races beta and delta on stripped and unstripped hypocotyls 36h after inoculation

	% of germinated spores[1] examined[2] forming different structures			
	Stripped hypocotyls		Unstripped hypocotyls	
Spores germinated to form :-	Race beta(AV)	Race delta(V)	Race beta(AV)	Race delta(V)
Appressorium	3.0	2.0	53.5	71.0
Superficial germ tube	33.5	20.0	46.5	29.0
Germ tube less than spore width	39.5	1.0	0.0	0.0
Thin walled hypha in cell	0.0	48.0	0.0	0.0
Reaction material produced	24.0	29.0	0.0	0.0
% spore germination on tissue[3]	27.8	86.5	58.1	85.2

Notes

1 Defined as spores with any outgrowth

2 Two replicate hypocotyls, giving a total of 200 germinated spores, examined for each race/treatment

3 Two replicate hypocotyls, giving a total of 200 spores, examined for germination /defined as in 1) for each race/treatment

It is clear from Table24 that on stripped hypocotyls appressoria were neither the main product of germination nor were they essential for penetration, which was effected directly by means of the germ tube. As mentioned earlier appressoria were always involved in cuticular penetration. At 36h, penetration was only detected in stripped hypocotyls inoculated with race delta (V) although the presence of reaction material in beta-inoculated (AV) stripped tissue suggested that some attempt at penetration may have been made by hyphae of this race. Reaction material was also present in delta-inoculated (V) tissue. Appressorial penetration did not normally take place until after 36h so no penetration was expected in unstripped hypocotyls at this time. Inhibition of race beta (AV) on the surface of stripped tissue was indicated by poor germination (compared with race delta and race beta on unstripped tissue) and the presence of spores with "stunted" germ tubes.

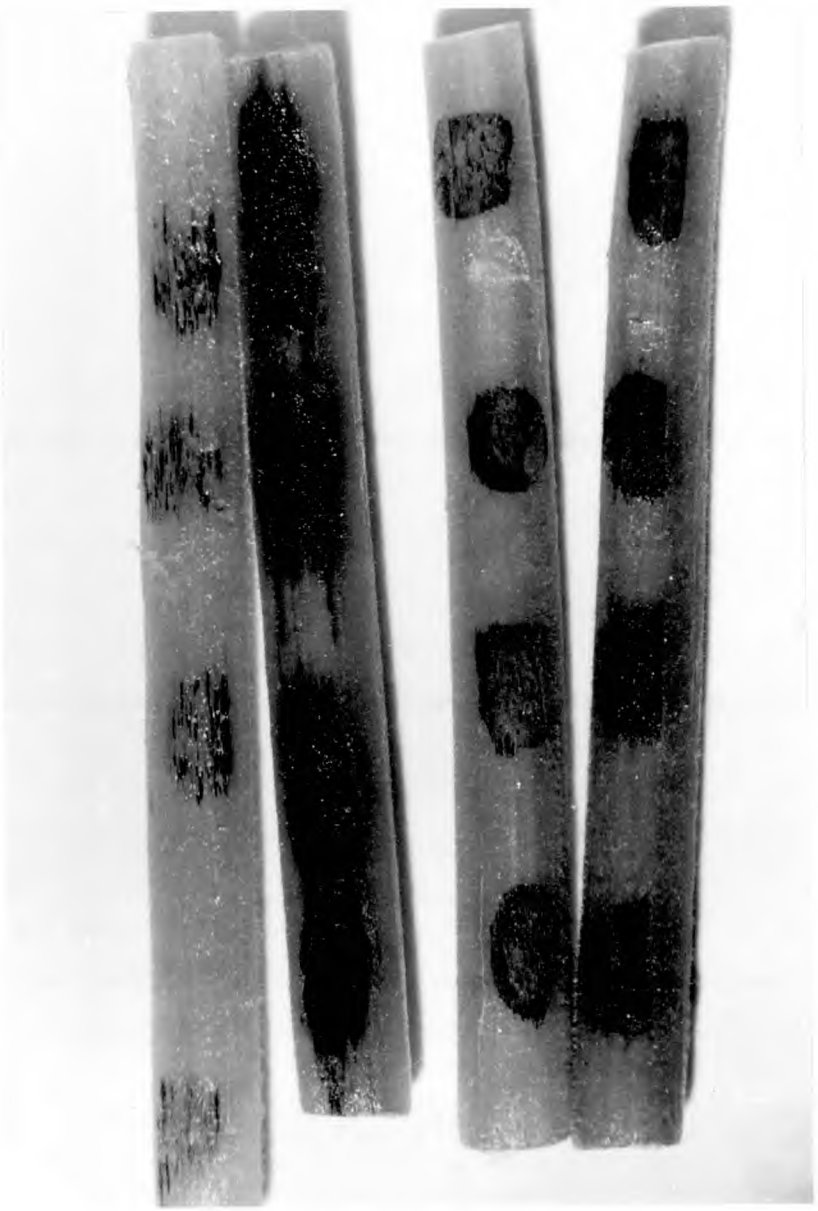
Further hyphal development in the susceptible reaction was investigated by examining strips cut at $3\frac{1}{2}$ and $5\frac{1}{2}$ days. Race delta (V) had penetrated 10 and 16% of epidermal cells of unstripped tissue at these respective times, only 1-2 cells being colonized for each infection at $5\frac{1}{2}$ days. Nearly all cells of stripped tissue beneath the inoculation drop contained hyphae at $3\frac{1}{2}$ days. Thus it appears that infection takes place earlier and to a greater extent in stripped tissue. This is reflected in the more rapid and more numerous development of lesions in this than in intact hypocotyls.

As with penetration, the response of infected cells of unstripped tissue to the avirulent race (beta), was not detected until the

examination at $3\frac{1}{2}$ days when granulation and browning was often observed. Very few brown cells were found in stripped tissue at any time, reaction material however being observed frequently (associated with 24% of spores at 36h) in stripped tissue and infrequently (associated with 2.5 and 5% of spores after $3\frac{1}{2}$ and $5\frac{1}{2}$ days) in unstripped. Although little cell browning occurred, inoculation drops containing both beta and delta spores became brown and after drying (12-36h) a brown film was present on the cut surface at the inoculation site (Plate 37). No browning was observed in drops of water or in any drops on unstripped tissue.

The conclusion reached was that when spores of a virulent race are applied directly to cortical tissue of the hypocotyl, direct penetration by germ tubes is followed by rapid intracellular colonization and leads to the development of necrotic lesions. Lesions may be formed later and less extensively in intact tissue because of the delay due to the development of appressoria and resistance to cuticular penetration. Resistance of stripped hypocotyls to an avirulent race may have been caused by an inhibitor produced by host cells which diffused into the drop and inhibited spore germination. That substances had diffused into the inoculation drops (of both avirulent and virulent races) was suggested by the fact that drops become brown during incubation. Little necrosis of host cells was detected, indicating that such diffusible inhibitors were produced by affected living, rather than dead brown, cells.

Plate 37 Infection of stripped hypocotyls



37) Stripped hypocotyls of c.v. Immuna 14 days after inoculation with races delta(V) and beta(AV). Note necrosis at site of delta inoculation (2 on left) and a brown film where beta was applied (2 on right)

General conclusion

Stripped hypocotyls (c.v. Immuna) were more susceptible to a virulent race than unstripped hypocotyls but remained resistant to an avirulent race. The processes of infection, and resistance to an avirulent race, of stripped hypocotyls appeared to differ from those of hypocotyls with an intact epidermis and cuticle.

12. Pod endocarp tissue

a) Infection of different varieties by three races

The responses of different bean varieties to the different fungal races was clearly and consistently distinguished on excised hypocotyl tissue. Pod endocarp, which has been used by a number of workers for biochemical studies, was tested to see if the same clear differentiations could be obtained.

Bean plants of the varieties: Dubbele Witte, Immuna, Kievit and Red Kidney were grown in Vermiculite, watered at intervals with Bio nutrient solution, for 88 days. Pods were collected, pieces containing 2 seeds were cut from each pod and these pieces split to reveal the 4 endocarp cavities. Only pieces with seeds less than 1 cm long in cavities, which were green and not lined with a white "felt", were used. The seeds were removed and 20 µl drops of water or beta, gamma and delta spore suspensions placed on the exposed surface. Thin surface sections of the areas beneath 2 drops were examined microscopically for each race/variety combination after 36h, 100 cells and their associated spores being examined for each. The macroscopic appearance of cavities was scored after 7 and 14 days, The results are summarised in Tables 25 and 26.

It is clear from Table 25 that, if the development of necrosis in endocarp tissue indicates susceptibility, then the differential reaction of bean varieties to races of C. lindemuthianum is not the same in pod tissue as in hypocotyls. The effect is of susceptibility

Table 25. Appearance of the endocarp cavities of 4 bean varieties 7 and 14 days after inoculation with three races of C. lindemuthianum

Race	Variety	Expected Response	No. of inoculated sites showing necrosis and browning		Total possible
			7 days	14 days	
Beta	Dubbele Witte	R	0	0	12
	Immuna	R	0	4	6
	Kievit	R	0	1	12
	Red Kidney	S	3	5	9
Gamma	Dubbele Witte	R	4	6	12
	Immuna	R	0	0	6
	Kievit	S	4	10	12
	Red Kidney	S	4	7	9
Delta	Dubbele Witte	S	7	10	12
	Immuna	S	1	5	6
	Kievit	R	0	0	12
	Red Kidney	R	1	7	9

Table 26. Germination and penetration of three races of C. lindemuthianum 36h after inoculating pod cavities of different bean varieties

Race	Variety	Expected response (from hypocotyl tests)	Results of observations[1] on :- Germination % groups[2]	Germination fate of germ tubes[3]	Penetration hyphae or reaction mat. present[4]	Cell browning[5]	Brown film (on endocarp surface)[6]
beta	Dubbele Witte	R	+	S	-	-	+++
	Immuna	R	+	PS	RM	-	+
	Kievit	R	++	P	RM	-	+
	Red Kidney	S	++	P	RM	-	++
gamma	Dubbele Witte	R	+	S	-	-	+++
	Immuna	R	+	S	-	+NP	+
	Kievit	S	++	P	RM	-	+
	Red Kidney	S	++	PS	RM	+NP	+
delta	Dubbele Witte	S	-			-	+++
	Immuna	S	++	PS	H	+NP	+
	Kievit	R	+++	P	NV	+P/NP	+++
	Red Kidney	R	++	P	RM	+NP	++

NOTES

1 For each combination; 100 cells on each of 2 strips examined for penetration and cell appearance, germination estimated during examination
2 Estimated to be: - absent, + less than 10%, ++ 10-50%, +++ over 50%
3 S superficial growth, P penetrate underlying cell directly
4 - absent, RM reaction material seen at point of germ tube penetration, H thin walled hypha, NV not visible because of cell browning
5 - absent, + some cells granular and brown, P brown cells penetrated, NP browning in absence of spore above
6 Arbitrary scale + pale brown, ++ light brown, +++ dark brown

in varieties previously classified as resistant. One explanation was that although a resistant response may have been initiated in all resistant varieties, in some it was not sufficiently rapid or intense to prevent the development of the avirulent race completely.

The results of observations on the infection of pod endocarp tissue (Table 26) showed some similarity to those obtained with stripped hypocotyls (avirulent race beta - reported earlier) in that:-

1 Germ tubes and not appressoria were produced during germination

2 Short germ tubes entered underlying cells directly

3 Reaction material was produced beneath most of such penetrating germ tubes

4 Little granulation and browning of cells took place

5 A brown film was present at the inoculated surface.

The brown film, originally found as a "skin" over the inoculation drop, came to lie on the endocarp surface when drops dried or were lost during sectioning. Although race delta (V) failed to germinate on Dubbele Witte, a very dark film developed suggesting that production of this brown material might have been stimulated by some spore product. No browning occurred in water drops on this or other varieties. Cell browning was not observed in Dubbele Witte but with other varieties, brown cells were found. These cells were often unpenetrated which also suggested the possible effect of substances released from spores.

There was little indication that infection events were similar to those taking place in the development of resistant and susceptible reactions on hypocotyls, or how these events might lead to the later development of the necrosis shown in Table 25. However in two cases

the hypocotyl type of infection and response was indicated. These combinations were Immuna(S)/delta, where thin walled hyphae (Plate38) were found in some cells and Kievit(R)/delta, where granulation and browning in addition to reaction material was found in many penetrated cells.

It was concluded that:-

1 The differential resistant and susceptible reactions of bean varieties to three races of C. lindemuthianum, previously shown in hypocotyl tests could not be demonstrated in pod cavities

2 Germ tubes, rather than appressoria, were the major product of spore germination and were involved in penetration

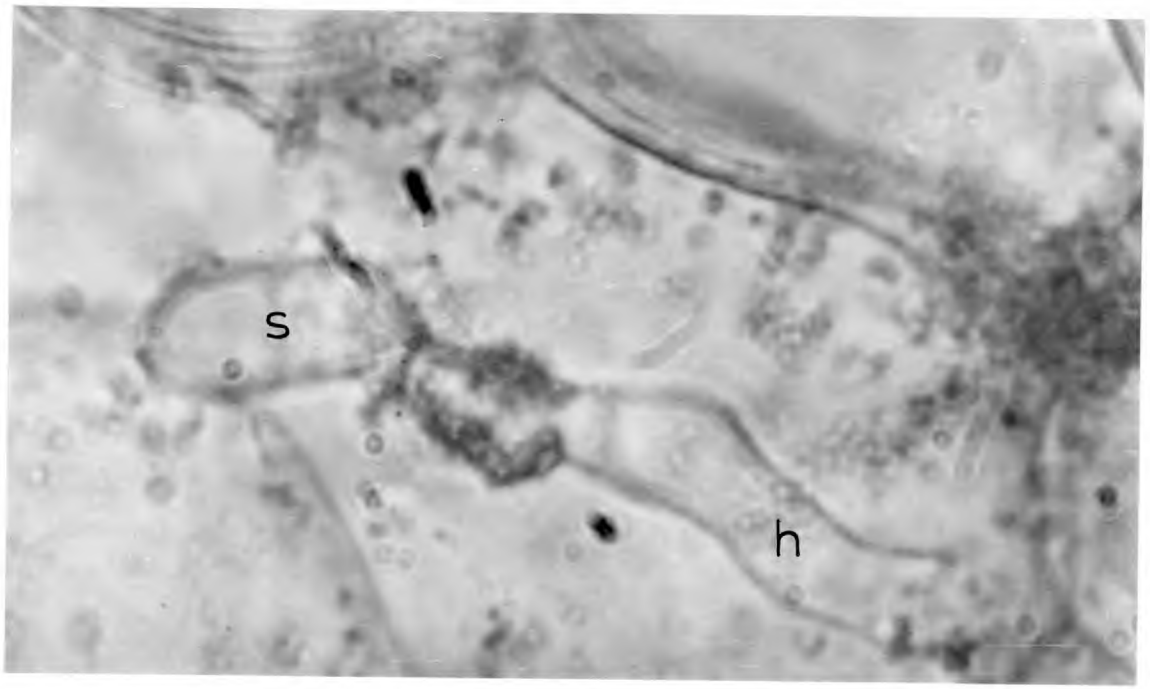
3 With the exception of Kievit(R)/delta and Immuna(S)/delta, infection events in pod cavities were unlike those which occurred during the resistant and susceptible reactions on hypocotyls reported earlier

4 With most races and varieties no hyphae had developed within endocarp cells at 36h. Spores often failed to germinate or developed germ tubes which grew superficially. However in many cases where germ tubes appeared to stop at or below a cell wall, reaction material was found in the cell directly beneath this point in the wall.

b) Races gamma and delta on c.v. Kievit

Although reaction material was often found in endocarp cells beneath a point where a germ tube appeared to enter the wall, no conclusive evidence was obtained to show that the production of reaction material was associated with penetration, neither was there any indication of how necrosis ultimately developed. In the experiment described below,

Plate 38 Infection of pod endocarp



38) Surface view of a pod endocarp cell showing a spore (s) and a thin walled intracellular hypha (h). Note deposit near point of hyphal entry (Immuna(S)/delta 36h x 3100)

observations were made at intervals during 14 days incubation to determine; at first, the relationship between germination, penetration and the development of reaction material, and later, whether hyphae might develop further to cause necrosis.

Kievit pod cavities (seeds less than 1 cm) were inoculated with 20 μl drops of gamma (V) and delta (AV) spore suspensions (5×10^4/ml) so that each pod provided one cavity inoculated with gamma and one with delta. At each of the times indicated, drops were allowed to dry (to prevent loss of spores during sectioning) and thin surface sections of inoculated tissue were taken and examined using an oil immersion objective.

The progress of infection is shown by the results in Table 27.

Less than 5% of spores produced appressoria, therefore "germination" in Table 27 refers mainly to the production of germ tubes. Germination of delta (AV) spores was more rapid than those of race gamma (V) and with the former near-maximum values were reached at 12h. Table 27 also shows that, as indicated previously, reaction material developed in cells which appeared to be penetrated by germ tubes, reaction material being first detected at the same time as this penetration. Drop and cell browning occurred with both races, the latter taking place in non-penetrated as well as penetrated cells. Drops of delta spore inoculum were first seen to be brown before any cell browning was evident. This confirmed the observations made previously and suggested that the brown substances are not necessarily the products of brown necrotic cells.

The relationship between penetration and the production of reaction material was shown more clearly by the following observations.

Table 27. Germination and penetration of spores of races gamma(V) and delta(AV) in bean pod endocarp and events associated with these processes

Observations	Results of observations made after (h) :- 3	6	12	24	36	60	168
Race gamma (V)							
Germination (%)[1]	0	17	43	56	70		
Behaviour of germ tube (%)[2]							
Grew superficially	0	0	0	0	0		
Penetrated cell, grew as thin walled hypha	0	0	0	0	0		
Penetrated cell(?), reaction material beneath	0	0	100	100	100		
Browning							
Penetrated brown cells (%)[3]	0.0	0.0	0.0	0.0	0.0	1.7	0.0
Total brown cells (%)[3]	0.0	0.0	0.0	0.0	0.0	5.4	0.0
Browning in drops	-	-	-	-	+	+	
Race delta (AV)							
Germination (%)[1]	0	50	96	99	100		
Behaviour of germ tube (%)[2]							
Grew superficially	0	5	0	1	0		
Penetrated cell, grew as thin walled hypha	0	0	0	0	0		
Penetrated cell (?), reaction material beneath	0	95	100	99	100		
Browning							
Penetrated brown cells (%)[3]	0.0	0.0	0.0	0.0	5.7	2.7	3.9
Total brown cells (%)[3]	0.0	0.0	0.0	0.0	5.7	8.1	6.8
Browning in drops	-	-	-	+	+	+	

NOTES 1 100 spores examined on either of 2 strips for germ tube or appressorium production. Results expressed as mean % for 2 strips

2 All spores in inoculation site examined for presence of some structure in the cell which appeared to arise from them. Results as mean % for 2 strips

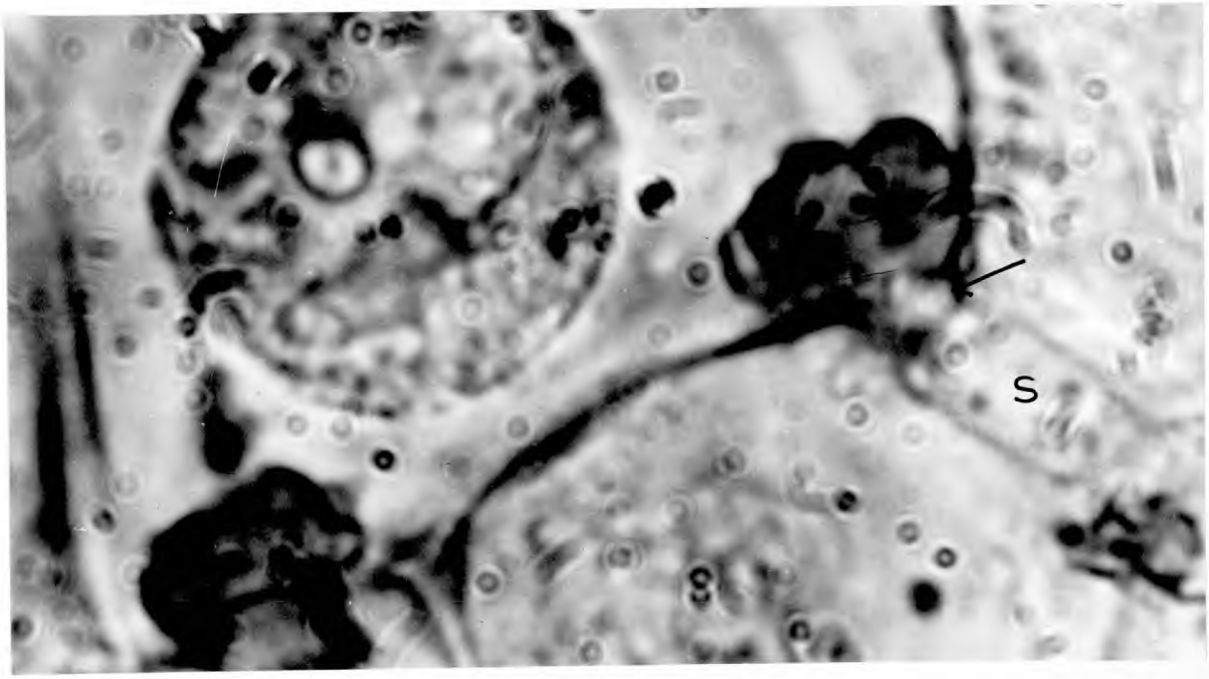
3 All cells in inoculated area examined and those showing granulation and browning were counted. Results as mean % brown in 2 strips.

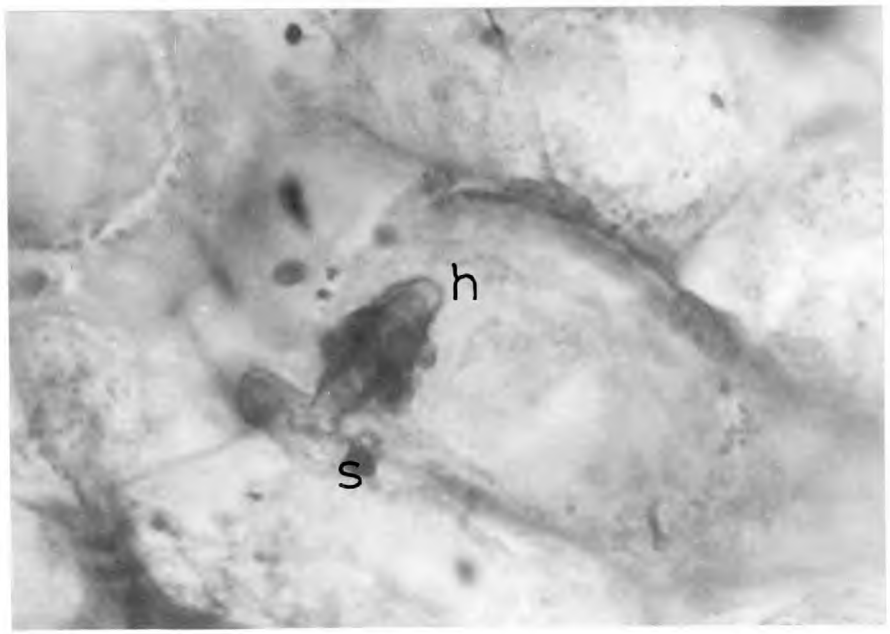
In tissue inoculated with race delta (AV), structures were seen beneath some spores at 3h and although too small to be clearly distinguished as hyphae these bodies were colourless, unthickened and more or less transparent. Later, structures which were slightly larger but were found in the same position, appeared dense, opaque and yellow-brown. Plate 39 shows that these bodies were more clearly visible after plasmolysing the cell with 0.8M sucrose and in this plate the connection between the base of the spore and the reaction material can be seen. In a few cells similar brown material was seen surrounding clearly recognisable hyphae (Plate 40) thus suggesting that reaction material was produced in a penetrated cell.

Cell browning in penetrated and non-penetrated cells is shown in Plates 41 and 42 respectively. During the observation period of Table 7, cell necrosis was rarely detected and most penetrated and non-penetrated cells plasmolysed in hypertonic sucrose solution (Plate 42). Examination of transverse sections cut after 60h and 7 days revealed that although the cell walls in the top few layers of cells were slightly brown, no necrosis was evident in any cell beyond the first layer. No hyphae were found in any cell apart from those which could be seen within the reaction material. These observations and results given earlier indicate that for at least 6 days after the time of penetration (the first day after inoculation) little or no intracellular hyphal growth took place and necrosis was only evident in a very few endocarp cells.

After 16 days however, slightly sunken, dark brown necrotic spots which were about the same size as the original drop were found in all

Plates 39 & 40 Infection of pod endocarp

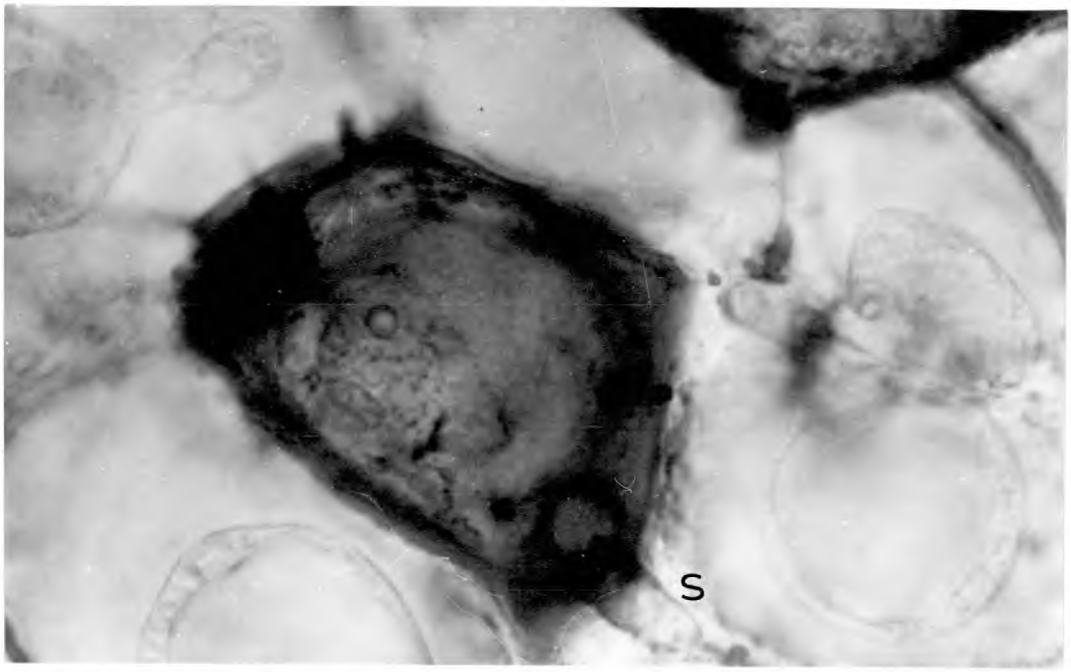


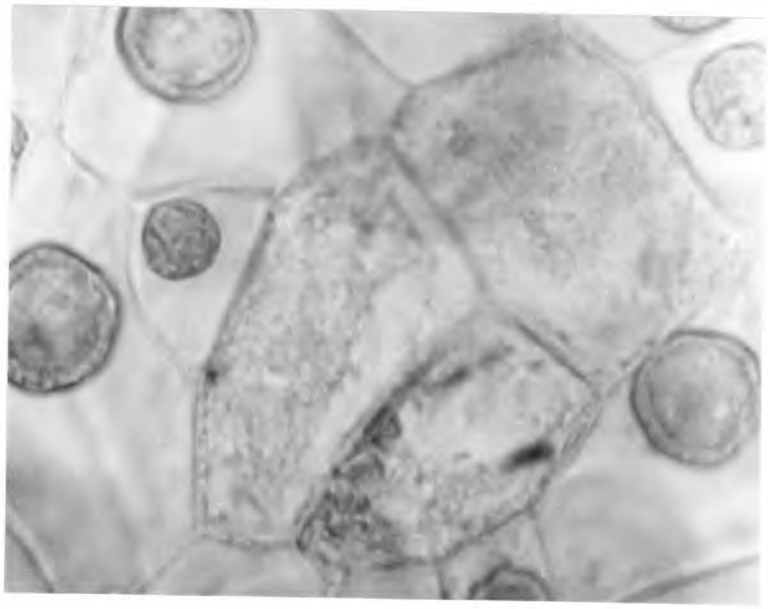


39) Pod endocarp cell with plasmolysed protoplast showing a dense deposit beneath the point where a short germ tube (arrowed) appeared to enter the cell from the spore(s) (Kievit(S)/gamma 60h x 3100)

40) Clearly recognizable hypha (h) in pod endocarp cell. Note marked deposit at site of penetration but lacking at hyphal apex. (Kievit(R)/delta 7 days x 1250)

Plates 41 & 42 Granulation and browning of pod endocarp cells





41) Penetrated pod endocarp cell showing granulation and browning. Note spore (s) giving rise to germ tube which penetrated cell. Adjacent cells plasmolysed in molar sucrose solution. (Kievit(R)/delta 7 days x 1250)

42) Non-penetrated pod endocarp showing granulation and browning. Note adjacent cells plasmolysed (Kievit(R)/delta 7 days x 1250)

the eight remaining cavities inoculated with race gamma (V). Hyphae were found throughout the necrotic region. Extensive superficial hyphal growth and acervulus production had also occurred. Necrosis was not apparent beyond the fibrous layer beneath the endocarp parenchyma. In delta-inoculated (AV) tissue, necrosis was limited to a few patches about 1 mm or less in diameter. The dead cells of which these necrotic patches were composed, occupied several successive layers of parenchyma, were commonly absent from the epidermis and were seen to contain thin walled transparent hyphae. Cells of the non-necrotic areas were similar in appearance to tissues at earlier examinations.

The following conclusion is suggested by the results of these observations:-

At about the time when germ tubes of races gamma (V) and delta (AV) penetrated the endocarp cells of c.v. Kievit the production of reaction material was initiated and hyphal growth ceased. Between 6 and 15 days after the initial penetration, hyphal growth (either of the few superficial hyphae or of those which had attempted penetration) was resumed; some colonization of tissue and the development of necrosis then took place.

No information was available to indicate why more necrosis developed in response to the virulent (gamma) than the avirulent race (delta).

Conclusion

Pod endocarp tissue does not always show the same differential resistant or susceptible reaction to C. lindemuthianum as intact hypocotyls. The processes of infection and cell response generally differed from those in intact hypocotyls and were more similar to those in stripped hypocotyls. After penetration by virulent and avirulent races, reaction material was produced and hyphal growth stopped. Where necrosis ultimately developed, intracellular growth was resumed a number of days after it had first ceased.

13. Leaf Tissue

Leaf tissue was used in some later experiments and thus it was of interest to examine infection and cell response in this tissue.

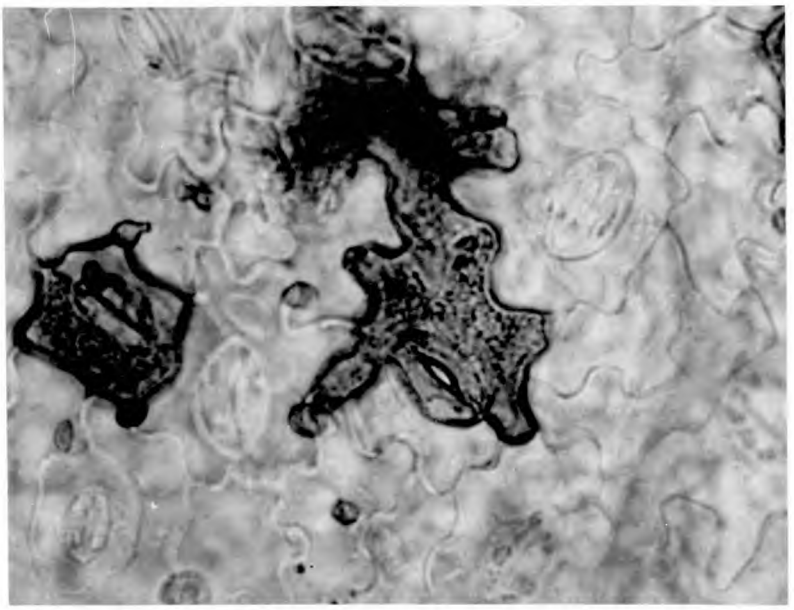
Leaflets from the first trifoliate leaves of c.v. Kievit were inoculated on their abaxial surface with 20μl drops of suspension of gamma (V) and delta (AV) spores (10^6/ml) and then floated on sterile distilled water in clean Petri dishes. After 10 days, the areas beneath the drops were removed with a cork borer, fixed, cleared and examined microscopically.

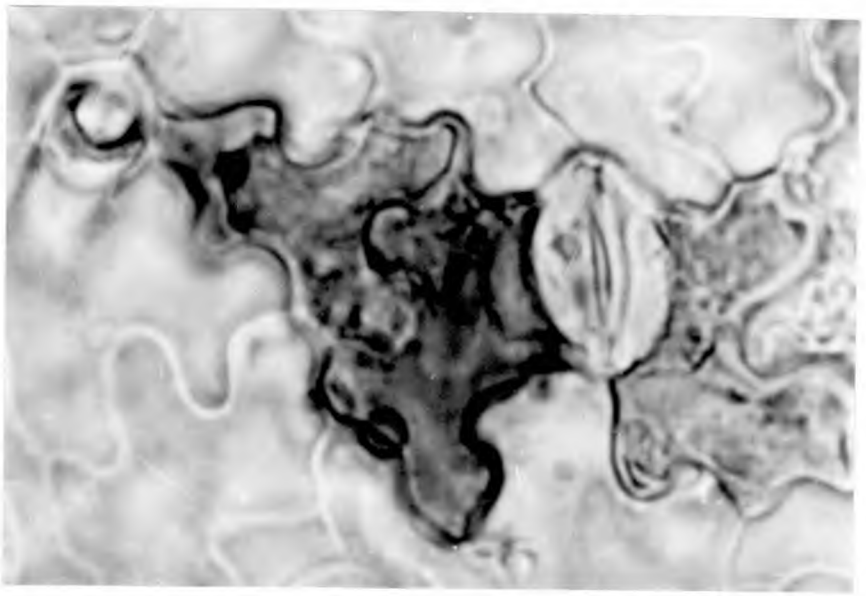
Brown granular cells, which occurred singly or in groups of 2 or 3 (Plates 43 and 44) were found in the epidermis of leaf tissue inoculated with either race. Necrosis of mesophyll cells and larger groups of epidermal cells only occurred in tissue inoculated with race gamma (V) (Plate 45). However these areas were very limited in extent and in interveinal tissue were often only just visible with the naked eye. More obvious necrosis was visible on small veins but this was seldom more than about 0.5mm in length. Necrotic tissue was rust-brown in the interveinal regions and dark brown on the veins.

Many cells with appressoria attached were not brown and showed no sign of fungal penetration. Other cells were seen with a vesicle like structure beneath the appressorium (Plate 46). These were transparent, smooth in outline and brown; they were found in both gamma and delta inoculated tissue. Apart from these structures there was no evidence of hyphal growth in epidermal cells.

Thus although the resistant and susceptible reactions could be clearly distinguished by the absence or presence of veinal necrosis,

Plates 43 & 44 Infection of leaf tissue

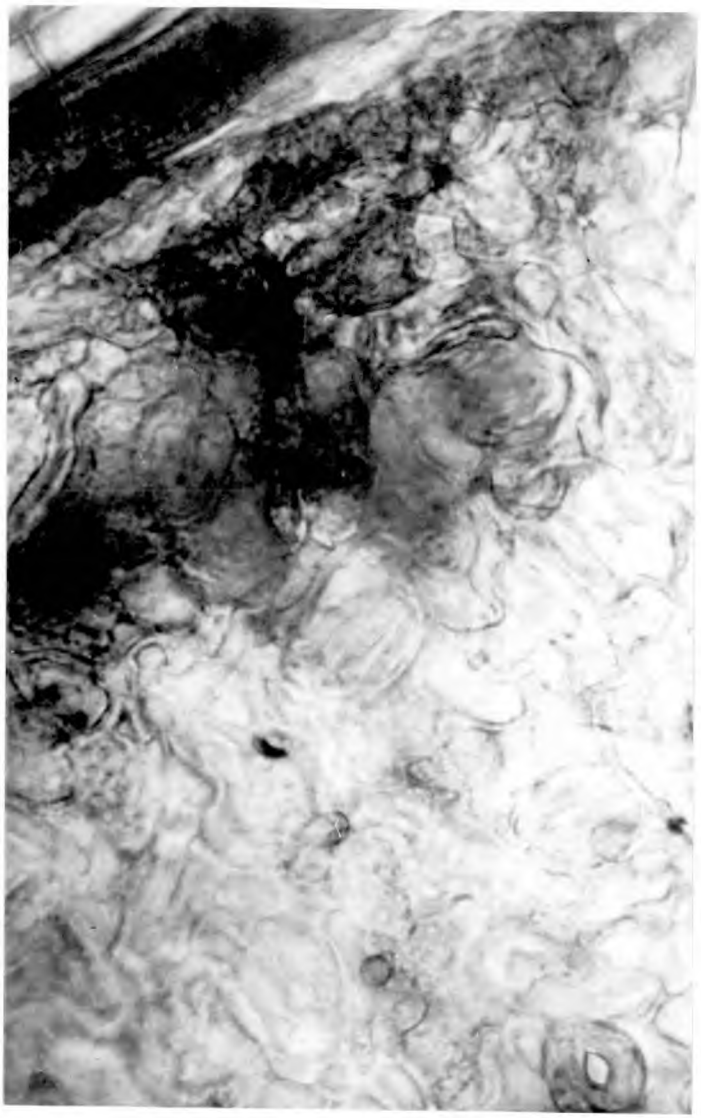


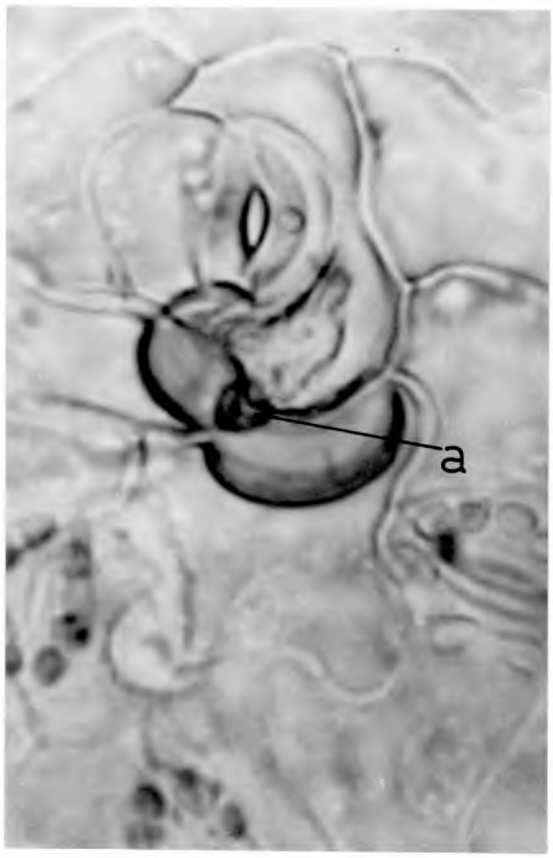


43) Epidermal cells of leaf lamina showing granulation and browning in response to an avirulent race of C. lindemuthianum (Kievit(R)/delta 13 days x 625)

44) Epidermal cells of leaf lamina showing granulation and browning in response to a virulent race (Kievit(S)/gamma 10 days x 1250)

Plates 45 & 46 Infection of leaf tissue





45) Necrosis of leaf mesophyll tissue near an infected vein (Kievit(S)/gamma 10 days cleared tissue x 675)

46) Dark "vesicles" beneath appressoria (a) in lamina epidermal cells (Kievit(S)/gamma 10 days cleared tissue x 1250)

restricted browning of lamina epidermal cells occurred with both races and probably indicated that lamina tissue is very resistant to even a virulent race.

Section II. Factors affecting the response of cells to infection and the number of lesions produced

This section describes the effect of factors which might be considered to be normal experimental variables.

1. Number of spores in drops of inoculum

In a number of experiments, drops of spore suspension of races of C. lindemuthianum were applied at different concentrations to hypocotyls of bean varieties giving susceptible and intermediate responses.

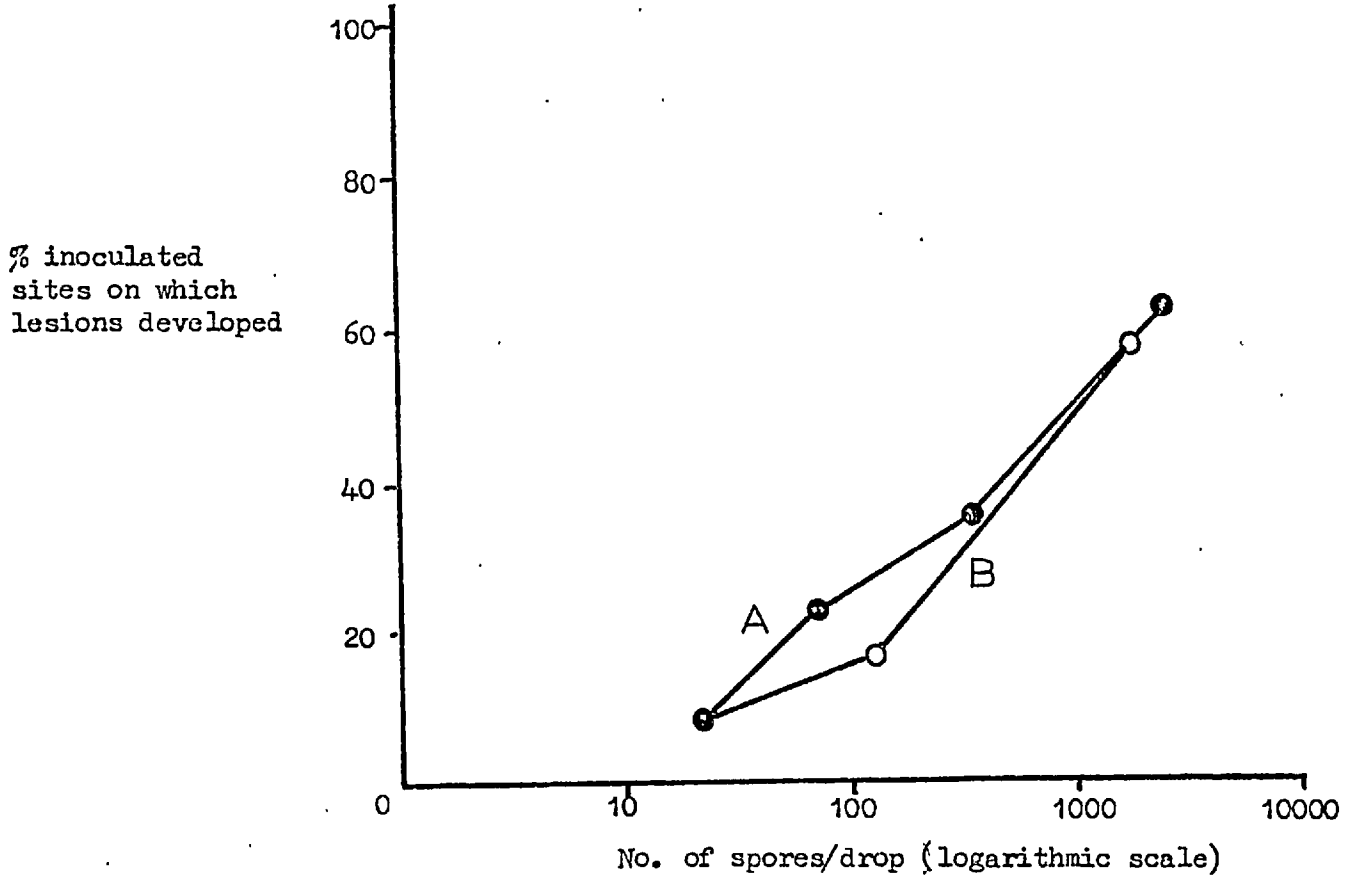
The mean number of spores applied per drop was determined by counting all spores in drops of known volume applied to microscope slides. After 12-16 days, when constant values were obtained, the number of inoculated sites in which lesions had developed was counted. The details and results of these experiments are summarized in Table 28 and Figs. 9, 10 and 11.

In experiments A-C, the percentage lesions produced increased in proportion to the logarithm of the numbers of spores applied. These race variety combinations had previously been classed as susceptible. In two experiments where the Pinto/beta combination (Intermediate) was used exactly the reverse occurred. At high concentrations most sites were covered with numerous minute brown flecks. The size of individual flecks and the number of necrotic areas classed as lesions increased as spore number decreased. This marked "concentration effect" in the Pinto/beta interaction is unlikely to be due to self inhibition by spores as, with higher spore concentrations than were required to produce this effect, no inhibition could be detected on susceptible

Table 28. Details of procedure for the experiments the results of which are summarized in Figs. 9, 10 and 11.

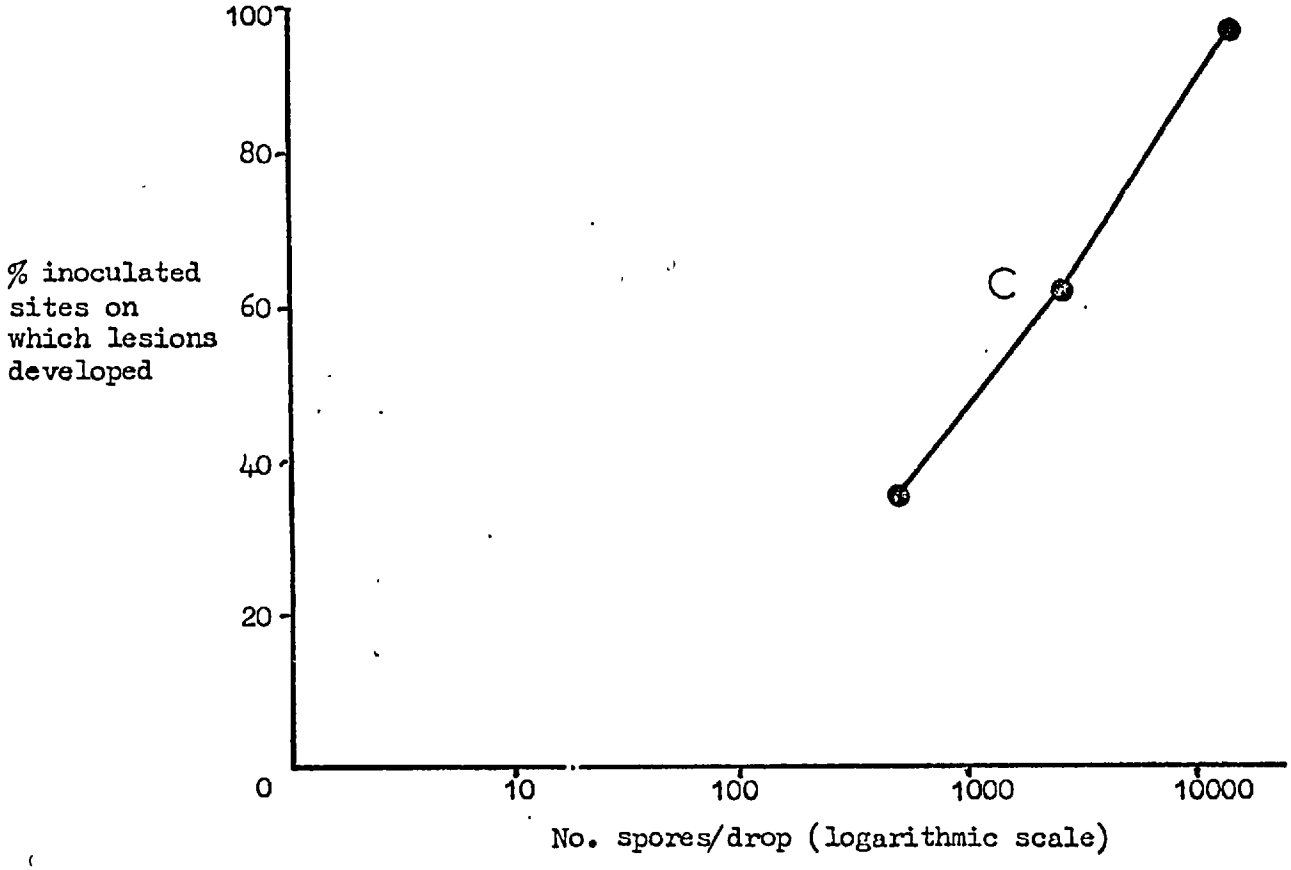
Experiment	Variety	Age of hypocotyl (days)	Race	Mean No. Spores/drop	No. drops of each concentration applied	Lesions counted after (days)
A	Kievit(S)	11	Gamma	24±9	45	14
				78±14	45	
				388±34	40	
				1950±172	35	
B	Kievit(S)	13	Gamma	29	200	16
				140	200	
				575	200	
				2750	175	
C	Pinto(S)	12	Delta	971±148	150	12
				1138±148	150	
				163400±5177	140	
D	Pinto(I)	9	Beta	62±24	105	9
				372±50	110	
				1890±400	105	
E	Pinto(I)	7	Beta	90	210	14
				290	210	
				1450	210	
				7500	210	

Fig. 9 Relationship between number of spores in 5 $\mu\ell$ drops of suspension and the number of lesions produced by race gamma on a variety giving a susceptible response c.v. Kievit.



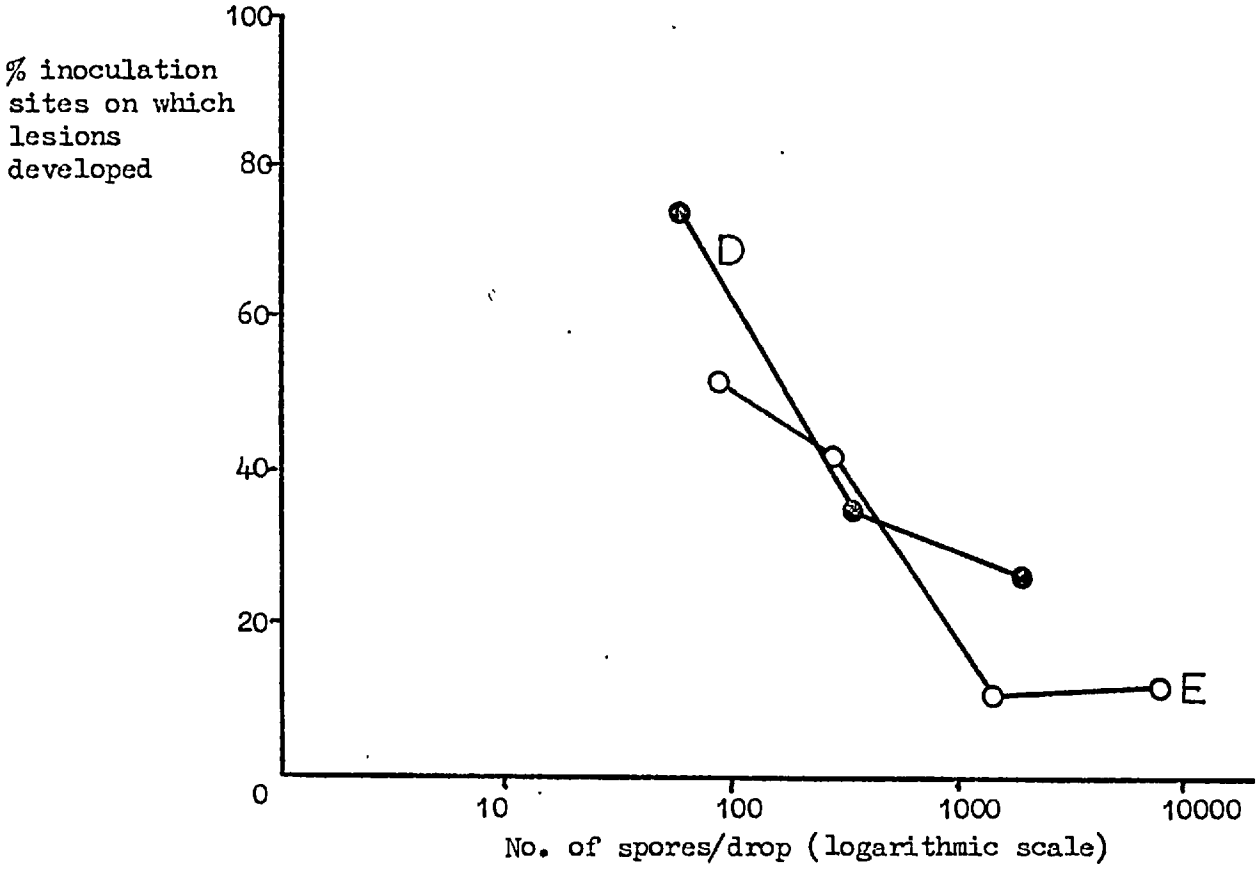
Letters refer to experiments listed in table. 28

Fig. 10. Relationship between number of spores in 5$\mu\ell$ drops of suspension and the number of lesions produced by race delta on a variety giving a susceptible response c.v. Pinto



Letter refers to experiment listed in Table 28

Fig. 11. Relationship between Number of spores in 5 µl drops of suspension and the number of lesions produced by race beta on a variety giving an intermediate reaction c.v. Pinto



Letters refer to Experiments listed in Table. 28

varieties. It is more likely that a host response was involved, possibly in an effect similar to the cross-protection obtained with a mixed inoculum of spores of avirulent and virulent races (Page222).

Conclusion

Increasing the number of spores in drops applied to a susceptible variety increased the number of lesions produced. Whereas, with the same range of spore concentration, the numbers of lesions produced on an intermediate variety decreased with increased spore number.

2. Virulence of the isolate

The original cultures of races gamma and delta appeared to be less pathogenic than those obtained after re-isolation from infected plants (see materials and methods). To test whether the re-isolate of race delta had retained differential pathogenicity on bean varieties, spore suspensions from cultures of both types of isolate were tested on hypocotyls of c.v. Immuna and c.v. Red Kidney. Spray inoculation and scoring of response were carried out as described by Smith (1967). Eleven days after inoculation, the eight replicate hypocotyls inoculated with either isolate on Red Kidney were classed as grade 1 (resistant), and a similar number of Immuna hypocotyls, were classed as grade 4 (susceptible). Thus re-isolation had not changed the differential pathogenicity of race delta.

To determine whether the ability to produce lesions on susceptible hypocotyls was greater in the re-isolated culture, 11 day Immuna and Pinto hypocotyls were inoculated, 5 drops on each of 14 and 20 hypocotyls respectively, and incubated for 11 days. Two lesions appeared on Immuna and 57 on Pinto with the original culture isolate whereas the re-isolated culture produced 0 and 91 lesions. Thus an increase in pathogenicity was demonstrated, at least in the Pinto/delta combination.

Conclusion

When a re-isolate from infected tissue was used race delta was more pathogenic to a susceptible variety while remaining avirulent on a resistant variety.

3. Hypocotyl age

a) Lesion development, infection and cell response in hypocotyls of different ages

It has often been found that when spores of a virulent race of C. lindemuthianum are applied to old hypocotyls, very few lesions develop. The following experiment was carried out to determine how the age of hypocotyls of a variety used in this study affected the number and size of lesions. In addition germination, penetration (and cell response to an avirulent race) and hyphal growth were studied to determine the stage of infection at which "age resistance" might operate. As it was found earlier that removal of the epidermis increased lesion development in the Immuna(S)/delta combination, this treatment was used here to see whether a similar increase in susceptibility could be detected.

Seeds of c.v. Kievit were sown in 4 successive batches at 3 day intervals. They were grown in the normal way and after 10 days most of the hypocotyls of a batch were extended. All hypocotyls were harvested 10 days after the final batch of beans had been sown. The following hypocotyls were inoculated with spores of race gamma (V) for each of the hypocotyl ages: 20 stripped hypocotyls, 20 freshly cut unstripped hypocotyls, 4 unstripped hypocotyls for penetration counts and 4 for germination counts (only first and last batches).

For race delta (AV) only germination and penetration were studied and appropriate numbers of replicate hypocotyls (same as in susceptible study) were inoculated. Hypocotyls of the 4 batches are hereafter referred to as 10, 13, 16 and 19 days old.

Germination was determined on 4 replicate hypocotyls (2 strips per hypocotyl) taken 3 days after inoculation. Penetration (race gamma) and cell response (race delta) were examined on another 4 hypocotyls (3 strips from each) after 5 days. Symptom development was examined at intervals. The results are summarized in Fig. 12 and in Tables 29, 30, 31 and 32.

Clearly (Fig.12) the age of hypocotyls at the time of inoculation affected both the rate of appearance of lesions and the numbers finally produced. The amount of necrosis (Table 29) which developed at inoculation sites decreased with increasing hypocotyl age and non-limited, watersoaked lesions were found only in 10 and 13 day hypocotyls. Numerous minute brown spots were found in inoculation sites of 16 and 19 day hypocotyls and these often resembled the flecking found in resistant reactions. Hyphae were found to be present within these brown cells when the tissue had been cleared with 0.1N caustic soda.

Plates 47,48 and 49 show the depth of necrosis and hyphal growth typical of tissue of different ages, 21 days after inoculation. Considerable hyphal growth was evident in 10 day tissue and had extended to the pith (Plate 47). The lesions were a green brown colour and averculi were abundant on the surface. Many lesions on 13 day hypocotyls were sunken and sharply delimited to an area approximately the same as the original drop. These areas were dark brown to black in colour. Plate 48 shows that necrosis was limited to the cortex and was bounded centripetally by a layer of fibrous tissue. Some acervuli were formed. Cells of the fibre layer are shown more clearly in Plate 49 which was taken from a very limited necrotic area of type 0 or 1 of 19 day hypocotyls.

Fig.12 The development of lesions on susceptible hypocotyls (c.v. Kievit) of different ages at various times after inoculation with spores of race gamma - hypocotyls cut just before inoculation

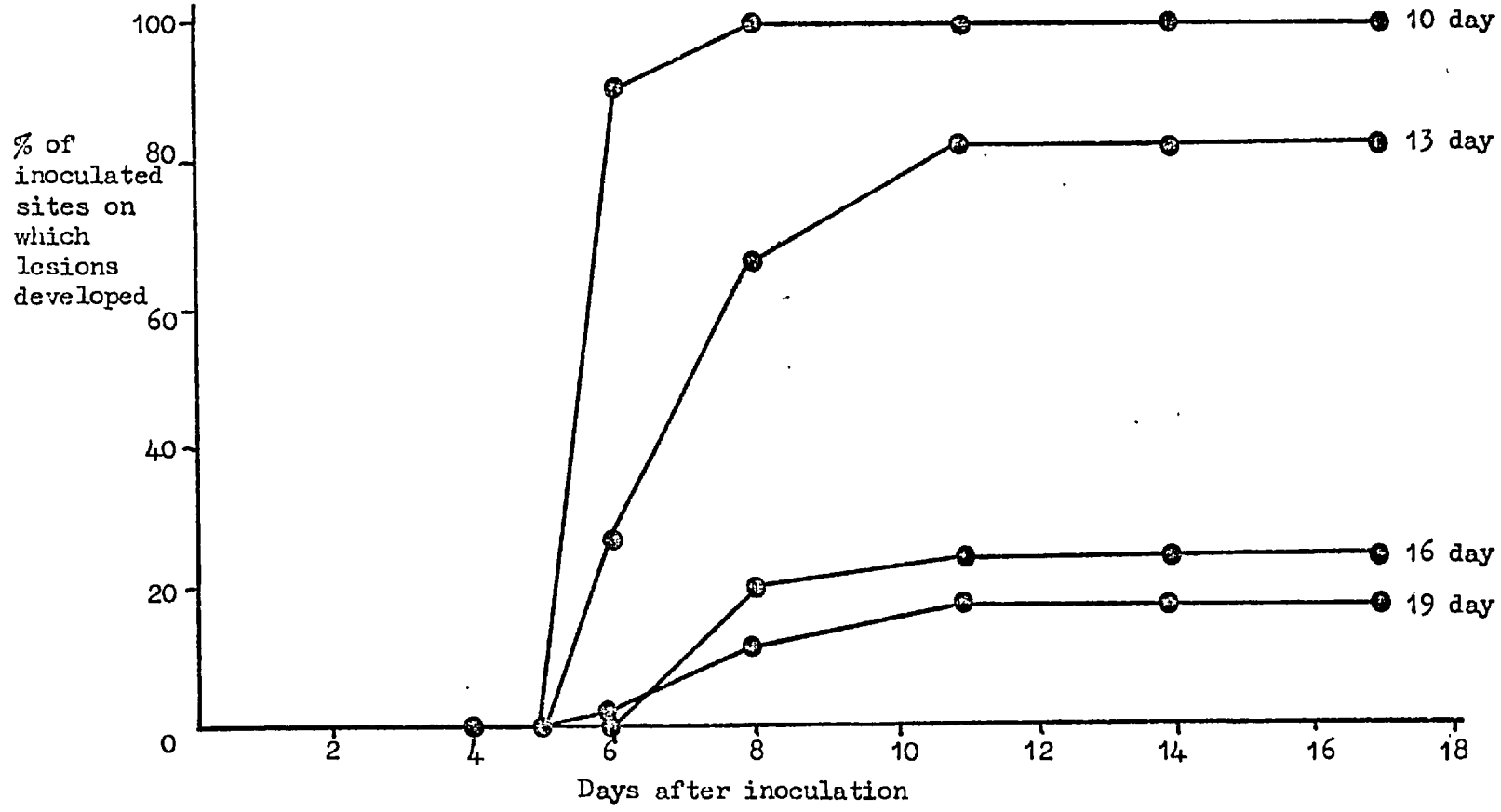
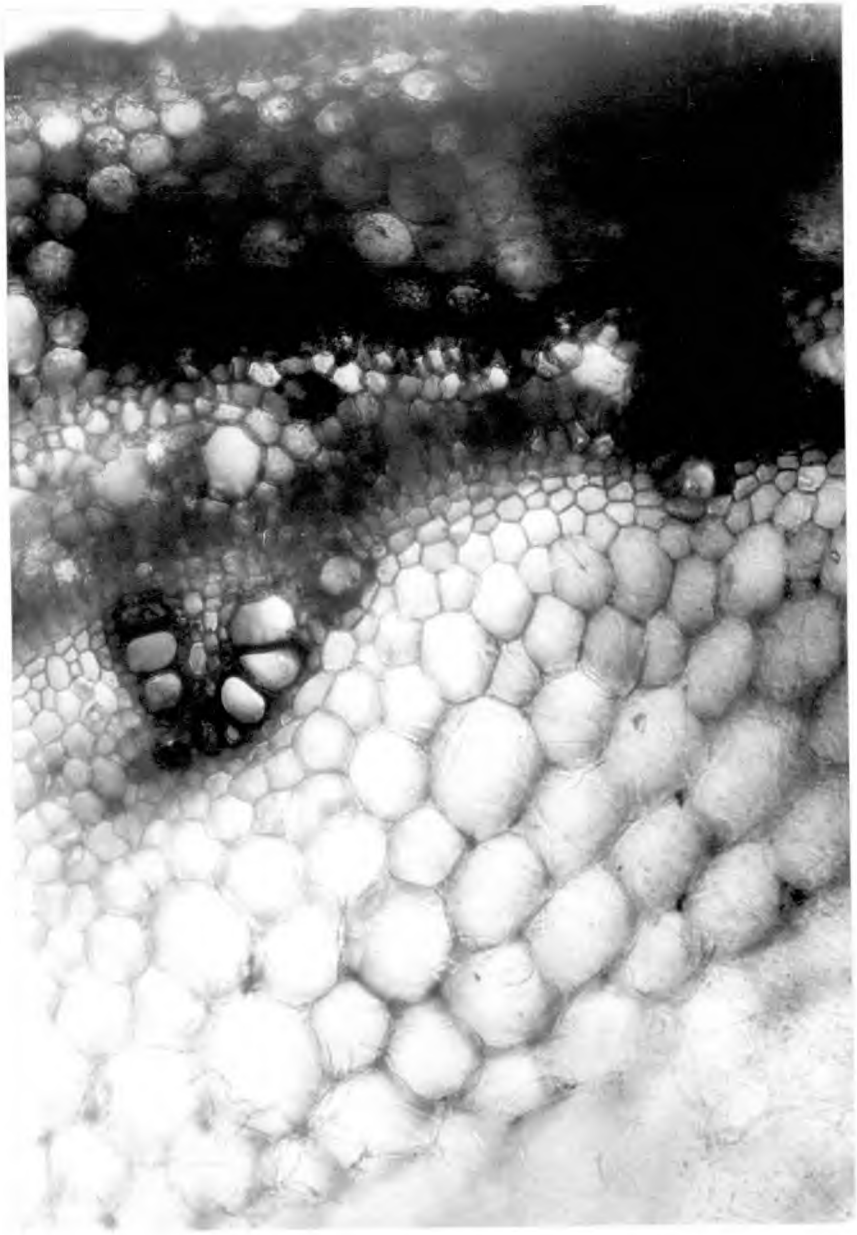


Table 29. Types of necrosis appearing on susceptible hypocotyls of different ages (c.v. Kievit) 21 days after inoculation with race gamma (- hypocotyls cut at time of inoculation)

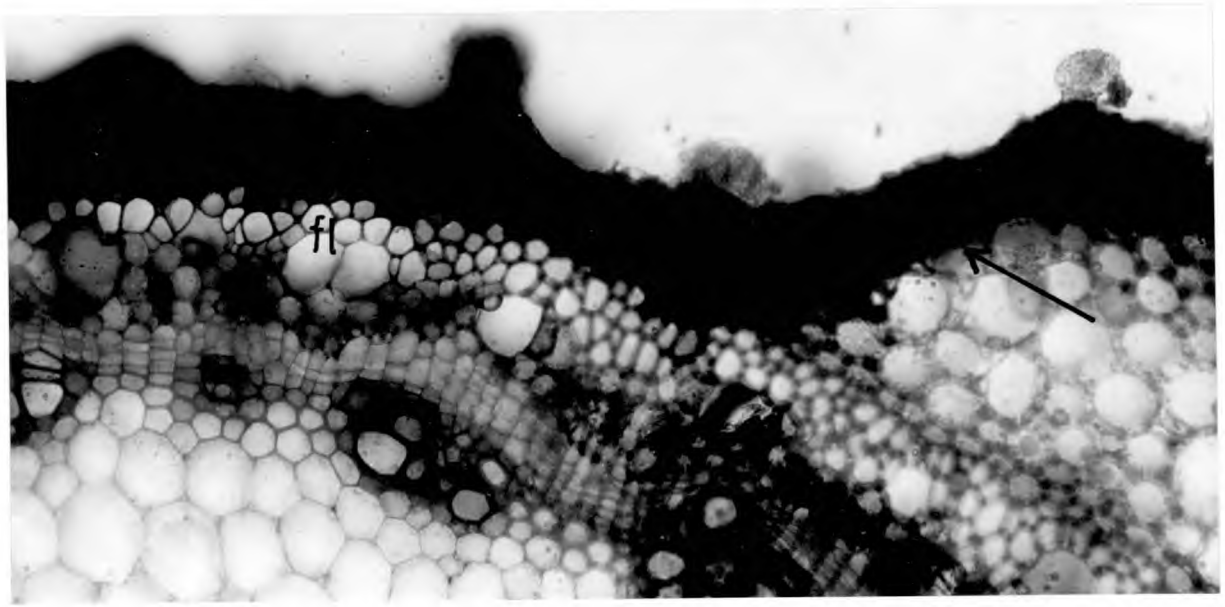
Grade	Description	% of sites assigned to different grades with tissue aged (days) :- 10	13	16	19
0	Inoculated site covered with minute brown flecks with occasional dark brown or black areas less than 1mm.	0	14	77	84
1	Dark brown or black necrosis greater than 1mm in length - superficial	0	7	18	13
2	Dark brown or black sunken lesion about area of drop in size	0	40	5	3
3	Green-brown water soaked lesions at inoculation site - collapse of rest of hypocotyl	100	39	0	0

Plate 47 Infection of old hypocotyls



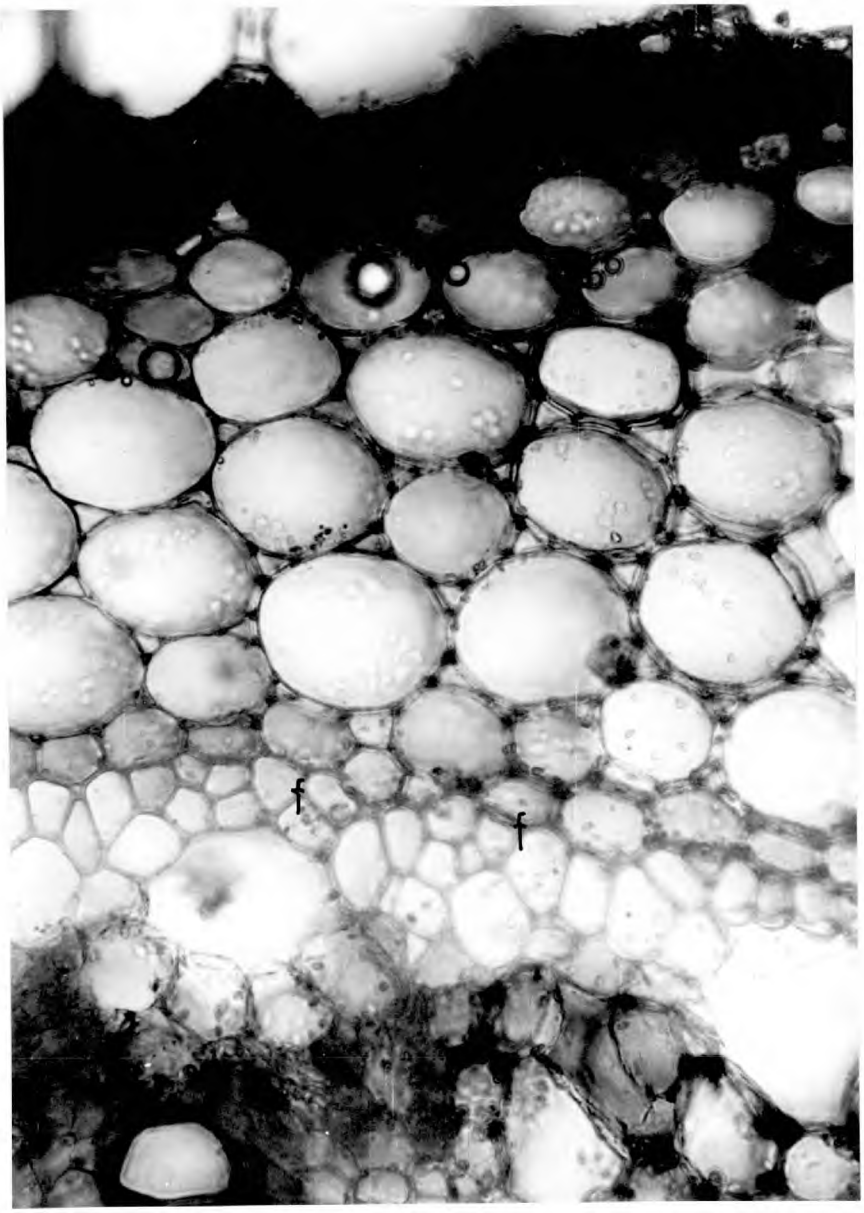
47) T.S. hypocotyl in the region of a lesion showing the extent of infection of hypocotyls inoculated when 10 days old. Note browning in cortex and hyphae in pith. (Kievit(S)/gamma 21 days x 160)

Plate 48 Infection of old hypocotyls



48) T.S. hypocotyl in region of sunken, "eye-shaped" lesion. Hypocotyls inoculated when 13 days old. Note blackening of tissue and sharply defined edge of lesion in cortex (arrowed) and at a layer of fibres (fl) above vascular tissue (Kievit(S)/ gamma 21 days x 160)

Plate 49 Infection of old hypocotyls



49) T.S. hypocotyl in region of small brown flecks. Hypocotyl inoculated when 19 days old. Note necrosis limited to epidermis and a few cortical cells. Fibres (f) shown above vascular tissue (this was region of lesion limitation in Plate 48).
(Kievit(S)/gamma 21 days x 390)

Necrosis was limited to epidermal and a few layers of cortical cells; it did not reach the layer of thickened cells. No marked collapse of the necrotic area of 19 day hypocotyls was observed.

The number of lesions produced on stripped hypocotyls after 11 days incubation was 89, 36, 43 and 16 for 10-19 day hypocotyls respectively. Stripped 16 and 19 day hypocotyls (Fig. 12) did not appear to be any more susceptible to race gamma than unstripped hypocotyls of the same age.

Hypocotyl age had no great effect on spore germination (Table 30) but both penetration and growth to a second cell was less in older than in young hypocotyls inoculated with race gamma (Table 31). Growth to other cells was progressively less in 10, 13 and 16 day old hypocotyls. The number of cells containing reaction material did not change much with increasing age although the proportion of affected cells which contained this material increased slightly. Cells of tissue of any age were not brown and granular when examined 5 days after inoculation.

In resistant hypocotyl tissue to which race delta (AV) had been applied (Table 32), there was a marked reduction in the proportion of cells that became brown and granular in 13 day plants compared with 10 day. There was little further decrease in the number of affected cells with increase in age.

It is evident from these results that the decrease in lesion number, size and rate of appearance with increasing hypocotyl age was not associated with decreased spore germination, increased production of reaction material or a more rapid cell death soon after penetration. As necrosis was often confined only to a few cells of the cortex in the

Table30. The production of appressoria by spores of races gamma and delta on Kievit hypocotyls of different ages

Race	% spores examined[1] which had produced appressoria on tissue aged: 10 days		19 days	
	%[2]	Range[3]	%[1]	Range[3]
Gamma (V)	78.5	59.5 - 96.0	68.9	53.5 - 86.0
Delta (AV)	59.8	52.0 - 67.2	72.5	60.4 - 84.6

1 All spores on strips (2 from each of 4 hypocotyls) giving a total of 4400 - 5100 spores, examined for each combination of race and age

2 % spores examined bearing appressoria

3 Highest and lowest values for individual replicates

Table 31. Penetration and cell response in susceptible hypocotyls (c.v. Kievit) of different ages 5 days after inoculating with spores of race gamma(V)

Hyphal Grade	Description	% of cells examined[1] in which hyphae were assigned to the following grades in tissue aged (days):- 10	13	16	19
2	Hypha penetrated one cell	2.7	7.2	6.0	8.0
3	" " second cell	46.5	25.3	2.3	2.2
	Total penetrated (%)[2]	49.2	32.5	8.3	10.2
1	Reaction material (%)	3.0	2.3	1.3	2.2

NOTES

1 600 cells (50 on each of 3 strips from 4 hypocotyls) examined for each determination

2 Reaction material containing cells excluded

Table 32. Penetration and cell response in resistant hypocotyls (c.v. Kievit) of different ages 5 days after inoculating with spore of race delta (AV)

Grades	Description	% cells examined[1] which were assigned to the following grades in tissue aged (days) :- 10	13	16	19
7,8,9,10	Granulation and browning of cells	21.5	8.0	2.3	9.0
1.2, 1.3	Hyphae in unaffected cells	2.5	0.0	0.2	0.3
2.1	Reaction material	3.2	0.3	0.5	2.0
	Total cells reacting or penetrated %	27.2	9.3	3.0	11.3

NOTE

1 600 cells (50 on each of 3 strips from 4 hypocotyls) examined for each determination

oldest tissue, fibres in the vascular ring could not have been important; neither could drying of necrotic tissue as the hypocotyls were incubated at high humidity. Less frequent penetration and the slower growth of hyphae from penetrated cells suggested that age resistance was due to a decrease in hyphal growth rate and might be related to an increased resistance of cell walls of older tissue to hyphal penetration. The reduction in the proportion of cells responding to an avirulent race (delta) was also consistent with this view.

b) Cell death in old tissue inoculated with a virulent race

The results presented in sub-section 3a suggested that old hypocotyls were less susceptible because of an increased resistance of cell walls to hyphal penetration; age resistance thus being a passive phenomenon. However minute red-brown flecks, resembling those appearing after inoculation of resistant hypocotyls with an avirulent race, were found at inoculation sites and thus another possibility, that cell death and browning were also involved in an active resistance mechanism, was not ruled out. As no infected cell was dead at 5 days (Table 31), such a response would be likely to occur at a rather late stage in infection. There are two conflicting views on the origin and role of cell necrosis in older tissue. Sinden (1937) suggested that the response of old tissue was indistinguishable from the resistant reaction to an avirulent race; cell death occurring soon after penetration and before death of the hypha. Leach (1923) and Griffey and Leach (1965) suggest that hyphae whose growth was restricted in old tissue die and release products of autolysis which cause necrosis. To examine these possibilities further, the following investigation was conducted to determine whether: a) cell death occurs after that of the hypha, b) whether changes in the host cell during necrosis resemble those which occur in reactions to avirulent races and c) to confirm that cell death occurs some time after penetration.

Nine and 16 day Kievit hypocotyls were inoculated with spores of race gamma. After 6 days, water soaked, non-limited lesions had formed at all sites on the 9 day tissue but no sign of infection or browning was visible in 16 day plants. Strips were taken from 16 day plants

at intervals after inoculation and were examined. The results of these observations are summarized in Table 33 and below.

About half the cells that had been penetrated at 6 days (totals at foot of Table 33), and a few even after 13 days, were unaffected by the presence of hyphae, some of which had grown to a second cell (grades 1.2, 1.3 - body of table). Penetration increased for at least 13 days. The proportion of affected cells, nearly all containing hyphae, increased and most penetrated cells at 13 days were either brown or granular (cell grade 5). Other effects were Brownian motion of organelles and the active streaming of obvious particles. Most epidermal cells were colonized and all were dead in the lesions of 9 day plants at 6 days. These results confirm the earlier findings that infection was slow and that effects on cells do not occur frequently until the later stages of incubation.

The extent of hyphal colonization at 13 days is shown in Plates 50, 51 & 52. Usually the hyphae were confined to small groups of brown cells (Plate 50), mainly in the epidermis and often to only one cell which was rather similar in appearance to responding cells of resistant varieties (Plate 51). No secondary hyphae or acervuli were formed and no browning was observed outside the inoculation site. Not all hyphae shown in these plates terminated at a wall thus inhibition associated with cell necrosis as well as increased wall resistance was a possibility.

Epidermal strips removed at the above times were immersed in molar sucrose in order to determine whether cells and hyphae were able to plasmolyse. The protoplast of hyphae in granular cells (grade 5) normally plasmolysed although the host cell protoplast did not. In Plate 52,

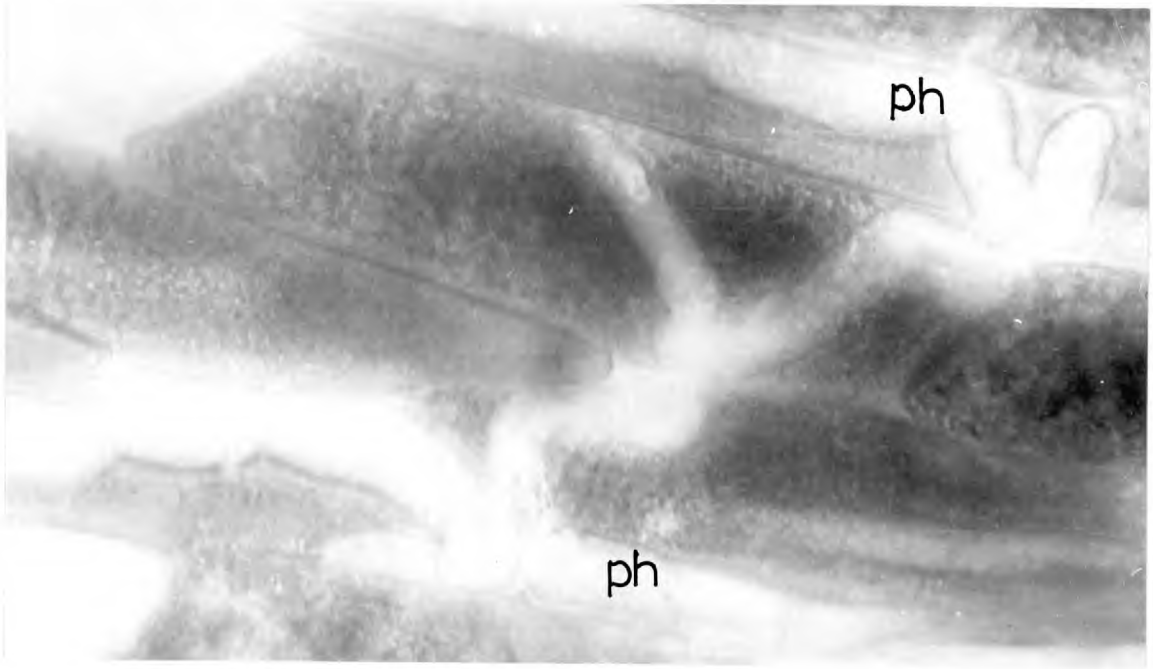
Table 33. Hyphal development and reaction of epidermal cells of 16 day old susceptible bean hypocotyls (c.v. Kievit) at various times after inoculating with race gamma(V)

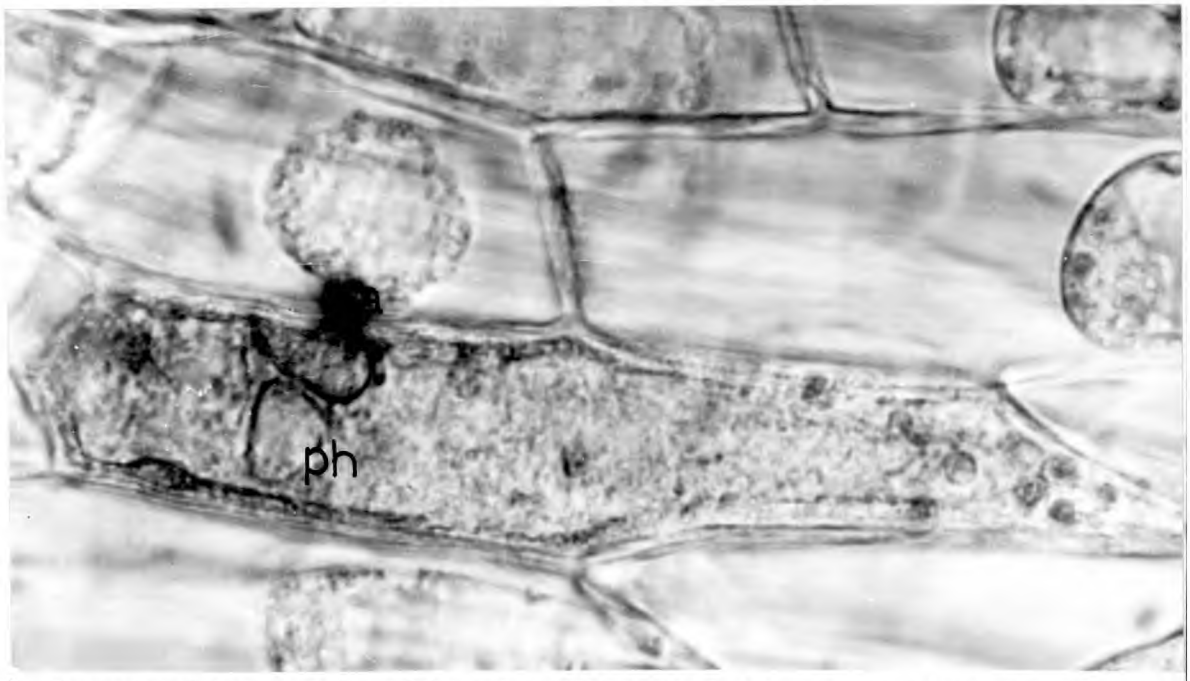
Grade	Description	% cells examined[1] assigned to grades after (days)- 6	7	9	13
	Cell unaffected				
1.2, 1.3	Hypha present	5.6	3.5	2.9	1.6
	Cell affected - colourless				
5.2, 5.3	Contents granular and motionless, membranes collapsed	3.0	2.2	4.1	5.1
6.2, 6.3	Brownian movement in cell	0.6	1.1	0.9	0.0
2.2,2.3,3.2,) 3.4,4.2,4.4)	Obvious particles in cell	1.9	0.1	0.3	0.0
	Cell affected - brown				
7.2,7.3,8.2, 8.3,9.2,9.3,9.4)	Browning throughout cell	0.8	2.4	7.2	12.9
10.4, 11.4	Browning in wall or part of cell	0.0	0.3	0.0	0.0
	Reaction material produced in cell				
2.1	Obvious particles streaming throughout cell	2.0	1.0	3.4	2.3
4.1,5.1,7.1, 8.1,9.1	Other grades	0.0	0.4	0.4	0.5
	Total hyphae penetrated second cell[2]	5.0	6.6	12.4	16.0
	Total penetrated cells[2]	10.9	9.1	14.8	18.7
	Total penetrated cells affected[2]	5.3	5.6	11.9	17.1
	Total affected cells[2]	6.3	6.1	12.5	18.0

1 800 cells (100 on each of 2 strips from 4 replicate hypocotyls)

2 Cells containing reaction material excluded

Plates 50 & 51 Infection of old hypocotyls





50) Groups of hypocotyl epidermal cells containing primary hyphae (ph) and showing marked granulation and browning. Hypocotyls inoculated when 16 days old. (Kievit(S)/gamma 13 days x 1600)

51) Single infected epidermal cell showing granulation and browning and containing a primary hypha (ph). Hypocotyls inoculated when 16 days old (Kievit(S)/gamma 13 days x 1600)

Plate 52 Infection of old hypocotyls



52) Hypocotyl epidermal cells treated with 1.0M sucrose showing granulation of non-plasmolysed cell (arrowed) containing a hypha(h). Note plasmolysis of fungal protoplast (fp) in unaffected cells (above) and no plasmolysis in granular cell. Hypocotyls inoculated when 16 days old. (Kievit(S)/gamma 7 days x 1600)

only that part of the hypha nearest a non-granular cell plasmolysed. However plasmolysis of hyphae in dark brown cells was occasionally demonstrated (Plate 53). Uninfected cells which were adjacent to granular and brown cells often failed to plasmolyse (Plate 54). The ability of hyphae to plasmolyse in dead host cells indicates that hyphae did not die before the host cell.

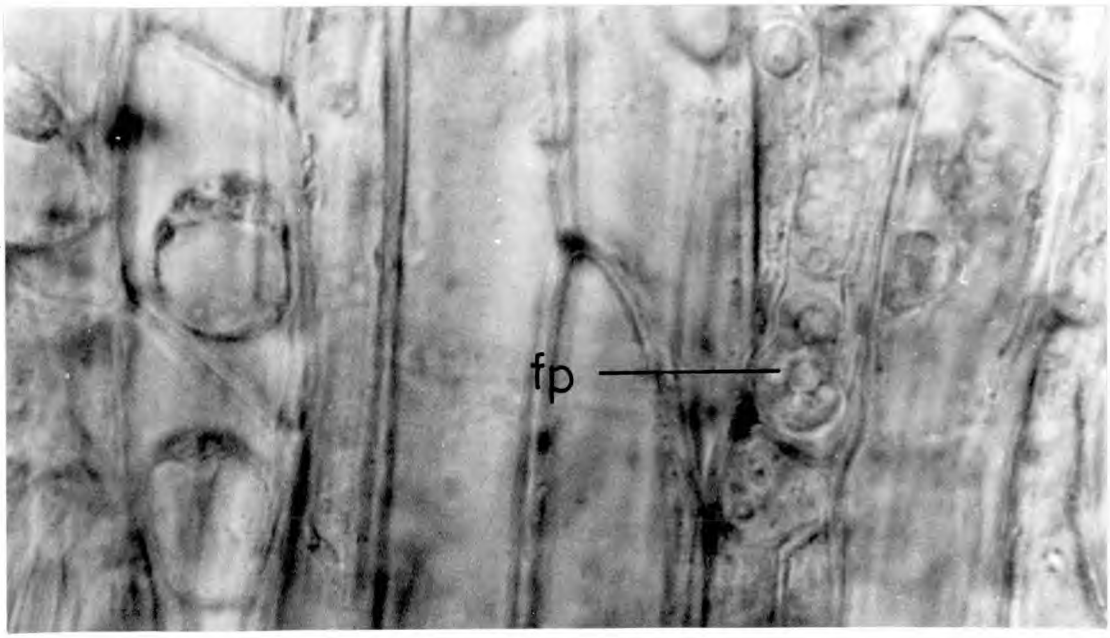
It was concluded that hyphal degeneration did not precede that of the cell and that this latter event did not take place for some time after initial penetration. Effects shown by colourless cells had previously been found in both resistant (grade 5 - Kievit/beta and grade 2 Kievit/delta) and susceptible (Kievit/gamma) reactions but the fact that the onset of such effects was delayed, suggests that necrosis was taking place as in a susceptible reaction in which only limited colonization had taken place. There was no evidence that this necrotic response was or was not associated with a further inhibition of hyphal development.

Conclusion (sub-section 3)

The observed decrease in susceptibility of old hypocotyl tissue, associated with reduced penetration and less extensive intracellular hyphal growth, was probably caused by the increased resistance of cell walls to hyphal penetration. A more active mechanism, associated with the death of infected host cells, may have been involved at the later stages of infection.

Plates 53 & 54 Infection of old hypocotyls





53) Brown granular epidermal cell treated with 1.0M sucrose showing plasmolysis of protoplast (fp) of primary hypha within cell. Hypocotyls inoculated when 16 days old. (Kievit(S)/gamma 13 days x 1600)

54) Infected and non-infected epidermal cells treated with 1.0M sucrose showing plasmolysis of fungal protoplast (fp) but inability of infected and adjacent epidermal cells to plasmolyse. Hypocotyls inoculated when 16 days old. (Kievit(S)/gamma 6 days x 1600)

4. Effect of Etiolation

Growing bean hypocotyls in the dark has been reported as a means of overcoming varietal resistance (Sinden, 1937) and etiolated hypocotyls have been used by Rahe et al(1969) for biochemical studies. Beans of c.v. Immuna, grown in the light and dark were examined for their macroscopic and microscopic response to avirulent and virulent races of C. lindemuthianum.

Seeds were germinated in the normal way for 4 days and then planted in moist Vermiculite. One box was maintained in the dark at 19-26°C and another in daylight (more than 10,000 lumens/sq.ft.) at 16-28°C. Nine days after sowing, the hypocotyls were harvested, the dark grown hypocotyls being 25-50cm and the light grown, ca 5cm long. They were trimmed to 5-7cm lengths, inoculated with races beta and delta and incubated at 17°C in the dark for 5 days. Strips were then examined and the number of cells with visible hyphae, cell reaction or reaction material were determined. The development of lesions was observed at intervals. Results of this experiment are summarized in Table 34.

After 14 days 80% of sites on etiolated hypocotyls inoculated with race delta had developed lesions compared with 20% light grown material. No lesions developed on beta-inoculated (AV) hypocotyls. Greater penetration (race delta -V) and more brown cells (race beta -AV) were observed in etiolated than non-etiolated hypocotyls. Fewer cells of etiolated hypocotyls developed reaction material when inoculated with either race.

Table 34. The effect of etiolation on hyphal development and the response of epidermal cells of bean hypocotyls (c.v. Immuna), 5 days after inoculation with races delta and beta

Grade	Description	% cells assigned* to grades in the following combinations:- Delta (V) Non-etiolated	Delta (V) Etiolated	Beta (AV) Non-etiolated	Beta (AV) Etiolated
1.2, 1.3	Hyphae developing in unaffected cell	33.2	39.8	0	0
5,7,8,9,10	Cells affected - granular and/or brown	0.0	0.0	5.7	11.5
2.1	Reaction material	12.6	1.9	5.0	0.8

* 4 replicate hypocotyls, giving a total of 400 cells, examined for each treatment/combination. Percentages to 1 decimal place.

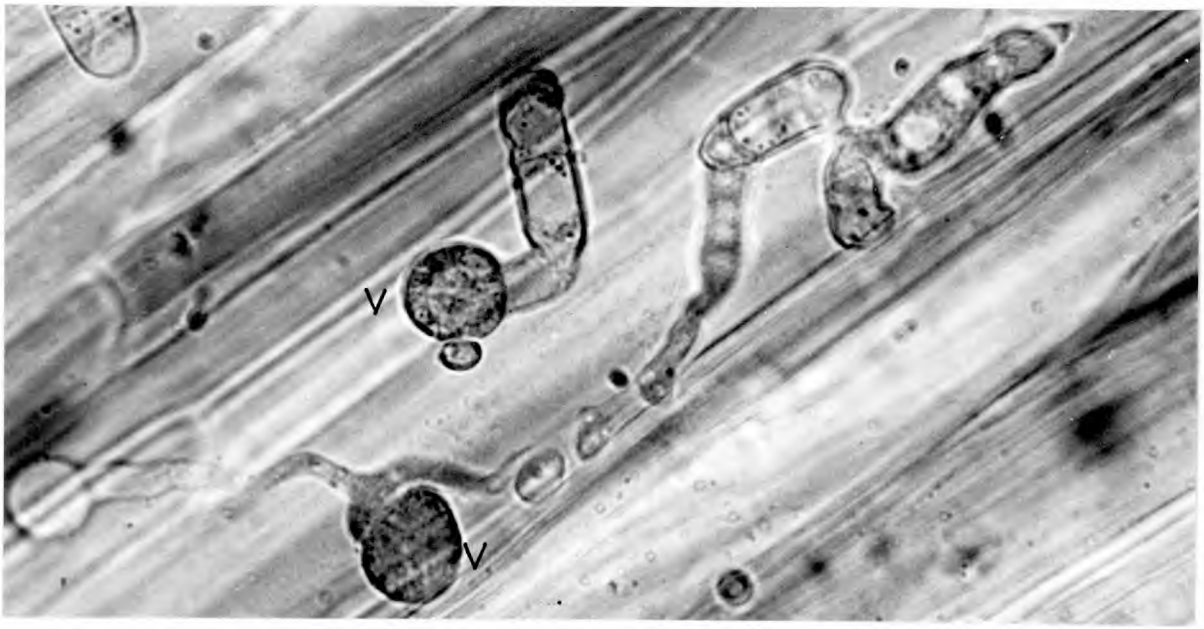
Examination of beta-inoculated (AV) hypocotyls, and those receiving race delta (V) where no lesions had developed, at 14 days, revealed that superficial hyphae were more commonly produced than appressoria on etiolated hypocotyls; swellings were often observed in the germ tubes of race beta (AV) (Plate 55). With both races inoculation drops dried to leave a thin brown film on the hypocotyl surface and cracks appeared as it shrank (Plate 56). Germ tubes were frequently seen embedded in this material and appeared to have made only limited growth. Streaming was observed in cells beneath the brown film.

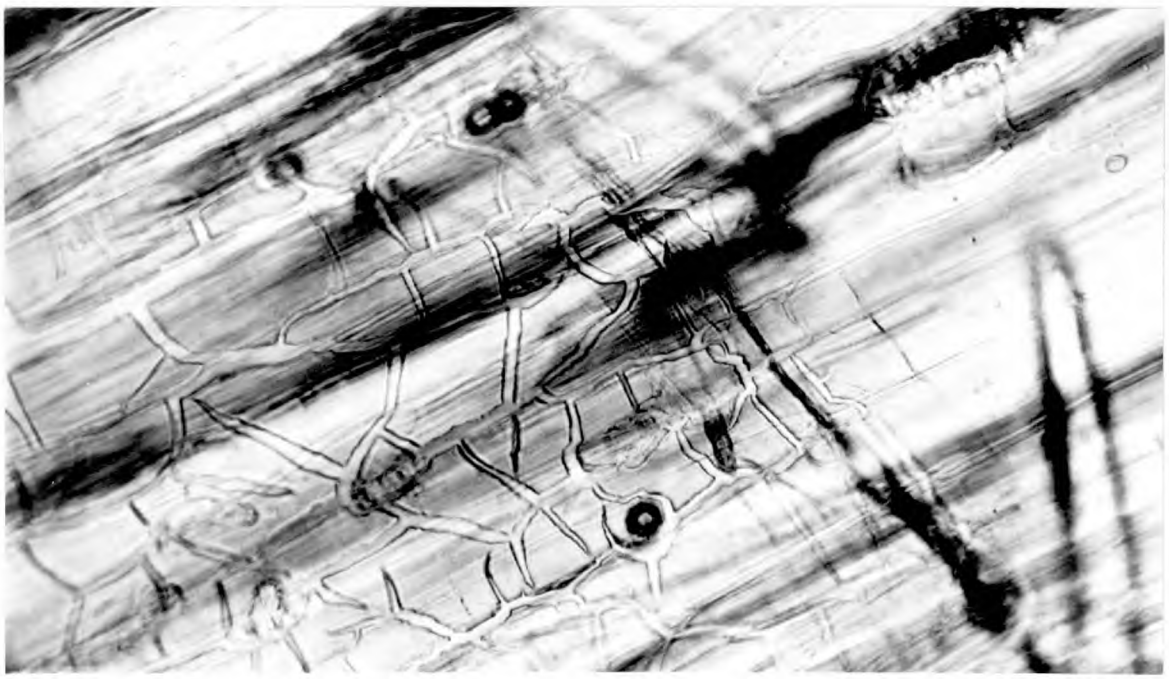
Brown granular cells were seen below, as well as in the epidermis of etiolated hypocotyls inoculated with race beta but the presence of hyphae in these was not investigated. Adjacent uninfected epidermal cells often contained larger particles.

Conclusion

Hypocotyls from dark-grown Immuna plants retained differential resistance to races beta (AV) and delta (V) but were more susceptible to this latter race than those grown in the light. Increased penetration of race delta (V) and cell response to race beta (AV) in etiolated plants was associated with a reduced development of reaction material. As with pods and stripped hypocotyls, substances appeared to diffuse from host cells and form a brown film on the etiolated hypocotyls.

Plates 55 & 56 Effects in inoculation drops on etiolated hypocotyls





55) Spores in inoculation drops showing malformed germ tubes. Note terminal vesicles (v) (Immuna(R)/beta 5 days x 1600)

56) Surface of hypocotyl at edge of area covered by inoculation drop showing the superficial film which has dried and cracked giving a "crazed" appearance. (Immuna(R)/beta 14 days x 630)

5. Effect of position of inoculation site

The following experiment was undertaken to determine whether the position of the inoculation site affected either the number of cells which responded to an avirulent race or the degree to which cellular response had progressed at a given time.

Ten day Kievit hypocotyls were inoculated with race delta and incubated at 17°C. Strips were removed from each of 8 hypocotyls at intervals, one from beneath the first drop (ca. 1.5cm from the position occupied by the cotyledons in untrimmed hypocotyls) and one from beneath the fifth drop (ca.6.5cm from the cotyledons). Cells were scored by means of the grading system and the results are summarized in Table 35.

The pattern of response of cells in strips taken from either position was remarkably similar 4 and 10 days after inoculation. Fewer cells were found to be affected in basal tissue after 3 days but the pattern of response was very similar to that of apical tissue. In a number of strips of this tissue germination was poor.

Conclusion

The position of the inoculation site appeared to have little effect on the proportion of affected cells and the rate of cell response.

Table 35. Cell reactions of tissue at the base and apex of resistant hypocotyls (c.v. Kievit) at intervals after inoculation with race delta

Cell grade	Description	% of cells examined[1] assigned to grades after (days):- 3 Apex	3 Base	4 Apex	4 Base	10 Apex	10 Base
1.2	Cell unaffected - hypha present	1.4	0.8	0.5	0.3	0.0	0.3
2.4,3.2,3.4	Cell affected - larger particles	1.2	0.0	1.4	2.1	0.0	0.3
4.2,4.4	" " - cytoplasm accumulated under appressorium	10.2	7.2	3.0	4.6	1.1	0.5
10.2,10.4	Cell brown - near appressoria	5.6	1.3	11.8	9.0	11.8	9.8
7.2,7.4	" " - all over - very pale brown	2.2	0.4	1.8	2.3	1.9	1.8
8.2,8.4	" " - " - light brown	2.2	0.4	5.6	4.4	9.0	11.0
9.2,9.4	" " - " - brown/dark brown	1.4	0.0	6.3	5.6	16.7	15.3
	Total affected and brown cells %	22.8	9.3	29.9	28.0	40.5	38.7
1.1,2.1,3.1	Reaction material - larger particles	1.9	4.6	6.6	5.4	10.6	18.8
4.1	" " - cytoplasm accumulated	2.8	5.1	3.0	4.4	0.3	0.3
7.1,8.1,9.1,) 10.1)	" " - cell brown	0.6	0.0	1.8	0.3	1.1	1.0
	Total cells with reaction material %	5.3	9.7	11.4	10.1	12.0	20.1
	Other grades	0.0	0.8	0.3	0.0	0.0	0.0

1 50 appressoria examined on each strip taken from the base or apex of 8 hypocotyls. The total number of spores examined was 361, 237, 400, 390, 367 and 400. Where there was poor appressoria production less than 50 were found on a strip; thus less than 400 cells were examined overall.

6. Variation in cellular response between individual epidermal strips

The validity of results obtained using the cell grading system depends largely on the variation in degree of cell response found in different epidermal strips. Table 36 shows the results from the individual strips used in the calculations of values for 3 of the times shown in Table 14. They have been expressed as % affected or penetrated cells to remove the effect of variation in the numbers of infected cells.

It is clear from these results that although there was much between-strip variation in the total number of graded cells, the major cell grades for each of the different times were the same for most hypocotyls. Thus the trends indicating the course of cell response, i.e. from grade 2, 3, 4 to 10 to 7, 8, 9 were reflected in the results for individual strips. Only rarely did a strip (e.g. 82h, 2a) show a pattern of response typical of another time of incubation.

Thus results obtained using the cell grading system indicate the major stages of cell response to be found in individual inoculation sites on different hypocotyls at any one time. Conclusions drawn concerning the course of cell response, as indicated by changes in the proportions of cells in different grades at different times, do not seem to be invalidated by between strip variation.

Conclusion

The cell grading system is a reliable method of examining the course of events following inoculation of bean hypocotyls with C. lindemuthianum.

Table 36. Counts made on individual strips at three of the times given in Table

	% affected or penetrated cells assigned to grades after (h):-																	
	50						66						82					
Cell grades	1a	1b	2a	2b	3a	3b	1a	1b	2a	2b	3a	3b	1a	1b	2a	2b	3a	3b
Cell unaffected - hypha																		
1	6	0	31	29	0	0	0	0	0	8	0	6	0	7	5	0	0	11
Cell affected - colourless																		
2	0	5	15	14	17	13	14	21	0	15	17	11	0	7	0	0	0	0
3	41	0	0	0	0	0	32	8	5	8	6	0	4	0	0	0	0	0
4	23	76	38	36	35	67	14	21	35	8	33	33	35	13	21	42	0	0
5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Cell affected - brown																		
10	0	0	8	14	9	0	27	25	35	31	28	33	19	13	26	17	31	15
7	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	8	3	0
8	0	5	0	0	4	0	5	0	0	0	0	0	12	13	0	17	3	11
9	0	0	0	7	4	0	9	8	10	0	0	17	15	40	5	0	44	41
Reaction material																		
2	18	14	0	0	9	13	0	4	5	23	11	0	4	7	32	17	6	7
Other grades	6	0	8	0	17	7	0	8	10	8	6	0	12	0	11	0	9	7
Total number graded cells on strip	17	21	13	14	23	15	22	24	20	13	18	18	26	15	19	12	32	37

SECTION III Cross protection studies

Section III is concerned with the demonstration of cross protection in different bean tissues, an investigation into the role of fungal products in its initiation and the possible causes of protection.

1. Confirmation of the protection demonstrated by Smith (1967)

Two experiments were carried out using the detached trifoliate leaflet technique of Smith (1967).

Leaflets (4.5-9.0 x 4.0-7.0cm) from the first trifoliate leaves of 39 day old plants (c.v. Kievit) were detached, washed in distilled water and allowed to dry. They were placed adaxial surface downwards on crossed microscope slides in Petri dishes containing sterile water.

The same total numbers of drops were used for the protected and non-protected treatments and the water and protectant controls (see Materials and Methods, 2e). Treatments and controls were arranged on the $\frac{1}{2}$-leaflets of 4 leaves in the form of 4 x 4 latin square design where each leaf constituted a "row". Three complete latin squares were used for each experiment.

Six, 20$\mu\ell$ drops of water or protectant spore suspension (delta 10^6/ml) were applied to $\frac{1}{2}$-leaflets and the leaves incubated at 17°C in the light for 3 days. All drops were then removed and replaced with water. After 1-2h they were replaced with drops of gamma spore suspension (10^6/ml) or water.

Leaflets were reincubated for a further 7 days (Experiment 1) and 11 days (Experiment 2) and then examined with a stereo-microscope

fitted with an eyepiece micrometer. Brown patches more than 0.1mm were counted and the results are summarized in Table 38.

Significant protection was obtained in both experiments and this confirmed the results of Smith (1967).

Detached leaf tissue was not ideal for a further study of the mechanism of cross protection. Leaves were extremely resistant even to a virulent race (only about 20 countable lesions were found in each site where 20,000 spores had been applied) and the resulting necrosis was only just visible with the naked eye. The infected tissue showed heterogeneity in the degree of its response. Quite large areas of necrosis (up to 1mm) developed in the parenchyma associated with the small veins, while only minute areas of the lamina were affected. Other bean tissues were examined in the hope of finding a better model system.

Table 38. Demonstration of cross protection in detached bean leaflets (c.v. Kievit) inoculated with race delta (AV) then race gamma (V)

	Mean No. of necrotic areas (0.1mm) per $\frac{1}{2}$-leaflet.		
Treatment/Control	Experiment 1	Experiment 2	Smith (1967)
Non protected	88.9	112.7	91.8
Protected	50.1*	56.3*	26.4*
Protectant control	1.3	1.0	0.0
Water control	0.0	0.0	0.0

* P less than 5% for difference between means

2. Demonstration of cross protection in different bean tissues

a) Exterior of young pods

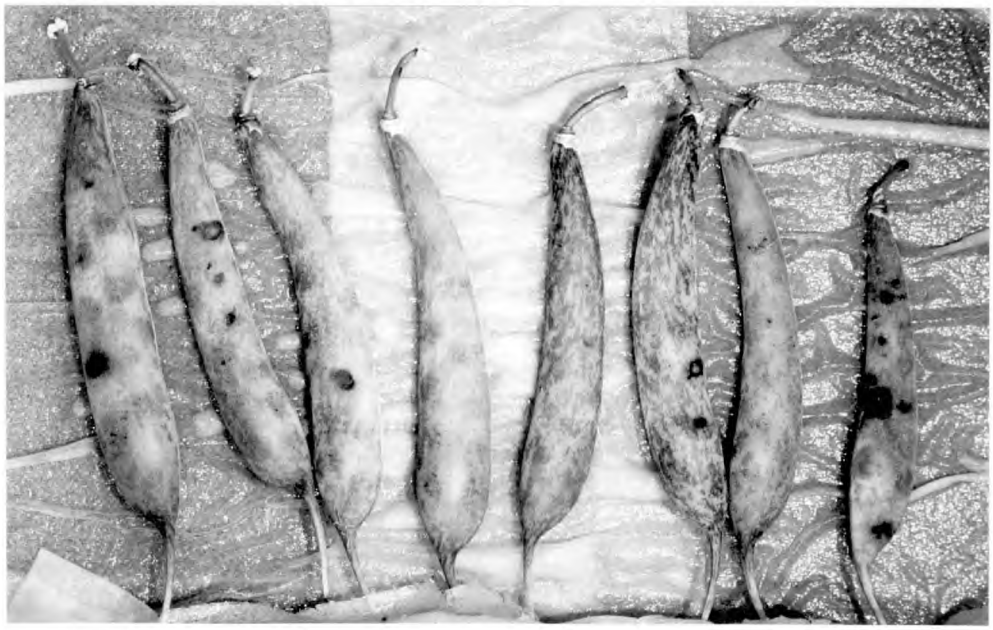
Pods, 5-8cm in length were taken from plants of c.v. Kievit which had been grown in John Innes No. 1 compost in a greenhouse. Pods were selected for uniformity of size and colour and 8 were assigned to each treatment and the protectant control. Three were used for the water control. Races delta (AV) and gamma (V) were used as the protectant and virulent races respectively and these were applied at 10^6 spores/ml, 3 days elapsing between the 2 inoculations. Ten, 10$\mu\ell$ drops of spores or water were applied to each pod on both occasions. After the second inoculation the boxes were re-incubated and examined at intervals for the development of lesions (pale brown sunken areas more than 1mm in diameter).

Lesions were formed from small, superficial light brown spots which increased in size and sank. After 10, 13 and 17 days; 12, 24 and 33 lesions had developed on non-protected and 2, 13 and 20 on protected pods. A maximum of 80 lesions was possible. No lesions developed on either control. Plates 57 and 58 show pods of the 2 treatments 13 days after the second inoculation.

Protection was therefore demonstrated in this tissue and it appeared to be related to a slower rate of lesion development in the protected tissue.

Plates 57 & 58 Protection of bean pods from infection by a virulent race by prior inoculation with an avirulent race





57) Non-protected Young pods (c.v.Kievit) showing dark sunken lesions at sites to which drops containing a virulent race (gamma) of Colletotrichum lindemuthianum had been applied 13 days previously; 3 days before virulent inoculation drops of water were applied at the same site. x 0.75

58) Protected Details as in 57 but tissue inoculated with spores of an avirulent race (delta) 3 days before the virulent race at the same site. Note fewer lesions compared with pods in Plate 57. x 0.75

b) Pod endocarp tissue

Kievit pods, grown like those above and having seeds less than 1cm in length, were trimmed to 2-seed pieces. These were split open and the seeds removed to give 4 cavities, 2 in each half. One half was used for the treatments and the other for the controls. Twenty microlitre drops of water or protectant spores (delta - 5×10^5/ml) were applied to 8 pods. After 24h incubation the inoculated area was washed 3x before applying spores of the virulent race (gamma - 5×10^5) or water to complete the controls and treatments.

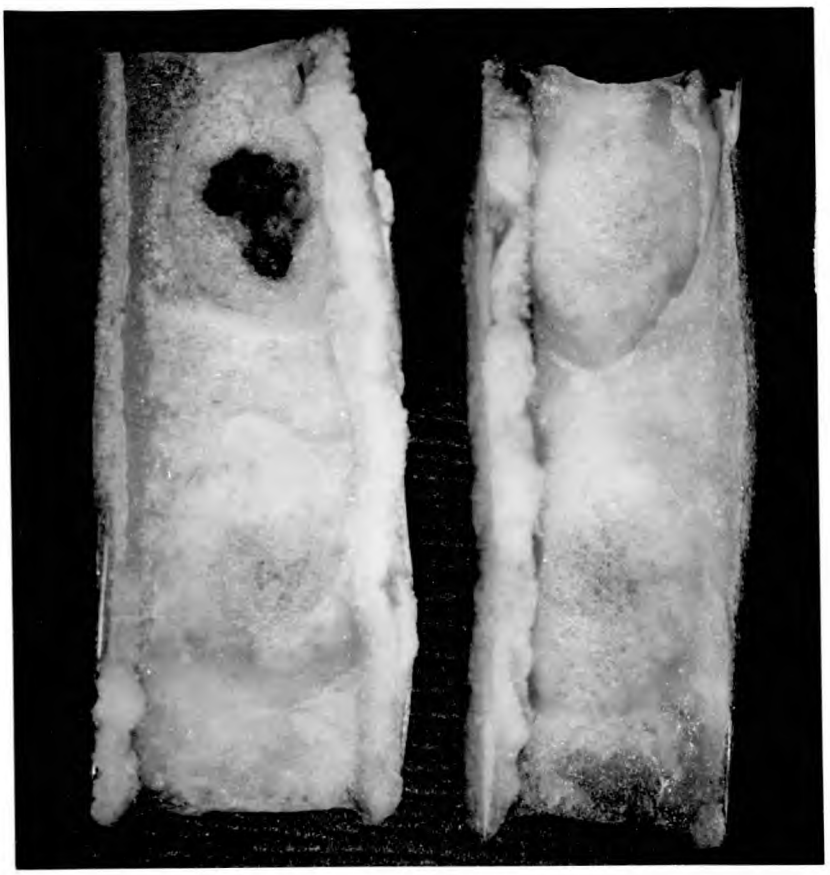
Twelve days later dark brown, non-superficial necrosis was found in all non-protected cavities (8) compared with 3 in the protected. Similar areas of necrosis were also visible in 3 cavities of the protectant control indicating that the pods were only partially resistant to race delta. The type of protection obtained in a pod which showed complete resistance to delta is illustrated by Plate 59.

c) Excised hypocotyls

Drops of water or of spores of race delta (10^6/ml) were applied to Kievit hypocotyls (5 on each) so that the 280 drops (5$\mu\ell$) were used for either treatment and 50 for the controls. After 3 days at 17°C all sites were washed once before inoculating with gamma (5×10^5/ml) or applying water drops. The boxes were examined at intervals after the second inoculation and the number of lesions (see Fig. 13) counted. No lesions developed on control hypocotyls.

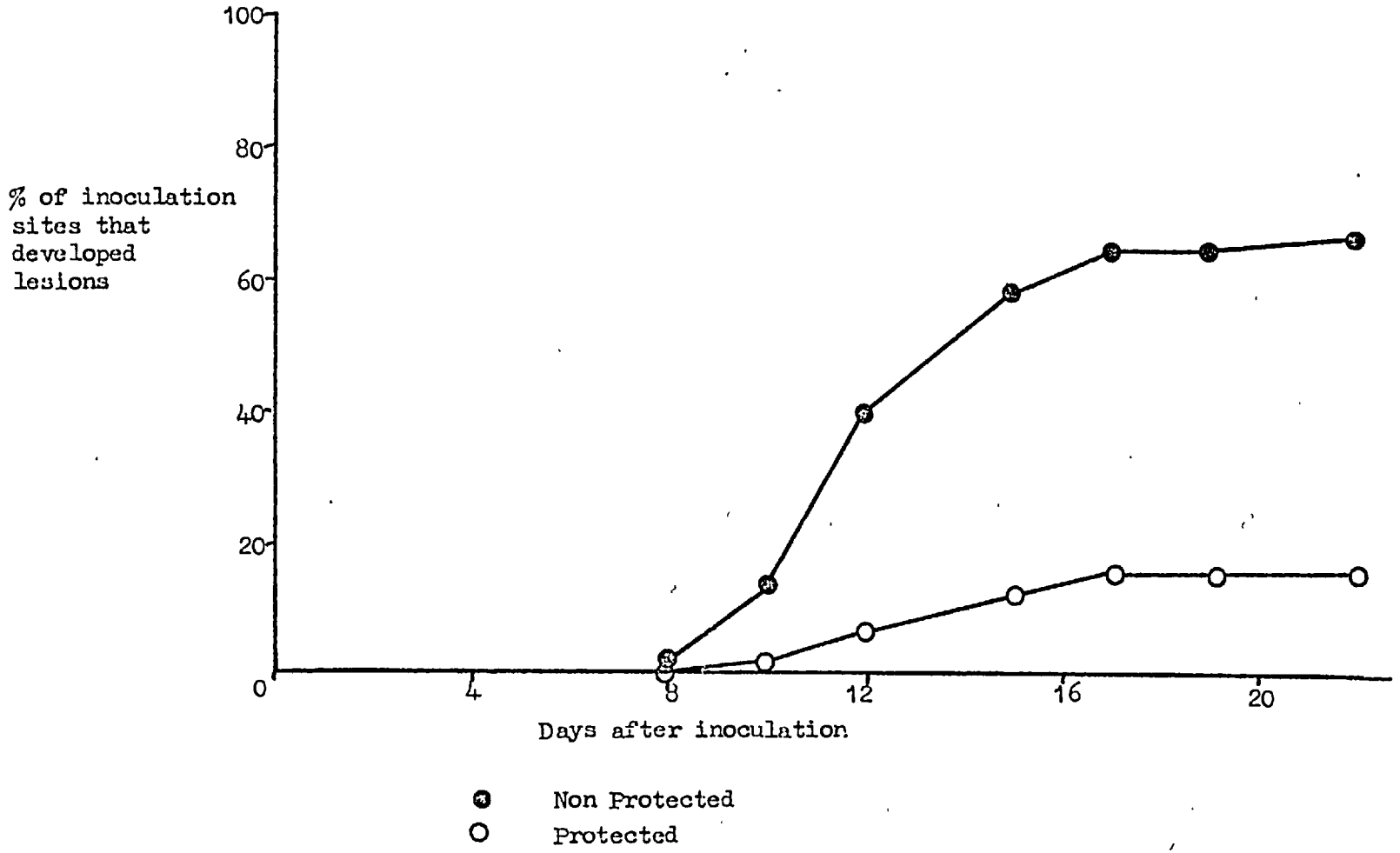
Clear evidence of a reduction in the number and rate of appearance of lesions was obtained. Lesions did not appear until 8-10 days and

Plate 59 Protection of pod endocarp tissue from infection by a virulent race by prior inoculation with an avirulent race



59) Four seed cavities of young bean pods (c.v. Kievit) are shown; the cavities in the top of each half pod received drops of water and those in the bottom received drops containing spores of an avirulent race (delta). One day later all drops were removed and those in the half pod on the left were replaced with drops containing a virulent race (gamma); those on the right received water. Photograph taken 18 days after second inoculation. Note in half pod on left necrosis in cavity (top) where water preceded virulent spores and no necrosis (bottom) in tissue protected with an avirulent race. x 3.5

Fig. 13 Development of lesions on protected and non-protected excised hypocotyls of c.v. Kievit inoculated with race delta (AV) then race gamma (V)



were of the limited type. However it should be noted that at the time of the second inoculation the hypocotyls were 12 days old.

d) Stripped hypocotyls

Drops of water or spores of race beta (AV) were applied to stripped Immuna hypocotyls. In addition to the usual treatments and controls, spores killed by boiling in water for 2 min were also applied in the first inoculation. After 16h the drops were removed and the area beneath washed twice before applying delta spores (2×10^6/ml) or water. The results are summarized in Table 39.

Protection was evident in hypocotyls treated with living spores of an avirulent race but not in those where the protectant spores had been killed by heat.

All the types of tissue tested in the experiments described above became more resistant to a virulent race when they were pre-inoculated with an avirulent race. However, two types of material seemed the most appropriate for use in future experiments, namely stripped and intact excised hypocotyls. These tissues gave clear race differentiation, developed easily countable lesions after inoculation with a virulent race, were easy to prepare and were likely to be homogeneous with regard to age and physiological state. Stripped hypocotyls could readily be used for tests on the effects of fungal products and gave greater lesion development with race delta on c.v. Immuna.

Table 39. Demonstration of cross protection in stripped hypocotyls (c.v. Immuna) inoculated with race beta (AV) then race delta (V)

	% inoculation sites* at which lesions or other necrosis developed							
	Lesions 1mm				Any visible necrosis			
	a	b	c	mean	a	b	c	mean
Non-protected	28	52	38	38	98	98	98	98
Protected	0	0	2	0.7	14	16	24	18
Killed spores	30	42	22	31	90	100	100	97
Water control		0				0		
Heat-killed spore control		0				0		
Protectant control		0				10		

* A total of 50 drops on 10 hypocotyls used for each replicate box. Three replicates were used for the treatments and one for the controls. Results after 7 days.

3. Factors affecting the expression of protection

Some experiments were conducted to determine the optimum conditions of protectant concentration, interval between inoculations and the degree of localization of protectant in order to obtain the best system for studies on the cause of protection.

a) Effect of protectant concentrations - excised hypocotyls

An experiment was carried out in which different avirulent spore concentrations were applied to determine that which gave greatest protection. By varying spore number, the proportion of brown epidermal cells would also be changed. If protection were demonstrated when few epidermal cells were affected this would suggest that the direct physical or chemical barrier formed by the individual brown cells was unlikely to be a cause of cross protection.

Three levels of the protectant, race delta, were used to inoculate detached hypocotyls of c.v. Kievit. These were 500, 2500 and 10,000 spores per 5 $\mu\ell$ drop. The spores of the virulent race (gamma 2500/drop) were applied 3 days later to complete the two treatments (50 hypocotyls each), 10 hypocotyls being used for either control. Extra hypocotyls which had only been inoculated with protectant were examined after 9 days to determine how many cells had shown a resistant response.

The relationship between spore number, brown cells and degree of protection is shown in Table 40. No lesions developed in controls.

At the two highest concentrations the position of the site of protectant inoculation was made visible by the minute flecks on the surface. Many of the lesions on the protected hypocotyls had developed

Table 40. The effect of number of spores of protectant on the number of brown cells and amount of protection obtained in excised hypocotyls (c.v. Kievit) inoculated with race delta then race gamma

Number of spores/drop	% brown cells of those examined[1]	% sites with lesions[2]	% protection[3]
0	0	76.8	-
500	7.2	35.6	53.6
2500	13.3	18.9	75.4
10000	27.0	9.2	88.0

1 A total of 4000 cells were examined (2 strips each from 5 hypocotyls, 400 spores/strip) 9 days after inoculating with protectant

2 15 days after second inoculation

3 $\frac{\text{Control-protected}}{\text{control}} \times 100$

at the edge of, or just outside this area (21 out of 47 at 5 x 10^5/ml and 17 out of 23 at 2 x 10^6/ml). The localization of protection is examined later, Page 224. Taking this into account it is seen that the highest level of protectant gave almost complete protection although only 25% of the epidermal cells were brown. The protection observed with only 7% of epidermal cells brown suggests, as indicated previously, that the direct effect of brown cells is not responsible for protection.

b) Interval between inoculation with protectant and virulent races

(i) Stripped hypocotyls

Two sets of stripped Immuna hypocotyls were used. Race beta (AV - 5 x 10^5/ml) was used as the protectant and race delta (V - 10^6/ml) as the virulent race. At 0h, half of one set was inoculated with virulent alone, the other half with virulent and protectant in a mixed inoculum. With the second set only the protectant or water were applied at time 0, the virulent inoculation being made after 18h. The development of necrosis at the inoculation sites 8 days after the first inoculation is shown in Table 41.

Although the greatest protection was demonstrated when avirulent was applied 18h before virulent spores, some protection was observed with the mixed inoculation. Fewer lesions developed on non-protected at 18h than at 0h suggesting that stripped hypocotyl tissue may naturally become more resistant with ageing.

Table 41. Cross protection on stripped hypocotyls (c.v. Immuna) inoculated with the protectant race(beta) 0 and 18h before inoculation with race delta (V)

	% inoculation sites* at which lesions or other necrosis developed	
	Lesions 1mm	Total necrosis
Water/delta after 18h	45	95
Beta/delta after 18h	1	5
Water and delta at 0h	83	95
Beta and delta at 0h	23	71
Controls	0	0

* Twenty hypocotyls giving a total of 100 drops each were used for each treatment Two hypocotyls were used as water controls and 4 as protectant controls. Results after 8 days.

(ii) Non-stripped hypocotyls - Protection with mixed inoculum

The application of protectant and virulent race in a mixed inoculum was attempted to see whether this type of protection shown above could be confirmed

Kievit hypocotyls were inoculated with race delta (AV) and race gamma (V) either together or separately, the final spore concentrations of these races being 10^5/ml and 5 x 10^5/ml respectively. Duplicate boxes were used for the non-protected and protected (mixed inoculum) treatments and for the protectant control. The drops were removed after 3 days and the appearance of sites examined at intervals. The proportion which were classified in the given categories of necrosis are shown in Table 42.

Flecking, more obvious than that in the protectant controls, appeared in the protected tissue at a time when there was very little necrosis in the non-protected (6 and $6\frac{1}{2}$ days). Compared with hypocotyls receiving only the virulent race, protection was shown by the majority of sites, which developed only small flecks or limited lesions. These non-limited lesions which did form appeared more slowly than in non-protected tissue.

Protection, therefore, was demonstrated when delta (AV) spores, at a fairly low concentration (2 x 10^5/ml) were mixed with gamma (V) spores.

In another experiment a higher protectant concentration (delta - 10^6/ml) and two concentrations of the virulent race (1.5 x 10^6 and 5 x 10^5 spores/ml) were used. This higher virulent concentration for a non-protected treatment was included to check

Table 4-2. The extent of necrosis in Kievit hypocotyls at intervals after inoculation with virulent race (gamma - non protected), avirulent race (delta) and both races as a mixed inoculum at 0h.

Days after inoculation	Treatment	% sites showing the following necrotic response: None visible	Flecking just visible	Obvious flecking up to 1mm	Limited Lesions	Non-limited lesions
5	Virulent alone	100	0	0	0	0
	Mixed inoculum	76	24	0	0	0
	Avirulent alone	76	24	0	0	0
6	Virulent alone	95	1	0	3	1
	Mixed inoculum	35	51	14	0	0
	Avirulent alone	34	66	0	0	0
6½	Virulent alone	85	3	1	1	10
	Mixed inoculum	23	50	27	0	0
	Avirulent alone	41	58	1	0	0
7	Virulent alone	38	0	14	15	33
	Mixed inoculum	13	14	59	14	0
	Avirulent alone	41	58	1	0	0
8	Virulent alone	5	0	8	9	78
	Mixed inoculum	11	5	43	32	8
	Avirulent alone	41	58	1	0	0
10	Virulent alone	0	0	0	1	99
	Mixed inoculum	1	0	38	20	41
	Avirulent alone	38	61	1	0	0

whether inhibition occurred at the spore density used in the protected treatment.

After 9 days, 2% of protected sites had developed lesions compared with 73% and 83% of the non-protected sites inoculated with the lower and higher concentrations respectively. The percentage of sites where lesions had formed in the non-protected tissue had increased to 97 and 100 by 15 days but only to 11 in the protected tissue. Even after 29 days incubation only 20% of protected sites bore lesions. No lesions developed on controls at any time.

Thus a very marked protective effect was demonstrated when a mixed inoculum was used. This system probably provides the most convenient model for studies on protection because of the extent of the effect and because there was no difficulty in applying spores of the two races to the same site.

Different types of experiment where the virulent race was applied first are considered later (5c).

c) Localization of the protective effect

To determine whether protection was localized at the site of protectant inoculation or was effective over a distance two experiments were carried out.

(i) Stripped hypocotyls

Drops of water or spores of race beta (5×10^5/ml) were applied to stripped 9 day old Immuna hypocotyls and their position marked with adjacent small spots of water insoluble ink. At the first

inoculation, 2 groups of 3 boxes were used for both inoculum and water. After 18h incubation, half the inoculated and uninoculated hypocotyls were removed and thin slices of tissue cut away from beneath the inoculation site without removing the marker ink. The other hypocotyls were left untouched. All hypocotyls were then washed in running tap water for 10 min, rinsed in sterile distilled water and allowed to dry. Water or spores of race delta (V - 2×10^6 /ml) were applied to the dry, marked areas to complete the treatments and controls shown in Table 43. The hypocotyls were re-incubated at 17°C and at 6 days the numbers of lesions and sites showing any necrosis were as shown in Table 43.

(ii) Non-stripped hypocotyls

In the second experiment non-stripped hypocotyls of c.v. Kievit were used and the inoculations with the avirulent (delta) and virulent (gamma) races were carried out at the same time. Two sets of 30 hypocotyls were inoculated with gamma spores (5 drops of 5×10^5/ml) at 1cm intervals along the hypocotyl. Drops of delta spores (5 drops of 5×10^5/ml) or water were applied between the gamma drops in one set or the other respectively. A further set was inoculated with drops containing a mixture of gamma and delta spores, both at the above concentration. Ten hypocotyls acted as water or protectant controls.

After 11 days, 0.7% of the sites treated with mixed inoculum had developed lesions, whereas 46% and 67% of those adjacent to drops of protectant or water respectively had developed lesions. The two latter figures increased to 81 and 87% by the 18th day while the

Table 43. The effect of removing tissue just beneath the site of protectant inoculation on the protection by race beta against race delta on c.v. Immuna

	% inoculation sites[1] in which lesions or other necrosis developed	
	Lesions	Visible necrosis
Non-protected - site removed	68	99
Protected - site removed	43	98
Non-protected - untouched	96	100
Protected - untouched	8	33
Controls[2]	0	0

1 A total of 50 drops, on 10 hypocotyls were used for each replicate box half a box was used for the controls and $2\frac{1}{2}$ for each treatment

2 Water control, water control - site removed, protectant control and protectant control - site removed

Results after 6 days

former was only 2%. No lesions were produced on controls.

These experiments indicated that only tissue up to a few cells in depth from the inoculated surface and less than 4 - 6mm from the centres of sites inoculated with protectant (drops of suspension were 3mm dia. on unstripped hypocotyls and placed between drops at 1cm intervals) were protected from infection by a virulent race. Cross protection was therefore very localized.

Conclusion

In the experiments described in sub section 3 the greatest protection was demonstrated when the following conditions were used:-

Intact excised hypocotyls (c.v. Kievit)

Mixed inoculum: avirulent race, 1.5×10^6/ml; virulent race, 5×10^5/ml

Stripped hypocotyls (c.v. Immuna)

Avirulent race at 0h, 5×10^5/ml; virulent race at 18h 2×10^6/ml

Cross protection was very localized.

4. Use of fungal products to initiate protection

The ability of extracts and culture filtrates of C. lindemuthianum to protect bean tissue from attack by a virulent race was studied to determine:-

1 Whether any fungal product caused protection while failing to inhibit the in vitro growth of the pathogen (thus suggesting their role in initiating an active host resistance response)

2 Whether only products of the avirulent race were effective (thus suggesting that race/variety specificity might be due to the differential ability of virulent and avirulent races to produce substances which stimulate a resistant reaction)

3 Whether host tissue was affected in a similar way by fungal products, and during reaction to an avirulent race

4 Some of the properties of these substances.

a) Demonstration of protection.

(i) Choice of medium

Smith (1967) found that after applying a liquid version of medium A (Table 2) to the seed cavities of bean pods, the diffusate became inhibitory in a slide germination test. This medium was therefore not suitable as a culture solution for the attempted detection of fungal components which might protect bean tissue from C. lindemuthianum. It was suggested by Smith that some element of the complex peptone portion of the medium might have been responsible for this effect, and thus the media used here contained acid hydrolysates of

casein (protein almost completely as amino acids) as the nitrogen source. Both germination fluids and the filtrate from mature cultures were used.

(ii) Use of germination fluids

As suggested earlier (Page160) substances diffusing from spores may stimulate the liberation of brown compounds and possibly fungal inhibitors from pod endocarp cells into the spore drop. The ability of spore diffusates to cause protection was tested below.

Portions (20ml/150ml plugged conical flask) of a medium consisting of 0.1% glucose and 0.1% Casamino Acids (Difco, Ltd.) were inoculated with aseptically prepared gamma and delta spore suspensions to give a final spore concentration of 2.5×10^5/ml. After incubation for 24h at 25°C on a platform shaker, the suspensions were passed through a sterile Oxoid membrane and the cell free filtrate obtained was kept at -20°C until use. Germ tubes had been produced by many spores during incubation.

Drops of germination fluid, water, medium or beta (AV) spores were applied to stripped Immuna hypocotyls and were replaced with water or delta (V) spores after 18h. Only those hypocotyls pre-inoculated with beta spores showed any evidence of protection.

Similar results were obtained with diffusates which had been concentrated 10x (reduced pressure at 40°C using a rotary film evaporator). The failure of these fluids to initiate protection could be caused by the destruction of an active principle e.g. in the deep freeze or the evaporator, an adsorption on to glassware,

its presence in ineffective concentrations, as well as its absence from germination fluids. A more concentrated type of medium and a longer incubation time were used below to see if this provided a cell free extract which caused protection.

(iii) Use of culture filtrates and other fungal products

A liquid culture medium (B, Table 2) and stripped hypocotyls were used in the following experiments.

A culture filtrate and mycelial extract of race beta was prepared as described in Materials and Methods. Following the observations of Sinha and Trevidi (1969 - see Review of Literature) that heated spores of Helminthosporium oryzae caused protection in rice plants, a suspension of spores of race beta (AV), which had been heated at 50°C for 10 min and then cooled, was also prepared. These 3 fungal preparations, together with unheated spores, medium and water were applied to stripped Immuna hypocotyls. After 18h at 17°C, the area beneath the drops was washed once before applying water or delta (V) spores (10^6/ml). The results obtained after 8 days re-incubation are shown in Table 44.

Culture filtrate of race beta (AV) gave protection comparable to that given by living spores of the same race. Heated spores and medium gave no protection but some was afforded by the mycelial extract.

(iv) Effect of culture filtrates on spore germination in vitro

Culture filtrates, prepared as described in Materials and Methods were tested for their effect on the germination of spores of races gamma and delta. Many spores germinated to give appressoria especially in the control medium, so germ tube growth could not be

Table 44. Use of different fungal products to obtain protection of stripped Immuna hypocotyls against delta inoculated 18h later

First treatment	% inoculated sites[1] which had developed lesions or other necrosis at 8 days	
	Lesions	Any necrosis
Water	25	71
Beta spores	0	6
Culture filtrate (beta)	0	14
Mycelial extract (beta)	4	90
Heated spores (beta)	41	83
Medium	48	89
Controls[2]	0	0

1 Two boxes of 10 hypocotyls giving a total of 100 drops, used for each treatment
Four hypocotyls used for controls

2 As in treatments but drops of water instead of spores applied in second inoculation

measured. The results are shown in Table 45.

Spores of race gamma germinated poorly, and those of race delta, readily in water. All culture filtrates caused stimulation of germination and when the assay with delta spores was repeated, similar results were obtained. These same filtrates initiated a protective response in hypocotyls when used at times before and after this experiment. It was shown later that the protective response was associated with the inhibition of germination in some cases.

Thus filtrates from cultures grown on the fungal growth medium are useful for the study of the role of fungal products in initiating the host reaction which leads to protection of tissue from attack by a virulent race.

b) Specific action of filtrates of different races

To determine whether initiation of protection was specific to filtrates of avirulent races of C. lindemuthianum, culture filtrates from 2 avirulent races (beta and gamma) and from a virulent race (delta) were applied to stripped Immuna hypocotyls. After incubation for 16h, the sites were washed once before applying spores of race delta (2×10^6/ml). Two days after the second inoculation, strips were taken (3 from each of 3 hypocotyls) from all the delta-inoculated hypocotyls which had previously been treated with water or the 3 filtrates. Germination counts were made on these, 100 spores being examined per strip. The % lesion production in the remaining hypocotyls treated with different filtrates is given in Table 46.

Culture filtrates from all races gave marked protection on

Table 45. Germination of races gamma and delta in growth medium and culture filtrates of three races of C. lindemuthianum

Source of Culture filtrate	Mean* % germination of :- gamma spores	delta spores
Beta	81.2	69.2
Gamma	79.8	85.0
Delta	75.7	75.5
Medium control	71.0	74.3
Water control	13.2	66.3

* of 3 drops, 200 spores examined per drop

Table 46. Protection of stripped hypocotyls (c.v. Immuna), by spores of avirulent races, and culture filtrates of virulent and avirulent races of C. lindemuthianum, against race delta inoculated 16h later.

First treatment	% inoculated sites[1] which had developed lesions or other necrosis after 15 days: Lesions	Any necrosis
Water	39	87
Beta spores (AV)	0	0
Gamma spores (AV)	0	8
Culture filtrate - beta (AV)	6	14
Culture filtrate - gamma (AV)	1	5
Culture filtrate - delta (V)	2	30
Controls[2]	0	0

1 Two boxes of 10 hypocotyls, giving a total of 100 drops, used for each treatment Four hypocotyls used for controls

2 As in treatments but with drops of water instead of spores applied in the second inoculation

stripped hypocotyls as did the spores of the 2 avirulent races. The mean percentage germination of race delta in unprotected tissue and that treated with filtrates of races beta, gamma and delta was 35.8, 0.6, 1.1 and 5.3 respectively. Thus there was a considerable inhibition of spore germination on hypocotyls treated with culture filtrates. Browning was apparent on the surface of these strips and there was no evidence of any marked bacterial contamination. As mentioned above, germination of race delta on glass slides was not inhibited by these culture filtrates.

It was concluded that the ability to initiate a response leading to the protection of stripped hypocotyls was not a specific property of filtrates of avirulent races. Culture filtrates probably acted on stripped hypocotyl tissue by stimulating the production of an inhibitor of spore germination.

c) Similarity between effect of culture filtrate and spores of an avirulent race on host tissue

Although with stripped hypocotyl tissue, some similarities were apparent; drop browning and the absence of much cellular necrosis being typical of the effect of both spores and filtrate; intact hypocotyls seemed to provide a more critical test. As described earlier characteristic visible changes occurred during the response of epidermal cells of an avirulent race and if similar events took place with culture filtrate a role of fungal substances might be suggested. The following observations were made:-

Brown flecking, resembling but being more obvious than that normally found in a resistant reaction, appeared 3-4 days after applying drops of gamma (V) and delta (AV) filtrate to intact Kievit

hypocotyls. Necrosis was found in cells adjacent to small superficial wounds and in groups of cells near the stomata. Thirty-four and 41% of sites inoculated with race gamma (V) 3 days after the gamma and delta filtrates were applied had developed lesions 14 days after inoculation compared with 65% on non-protected tissue. Thus some protection was evident with intact hypocotyls.

As it seemed likely that some wounding was necessary to allow filtrates to act on epidermal cells, abraded hypocotyls were used in later experiments. Protection was demonstrated in abraded Kievit hypocotyls treated with delta (AV) filtrate where 5% of protected and 74% of non-protected sites developed lesions.

Observations on abraded tissue (c.v. Kievit) to which only water drops had been applied indicated that cells around hairs, which had been broken during treatment, and around stomata, were occasionally plasmolysed and granular. No brown cells were seen. Similarly affected colourless cells were seen in medium-treated hypocotyls although a few brown cells were found after 36 and 60h. At these times, cells of unabraded hypocotyls were unaffected apart from some guard cells of delta filtrate treated tissue which showed obvious particles, granulation or apparent disruption of chloroplast membranes. Some cells around small wounds were brown and granular(delta filtrate).

Twelve hours after treatment of abraded hypocotyls with delta culture filtrate, numbers of colourless granular cells were seen, their appearance being similar to those of grade 5 but with no obvious shrunken nucleus. At this time but more commonly after 36 and 60h brown granular cells were also seen. These cells were

usually near stomata (Plate 60) or broken hairs but were occasionally found at other sites (Plate 61) where they resembled cells showing a resistant response. The cell walls of living cells near granular ones were often brown, and diffuse browning was seen in some areas of these cells. This browning was confined to cell walls rather than the hypocotyl surface and the cells often contained obvious particles in active motion. Thickenings of the cell wall (Plate 62) were sometimes seen between wall and plasmalemma. Obvious, actively streaming particles were present in these cells.

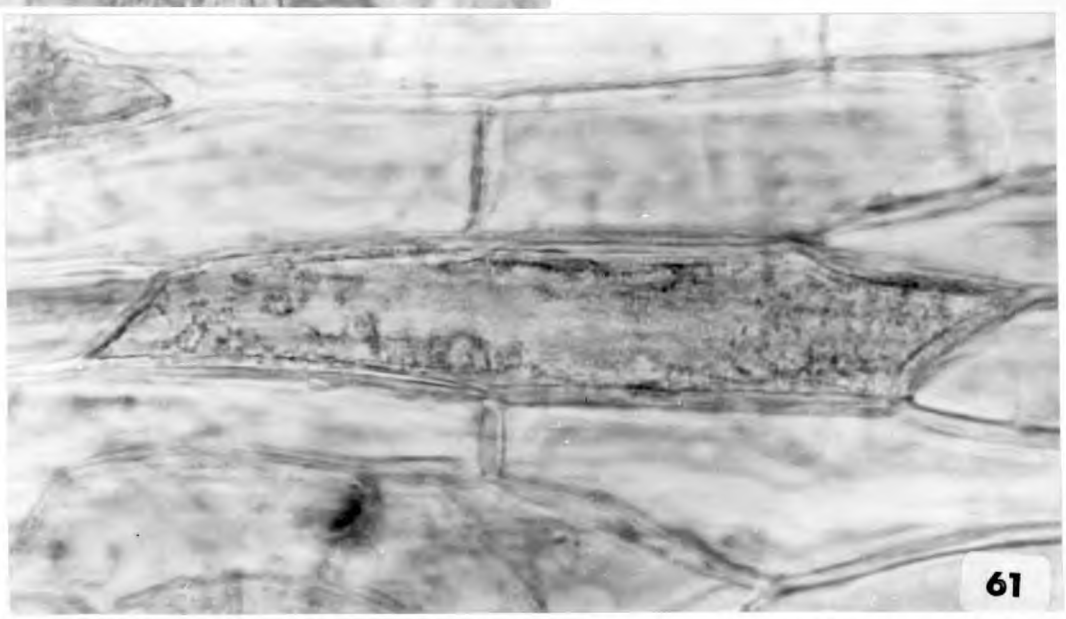
As some damage, as shown by granulation of cytoplasm, was detected in cells of abraded hypocotyls untreated with culture fluid, the role of filtrate in the process of cellular necrosis was not very clear. However the observations indicate that cell browning and possibly the initiation of granulation (more cells appeared affected in treated tissue), were stimulated by culture filtrate. Granular and brown cells were visibly similar to those showing the normal response to an avirulent race while the overall thickening and browning of cell walls was an effect not previously seen.

Thus there was no evidence that cell response was initiated by culture filtrate nor that response was exactly similar to that caused by an avirulent race of the pathogen however the outcome was the same - a brown granular cell.

Plates 60, 61 & 62 Effects of culture filtrate on epidermal cells of abraded hypocotyls







60) Brown granular epidermal cells seen around a stoma in an abraded hypocotyl (c.v. Kievit) 36h after treatment with culture filtrate (race delta-AV) x 1260)

61) Epidermal cell (seen to be brown) showing fine granulation. Details of treatment and magnification as in Plate 60.

62) Deposit of dense material (arrowed) against the wall within an epidermal cell. Details of treatment and magnification as in Plate 60.

d) Use of abraded hypocotyls to test effects of treated culture filtrates

Abraded hypocotyls (c.v. Kievit) were used to find out the effect of autoclaving, ether extraction, dilution and dialysis on the ability of culture filtrates of gamma and delta to cause necrosis and protection.

Ether extractions were carried out by shaking 10ml quantities of filtrate (pH 4.5-6.0) with 3 successive equal volumes of diethyl ether, the two phases being separated after each extraction. The bulked ether phases and the water phase were evaporated to dryness at 40°C and then redissolved in distilled water. Dialysis of 4ml quantities of filtrate against distilled water (2 changes of 500ml) were carried out with 8/32" Visking tubing (Scientific Instrument Centre Ltd., London) for 24h at 4°C. The contents of the dialysis tubing and all other test fluids were stored at -20°C for 24h before being applied to abraded hypocotyls (10/treatment and control). After 3 days the number of treated sites showing browning were recorded and these areas were washed 3 times before applying spores of race gamma (V). The results are summarized in Table 47.

Filtrates from virulent and avirulent races caused browning and protection, thus confirming earlier results. Autoclaving (121°C/15 min), extraction with ether or dilution up to 1:9 did not abolish protective activity or the ability to cause browning. It should be noted that the drop volumes decreased during incubation (some dried completely), thus concentration of substances in the filtrate would probably have occurred. Ether phases were totally inactive as

Table 47. The effect of autoclaving, ether extraction, dilution and dialysis on the protective activity of culture filtrates of races gamma and delta upon c.v. Kievit

Source of filtrate	Treatment given to filtrate	% sites with browning (3 days)	% sites with lesions (10 days)
Gamma	None	86	22
	Autoclaved	54	22
	Ether phase	0	82
	Water phase	60	34
	Diluted x 3	26	14
	Diluted x 9	54	22
	Dialysed 24h	30	20
	Maintained at 4°C, 24h	82	8
Delta	None	98	0
	Autoclaved	78	10
	Ether phase	0	84
	Water phase	96	8
	Diluted x 3	68	10
	Diluted x 9	36	16
	Dialysed 24h	18	76
	Maintained at 4°C, 24h	96	18
Medium	None	40	58
	Dialysed	0	48
	Ether phase	0	84
	Water phase	70	16
Water	None	0	68

protectants although both colour and smell of the filtrate were found in the ether phase.

Both the untreated and water phases of the culture medium caused browning to develop and with the latter few lesions were found. The drops on these hypocotyls were highly contaminated with bacteria. This contamination was not apparent in any other treatment.

With regard to the effect of dialysis relatively few lesions developed with gamma and many with delta filtrate. Browning was apparent with both. When strips were examined, few spores had germinated on dialysed gamma filtrate treated hypocotyls. Spores were brown and embedded in a brown film. A similar effect was observed with untreated and water-phase filtrates. In another experiment with dialysed filtrate, no protection was evident, 14 and 16 lesions out of 20 developing with gamma and delta filtrates respectively. Browning was observed.

It was concluded that the protecting activity of culture filtrates seemed to be caused by water soluble, thermostable substances, which retained their action on dilution. These protectant substances passed through dialysis membranes on 3 occasions out of 4, ability to cause some browning usually being retained. The protecting activity of control media seemed to be associated with quite a different process, possibly involving bacteria.

Conclusion (sub-section 4)

Hypocotyl tissue to which culture filtrates of either virulent or avirulent races had been applied was protected from subsequent attack by a virulent race of C. lindemuthianum. Protecting substances were water soluble, thermostable and probably dialysable. In some tissues the action of culture filtrate appeared to be different from that of spores of an avirulent race.

5. Attempts to find the cause of cross protection in intact hypocotyls

The process of lesion formation by a virulent race was described in Section I. Protection would result from the slowing or halting of development at 1 or more of the 3 main stages of infection :-

Appressorium formation

1 Antagonistic effect of spores of avirulent race:-

a) by producing an inhibitor

b) in causing depletion of nutrients stimulatory to germination

c) by producing substances stimulatory to germ tube, rather than appressorium, formation.

2 Production of a germination inhibitor by host:-

a) before cellular reaction to an avirulent race

b) during cellular reaction

Penetration

Brown granular cells might constitute a barrier to the initial entry and expansion of hyphae.

Intracellular hyphal growth

1 Brown granular cells might constitute a barrier to the colonization of epidermal cells after initial penetration of a non-brown cell

2 Death and browning of cells containing primary hyphae:-

a) caused by avirulent race infecting same cell

b) caused by cells near those affected by an avirulent race becoming sensitive to the virulent race and reacting as if to the avirulent

c) occurring with greater intensity but being caused by factors which lead to cell death in a normal susceptible reaction (cells could also be more sensitive to these factors)

3 Inhibition caused by some process not related to necrosis.

Of these possibilities, two seemed unlikely from preceding studies. It was seen in Section I that, penetration and visible host response to an avirulent race occurred 2 - 3 days after inoculation, the time of maximum germination. Thus protection obtained in a mixed inoculum experiment (Page 222) could not be caused by an inhibitor of virulent spore germination produced at the time of a visible response to the avirulent race. Protection was obtained (Page 218) when few spores of an avirulent race were applied. This protection was not likely to be caused by brown epidermal cells acting as a barrier to penetration because only a small proportion were affected. The other possible causes of cross protection were investigated in experiments described below.

a) Antagonism between races of C. lindemuthianum

It has already been shown that filtrates from 7 day cultures of three races were not inhibitory to germination of spores of this organism. To confirm that there was no specific antagonism of one race towards another the following agar plate experiment was carried out.

Duplicate plates of potato dextrose agar were inoculated with loopfuls of spore suspensions at 2 sites 2 cm apart to give all possible combinations of races. They were incubated at 25°C and after colony diameters had increased to about 2 cm the area of interface between the colonies was examined with a stereomicroscope. In all cases hyphae from either colony grew into the adjacent one without any evidence of inhibition.

Therefore no evidence of self-antagonism could be found in C. lindemuthianum

b) An attempt to detect germination inhibitors in drops of spores incubated on excised hypocotyls

Drops of water or of spores of races gamma (V) and delta (AV) (5×10^5/ml) were applied to 9 day Kievit hypocotyls. Three boxes of 10 hypocotyls with as many drops as possible were used for the collection of each type of diffusate 1, 3 and 5 days after inoculation. Two extra groups, one with delta (AV) spores and the other with water were inoculated to test whether inoculation with race delta actually gave protection in this test. The drops of both groups were replaced with drops of race gamma (V) (5×10^5) after 3 days.

At each of the collection times the drops were removed with a syringe and the bulked fluids from each treatment centrifuged at 5000g to precipitate spores and cell fragments. The supernatant was then decanted and stored at $-20^{\circ}C$. All glassware and the polythene centrifuge tubes were washed by the slide cleaning method before use. The results of germination assays on these diffusates are shown in Table 48.

Most germination led to the formation of appressoria thus germ tube growth was not examined. No evidence of inhibition of germination was obtained. After 13 days, 46% of non-protected sites had developed lesions compared with 8% in the protected.

Therefore although sites inoculated with the avirulent race were protected there was no evidence that an inhibitor was present in inoculation drops either before or after the time that the virulent race was applied. However the action of a host-produced germination inhibitor as a cause of protection was not completely ruled out because an active substance might have been adsorbed on to the cuticle, polythene tubes, glassware or lost during storage of the fluids. High percentage

Table 48. Germination of races gamma (V) and delta (AV) in distilled water and in the supernatant from drops of spores and water incubated on Kievit hypocotyls for different times

Source of diffusate	Mean * % germination of :- gamma spores	delta spores
1 day incubation		
Water drops	74	65
gamma "	72	71
delta "	73	77
3 days incubation		
Water drops	68	77
gamma "	68	56
delta "	72	62
5 days incubation		
Water drops	75	66
gamma "	69	59
delta "	80	60
Water control	69	70

* Mean of 3 drops, 100 spores examined in each

germination of both races was observed suggesting that depletion of germination stimulants was probably not involved. Clearly further in vivo experiments were necessary to give more conclusive evidence of the possible role of a germination inhibitor.

c) Application of avirulent (protectant) race after the virulent race

If protection could be demonstrated in an experiment where spores of the virulent race were applied before those of the avirulent the following information would be obtained:-

1 If many spores of the virulent race had germinated at the time of the second inoculation, inhibition of spore germination would not be the main cause of cross protection.

2 As penetration by a virulent race occurs at about the same time after inoculation as cell response to an avirulent race (Section I), when spores of the virulent race are applied first, one would expect intracellular hyphae of this latter race to be present in the tissue before any cellular response to the avirulent occurred. Therefore if protection of the tissue is associated with the development of this host response the protecting effect would be acting on established hyphae.

3 The effect of the relationship between time of cell response and extent of virulent hyphal growth at that time might give some indication of the role of the host response in the protection of the tissue.

In the following experiment spores of a virulent race (gamma) were applied 2, 1 and 0 days before adding protectant (delta) spores. Infection and cell response were followed to find out if demonstrable protection was related to the relative degree of advancement of

the two processes.

Batches of 80 Kievit hypocotyls (40 each for protected and non-protected treatments were inoculated with 4μl drops of gamma spore suspension (5 x 10^5/ml) at each of the inoculation times shown in Table 49. Other inoculated hypocotyls were used for germination counts and microscopic examinations which were carried out as described previously. The protectant was applied just after the third gamma inoculation. At this time 4μl drops of delta spores (10^6/ml) or water were added to those already present to complete the protected and non-protected treatments. Hypocotyls to which drops of water had been applied at the first inoculation and delta spores at the second were used to determine the course of the resistant reaction.

It was thought that the large drops (8μl) might reduce the oxygen tension in the epidermis sufficiently to reduce the amount of browning which occurred in affected cells. Therefore all drops were removed 3 days after the last inoculation. The results of observations on lesion development are summarized in Table 49 and microscopic studies in Table 50. The times quoted later refer to days after the first inoculation.

Table 49. Inoculation with a virulent race (gamma) at different times before an avirulent race (delta) at the same site on bean hypocotyls (c.v. Kievit) - Development of lesions at inoculation sites

Days pre-inoculation	Treatment	% sites[1] with lesions at (days after cutting):- 0	1	2	3	4	5	6	7	8	10	13
2	Non-protected	G[2]		W[2]				63	97	98	98	98
	Protected	G		D				88	95	98	98	98
1	Non-protected		G	W				0	43	71	98	99
	Protected		G	D				0	19	36	44	53
0	Non-protected			GW					0	4	55	77
	Protected			GD					0	0	13	18

1 Total of 200 possible for each treatment/time

2 Letters indicate drops of: G - gamma spores, D - delta spores and W - water, applied at this time

Table 50. Inoculation with a virulent race (gamma) at different times before an avirulent race (delta) at the same site on bean hypocotyls (c.v. Kievit) - Response of epidermal cells to race delta, intracellular hyphal growth of race gamma and germination of race gamma

Response studied	Days pre-inoculation	Observations	% cells showing response at (days after cutting hypocotyls) 0	1	2	3	4	5	6	7
Resistant reaction[1] (race delta)	2	Unaffected cells			D[2]	0.0	0.5	0.0	1.3	0.5
		Affected - colourless			D	0.0	6.5	4.5	2.4	0.5
		Affected - part or all brown			D	0.0	1.0	5.3	21.9	26.8
Virulent infection[1] (race gamma)	2	In one cell	G			35.3	18.5			
		In second cell	G			2.0	36.0			
	1	In one cell		G		0.3	19.2	3.9		
		In second cell		G		0.0	0.0	14.8		
	0	In one cell			G		1.0	5.6	1.8	
		In second cell			G		0.0	1.1	19.1	
Virulent germination[3] (race gamma)	1	Mean		G	24.4			46.8		
		Range of replicates		(12.2 - 52.9)				(34.2 - 83.2)		

1 Four replicate hypocotyls, giving a total of 400 cells examined at each time

2 Letters indicate drops of: D - delta spores, G - gamma spores applied at this time

3 All spores on 2 strips from each of 4 replicate hypocotyls examined at each time

Avirulent spores added 2 days after virulent

When avirulent spores or water were applied 2 days after inoculation with the virulent race, there was little difference between the numbers of lesions which ultimately developed (Table49). Considerable penetration (Table 50) by the virulent race had taken place before any response to the avirulent race was detectable (day 3) and by the time a high proportion of avirulent-infected cells were brown (day 6) more than 50% of sites on all hypocotyls had lesions. The lesions found in tissue treated with avirulent spores were darker and more abundant after 6 days than in tissue treated with water. However after 8 days all lesions were similar and non-limited.

Avirulent spores added 1 day after virulent

Avirulent spores added 1 day after inoculation caused clear protection (Table 49) At the time when the avirulent spores were applied, spore germination of the virulent race (Table 50) had already reached 24%; three days later percentage germination (on hypocotyls to which no spores of the avirulent race were applied) had only doubled. It was clear that the virulent race had penetrated and grown into a second cell before epidermal cells browned in response to infection by an avirulent race.

On day 6, although no lesions were present, brown flecking which was more obvious than that usually found in a resistant reaction, was apparent at the protected sites (22.4% compared with 0.5% in the non-protected). A microscopic examination of strips taken from sites

treated with water after inoculation showed that no cellular granulation or browning had taken place by day 6. However in strips taken from hypocotyls treated with the avirulent race after inoculation, areas which had been colonized to varying degrees by primary hyphae were often found to be granular and pale brown (Plate 63). Where extensive colonization had taken place sub-epidermal cells showed browning. Not many cells showed the typical response to an avirulent race.

On day 7, strips from sites of protected tissue which had not produced lesions were found with small areas of very dark brown cells containing hyphae (Plate 64). The limited nature of lesions of protected hypocotyls, and the non-limited lesions on non-protected hypocotyls on day 13 are shown in Plates 65 and 66 respectively.

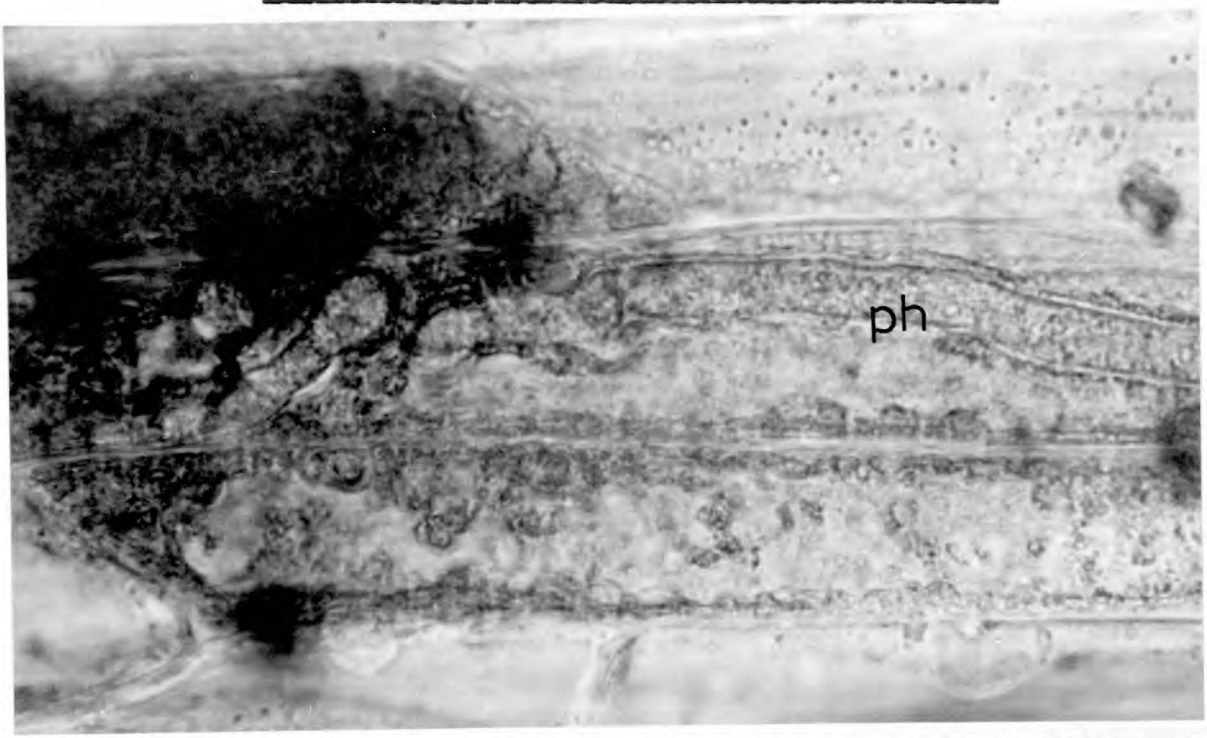
The very few hyphae which were found in tissue infected with the avirulent race alone were usually confined to 1 cell which was not necessarily brown. The flecking observed on this tissue was less pronounced than that in tissue inoculated also with the virulent race a day earlier.

Avirulent spores added at the time of virulent inoculation

A high degree of protection was also evident in hypocotyls receiving avirulent and virulent inoculation at the same time, confirming earlier results. Here, growth to a second cell by the virulent hyphae occurred at about the time of cell browning in response to the avirulent race (6 days).

The following conclusions can be drawn from this experiment:-

Plates 63 & 64 Protection of bean hypocotyls from a virulent race applied 24h before a protecting avirulent race

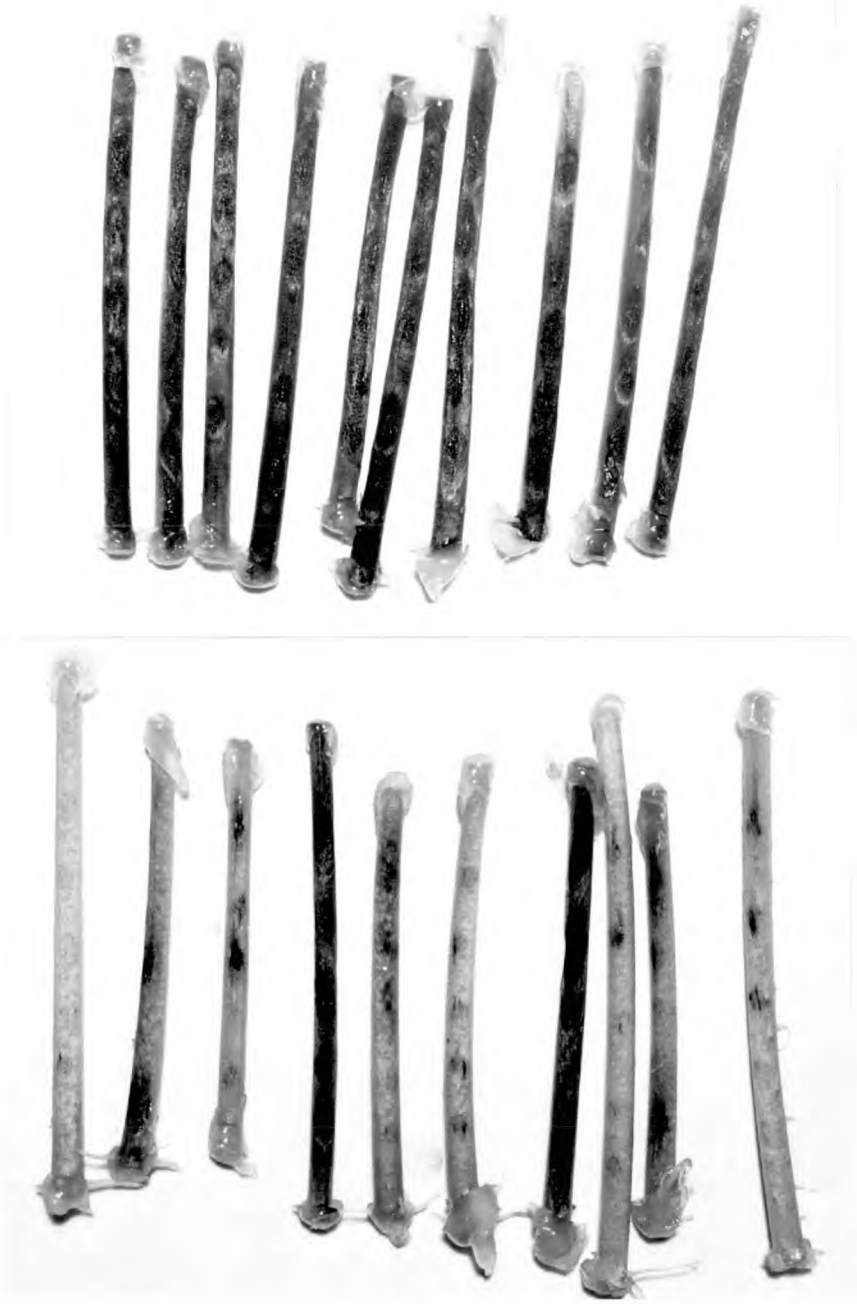




63) Granulation of cytoplasm in epidermal cells of c.v. Kievit hypocotyls containing primary hyphae (ph) of virulent race (gamma) 6 days after inoculation and 5 days after applying spores of race delta (avirulent to the same site) x 1600.

64) Dark brown epidermal cells (c.v. Kievit) containing hyphae of race gamma(V) 7 days after inoculation (6 after applying race delta-AV) x 1250.

Plates 65 & 66 Protection of bean hypocotyls from a virulent race applied 24h before a protecting avirulent race



65) Non-protected Bean hypocotyls (c.v. Kievit) 13 days after inoculation with race gamma(V) and 12 days after applying drops of water to the inoculation sites. Note sunken lesions at these sites and general collapse and darkening of hypocotyl x 1

66) Protected As Plate 65 but spores of race delta(AV) instead of water were applied 1 day after race gamma(V). Note fewer lesions; limited areas of necrosis. x 1

1 Protection can be achieved at a site where virulent spores have germinated before the addition of protectant spores

2 Hyphae of the virulent race were found in tissue showing protection (1 day pre-inoculation) and their growth to a second cell probably occurred before the tissue showed typical browning in response to the avirulent race.

3 Necrosis appears earlier in protected than non-protected tissue (1 day pre-inoculation) or was more obvious at an early stage of lesion development (2 days pre-inoculation)

4 Those lesions which did develop on tissue treated with avirulent spores 1 day after, or at the same time as virulent spores were usually of the limited type.

The overall conclusion is that the protective effect of spores of an avirulent race, applied to tissue previously inoculated with a virulent race, acted on established intracellular hyphae, was associated with an earlier necrosis of infected cells and resulted in limited symptoms of infection. The exact causal relationship between response of cells to an avirulent race and the initiation of this early necrosis of infected cells was not clear and was the subject of later observations.

d) The cause of necrosis in cells of protected tissue containing primary hyphae

As noted above, although symptoms were rarely absent from protected tissue they were reduced, often to small brown flecks and limited lesions. Such symptoms could occur if cells, colonized to a limited extent by primary hyphae, underwent a necrotic reaction during which hyphal growth was inhibited (i.e. as in the resistant reaction to an avirulent race). Three ways in which necrosis could develop in cells containing primary hyphae of the virulent race were outlined on Page 243 (Intracellular hyphal growth, 2). The operation of the first, second and third mechanisms respectively would be suggested if:-

a) more than 1 appressorium were always present

b) (i) a cell responding to an avirulent race were always found near the cell undergoing necrosis

(ii) few unaffected cells containing primary hyphae were found adjacent to those responding to an avirulent race

c) neither of the above situations always applied and death of cells infected with the virulent race did not occur until some time after penetration.

These possibilities were examined in the following experiment. Hypocotyls were pre-inoculated with the virulent race to allow a reasonable development of primary hyphae which could easily be distinguished from the limited growth of the avirulent race.

Inoculation of Kievit hypocotyls was carried out as described in the previous experiment. Drops of water or race gamma (V) were

applied to the freshly prepared hypocotyls; 24h later drops of water or race delta (AV) were added. Four and 5 days after the first inoculation, strips (2 from each of 4 hypocotyls) from tissue receiving treatments; water/delta, gamma/delta and gamma/water were examined. Tissue treated with the avirulent race (delta) alone was examined first to determine the degree of cell response and the maximum length of hyphae in any cell of the strips. Then cells of tissue receiving both races which contained hyphae of a greater length were examined for the presence of; any visible effect, a second appressorium and an adjacent affected cell. Other hypocotyls (30/treatment and 5 for the avirulent and water controls) were inoculated at the same time and observed at intervals for the development of symptoms.

Four days after the first inoculation, 82% of affected cells in tissue treated only with the avirulent race showed browning in part or all of their cytoplasm. Very few cells containing hyphae were unaffected and the maximum length of hyphae of the avirulent race was 22 μm. In strips from tissue receiving both races, 66 cells contained hyphae greater than this length and were presumed to be race gamma (V). Fifty-seven were apparently unaffected, 3 being adjacent to brown cells. Two affected cells neither had a second appressorium nor were adjacent to affected cells, 4 had both properties and 3 had only a second appressorium attached.

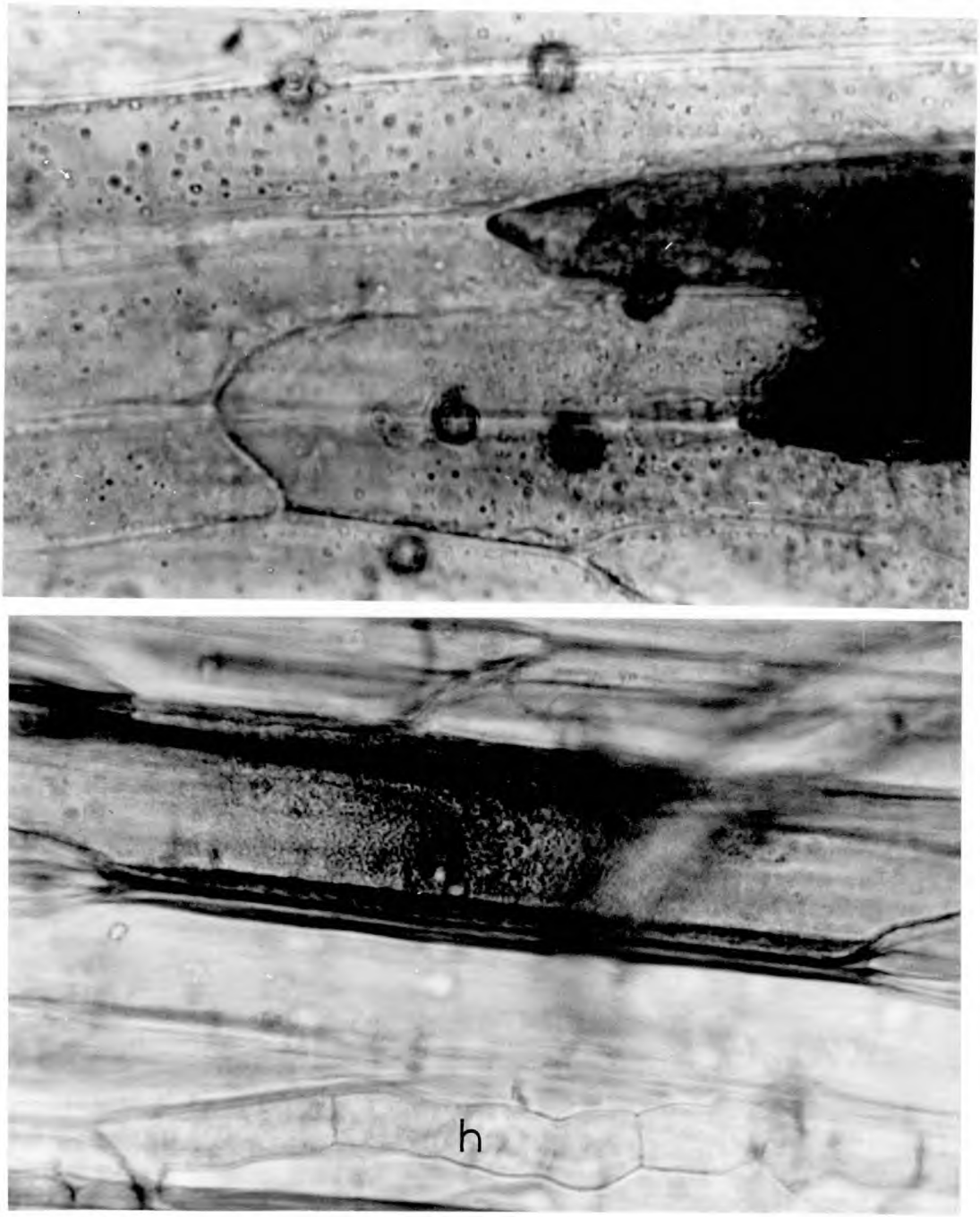
After 5 days, some cells on 2 strips taken from tissue inoculated with only the virulent race showed signs of granulation, but no lesions had yet appeared. More necrosis of hypha-containing cells was evident

in tissue treated with both races. Thirty-five out of 66 cells containing hyphae of greater length than the maximum avirulent hypha (101μm), however were unaffected. Colonization of 3 or more unaffected or affected epidermal cells was common but in a strip where a larger proportion of cells than normal appeared to have responded to the avirulent race, no long hyphae were found. On strips other than this, necrotic cells containing primary hyphae were found with other appressoria, adjacent to brown cells and having both or neither of these properties. In the 2 strips where most of such necrotic cells were found, there were large areas of epidermal cells like those shown in Plate 67 where marked cellular disentegration nad taken place. However the situation shown in Plate 68 was also common, with hyphae present in unaffected cells adjacent to those showing browning and granulation.

Six days after the first inoculation pronounced flecking was visible in 52 and 18% of sites which had not developed lesions, receiving gamma(V)/delta(AV) (protected) and gamma/water (non-protected) respectively.

Therefore at the time when the visible cellular response to the avirulent race was almost complete (4 days), the majority of cells containing virulent-race hyphae, in tissue receiving both races, were apparently unaffected. Necrosis occurred a little earlier than in tissue inoculated with only the virulent race and often no second appressorium or an adjacent brown/granular cell was present. Thus the first two possibilities on Page 243 seemed unlikely and the third more probable, i.e. that the response of some cells to the avirulent

Plates 67 & 68 Observations on hypocotyl tissue showing cross protection



67) Epidermal cells of c.v. Kievit hypocotyls, 5 days after inoculation with race gamma(V) and 4 days after spores of race delta(AV) had been applied to the same site, showing numerous particles or vesicles in uninfected cells. No motion was visible. Note brown cells typical of response to avirulent race (on right) x 1275

68) Two epidermal cells of c.v. Kievit hypocotyls 5 days after inoculation with race gamma(V) and 4 days after spores of race delta had been applied to the same site. One shows granulation and darkening typical of a response to an avirulent race the other appears unaffected and contains a hypha(h)(virulent) x 1600

race caused other cells, which were not visibly affected, to undergo an earlier and more intense necrotic reaction than that which normally occurs in the susceptible reaction.

Confirmation that the disorganization of cells occurs slightly earlier in cells inoculated with both virulent and avirulent races was obtained in another experiment. Race gamma(V) was applied to Kievit hypocotyls, 16h before race delta(AV). In the virulent control hypocotyls at 154h after inoculation, much of the epidermis beneath the drops was colonized but only a few cells on one strip were necrotic. At this time, in strips from tissue to which race delta(AV) had also been applied, many of the colonized cells were disrupted and some were pale brown. In this experiment protection was demonstrated, 39 and 93% of sites forming lesions on protected and non-protected hypocotyls respectively.

In several experiments, strips from tissue inoculated with both virulent and avirulent races often contained areas of cells with numerous large motionless particles and vacuoles (Plate 67). This effect was first seen just before flecking appeared and before any cells of the virulent control hypocotyls were noticeably affected. Such cells were also seen, although confined to much smaller areas, in avirulent control tissue especially when large numbers of brown cells were present. It was possible that this disorganization of uninfected cells may have been caused by the changes which led to the early necrosis of cells in protected tissue containing primary hyphae described above.

e) Hyphal growth in tissue showing marked protection

No direct evidence was available in experiments which did not specifically avoid the effects on spore germination and penetration, that protection was caused by inhibition of the growth of established hyphae. Long hyphae, clearly distinguishable from those of the avirulent race, were rarely seen in protected tissue where there was much granulation and browning in response to the avirulent race. To determine whether, in tissue showing marked avirulent response, intracellular hyphae were present and had grown to adjacent cells, the following experiment was carried out.

Spores of races gamma (V-5 x 10^5/ml) and delta (AV-10^6/ml) were applied separately or in mixed inoculum to Kievit hypocotyls. Six days later, protection was indicated by the fact that 32 and 0.6% (from 35 hypocotyls each) of sites bore lesions on non-protected and protected hypocotyls respectively. Strips were removed, mounted in 0.1N NaOH and examined for the presence of hypha: a) from an appressorium (i.e. = penetration) b) from an adjacent cell (i.e. = growth). The results are shown in Table 51.

In this experiment, where very marked protection was achieved, the proportion of cells which had been penetrated from appressoria was somewhat less after treatment with mixed inocula than with the virulent alone. However, protection seemed to have been mainly caused by prevention of hyphal growth from the cells penetrated first.

Table 51. Penetration, and growth to a second cell, of hyphae in Kievit hypocotyls 6 days after inoculation with races gamma(V) and delta(AV) separately or as a mixed inoculum

Effect studied	Inoculum	% cells examined[1] containing hyphae	
		Mean	Range[2]
Penetration	gamma	8.2	2.1 - 13.5
	gamma + delta	5.2	1.5 - 11.0
	delta	0.5	0.0 - 2.5
Growth to second cell	gamma	34.6	2.5 - 83.0
	gamma + delta	1.2	0.0 - 3.0
	delta	0.0	

NOTES
1 Two strips from each of 8 replicate hypocotyls scanned, 200 cells being examined on each for hypha derived from appressoria or an adjacent cell
2 Highest and lowest values for individual replicates

Conclusion (Sub-section 5)

Inhibition of appressorium formation and penetration were eliminated as major causes of cross protection in a number of experiments; hyphae presumed to be of the virulent race were found in tissue in which protection later became evident. Cells of protected tissue containing such primary hyphae degenerated and became brown sooner than those in non-protected tissue. Assuming that hyphal growth was inhibited in dead brown cells (as in cells which showed a normal resistant reaction to an avirulent race) then, because this reaction occurred earlier when relatively few cells were colonized, reduced symptoms of infection would have developed. Limited necrotic areas were typical of tissue showing protection. The exact cause of this early necrosis was unknown but in cells of protected tissue, fairly extensively colonized by primary hyphae, it occurred after the completion of response to the avirulent race and just before necrosis on non-protected hypocotyls. This suggested that cells near those which had responded to the avirulent race were more sensitive to the damaging effects of the virulent race.

Section IV Attempts to induce susceptibility to an avirulent race

Various treatments were used to determine whether lesions might develop in tissue which normally gave a resistant reaction to the race applied.

1. Volatile compounds

In a preliminary experiment, stripped and unstripped Immuna hypocotyls were treated for 1h with amounts of diethyl ether and chloroform varying from 0.02 to 2.5ml per bottle. Spores of an avirulent race (beta) were then applied. The only treatment which appeared to cause any increased susceptibility to race beta was that of 0.1ml of chloroform. After this treatment lesions resembling those caused by a virulent race were seen on stripped hypocotyls 8 days after inoculation (Plate 69). With greater amounts of chloroform and the highest ether concentration, hypocotyls collapsed before inoculation.

In 1 of 2 similar experiments using chloroform and stripped hypocotyls, lesions failed to develop on treated, inoculated hypocotyls but in the other experiment all sites on 10 hypocotyls bore lesions after treatment with 0.1ml chloroform for 1h. This effective chloroform concentration caused a slight darkening of the cut surface of uninoculated hypocotyls which became bronze later. Cells appeared unaffected just after treatment and contained intact

Plate 69 Effect of chloroform treatment on resistance of hypocotyls to an avirulent race



69) Stripped hypocotyls (c.v. Immuna) 8 days after treatment with chloroform vapour and subsequent inoculation with race beta(AV). No necrosis was evident in hypocotyls treated with 0.02ml chloroform/bottle; a brown film formed at the inoculation site. Some superficial necrosis can be seen in hypocotyls treated with 0.1 ml/bottle. ca. x 1

chloroplasts and vacuolar membrane. Cells in hypocotyls which had collapsed after being treated with greater chloroform concentrations were clearly disrupted. Nine days after treatment with 0.1ml chloroform for 1h most cells, of the 2 strips of uninoculated tissue examined, plasmolysed in molar sucrose. Thus there was no evidence that cells of tissue which became susceptible to the avirulent race beta were badly damaged after treatment with an effective concentration of chloroform.

Another experiment was carried out to determine whether hypocotyls on which lesions formed when an avirulent race was applied soon after treatment, recovered their ability to resist this race on further incubation. Such a recovery would suggest host cells at the inoculation site were not killed by the treatment. Chloroform treatments of 0.0, 0.075, 0.10, 0.125 and 0.15 ml per bottle for 1h were used; drops of water or beta spore suspension (50/treatment) were applied immediately or after 24h at 17°C. Lesions only developed on hypocotyls treated with 0.10ml, those receiving higher amounts were flacid before, and rotted after, inoculation. No lesions were found on the hypocotyls when inoculation was delayed for 24h after treatment and the sites were pale and superficially brown. Most sites where immediate inoculation was used were darker brown and necrosis which could be classified as a lesion was found in 6 out of 50. As few lesions developed it was difficult to say whether recovery of resistance had been shown especially as the 24h delay between inoculations meant that 2 different cultures had to be used in the preparation of spore suspension (both were at 5×10^5 spores/ml)

In these experiments the amount of chloroform used was critical and total hypocotyl collapse occurred with only small increases in concentration. This system seems unreliable for use in studies on the mechanism of resistance.

2. Hot water treatment

a) Pre-inoculation treatment

(i) Effect on infection by an avirulent race

Unstripped Immuna hypocotyls (10/treatment) were immersed in sterile water at 50°C for 0, 15, 45, 90, 150 and 300 seconds and then inoculated with race beta (AV). The results are shown in Table 52.

Many hypocotyls receiving the longer treatments (90-300s) appeared flacid soon after being removed from the water bath which suggested that heating had caused severe damage. Often, inoculated hypocotyls which had been treated for 90s or more had collapsed, rotted and showed a white bacterial ooze, 4 days after treatment. A few lesions formed on non-rotted hypocotyls or parts of hypocotyls with treatments of 90 and 150s. Treatment for 45s did not cause any rotting but only 2 lesions developed; some sites showed more obvious flecking than untreated controls.

Six days after inoculation, hyphae were found in lesions which had been cleared with 0.1N NaOH. However many of the larger flecks did not contain hyphae but were caused by browning in, and in cells around, small wounds where spores and superficial hyphae had accumulated. Thus some of the necrosis could not be directly related to an increase in hyphal growth within the tissue. To determine whether cells of tissue in which lesions were formed had been killed by the treatment, strips adjacent to lesions were removed, immersed in 0.5M sucrose and examined for plasmolysis. Most cells plasmolysed when

Table 52. Development of necrosis in heat-treated resistant hypocotyls 4 days after inoculation with race beta

Seconds at 50°C	No reaction	Flecking	Obvious Flecking*	Lesions	Rot
0	84	13	3	0	0
15	77	21	2	0	0
45	65	18	15	2	0
90	16	11	6	5	62
150	5	11	5	4	75
300	0	0	0	0	100

* More than was normally visible in a resistant response

the strips were taken from hypocotyls which showed no rot.

The role of the avirulent race in causing necrosis was examined in more detail in another experiment with the same race and variety. Heat was applied for 0, 30, 90 and 120s before inoculation; observations were made after 4 days. Strips from the 2 former treatments showed similar effects:- spore germination resulted in appressorium formation and occasionally thick (equal to spore width) germ tubes. No cells containing hyphae were unaffected. Browning was found in cells showing a typical resistant response, and in the cytoplasm and walls of cells around minute wounds. Streaming was visible in other cells and in cells of uninoculated tissue. No browning was seen round wounds. Thus these effects were typical of a normal resistant reaction to the avirulent race (Section I).

When strips from uninoculated 90 and 120s treated tissue were mounted in distilled water, most cells showed streaming, but colourless, granular plasmolysed cells were seen often in groups round stomata and hairs (Plate 70). Cortical cells did not appear to be affected. Most spores on the inoculated tissue had produced thickened germ tubes (Plate 71) not appressoria. Some hyphae derived from appressoria were found in unaffected cells. Granular cells often near stomata and hairs were usually brown and associated with superficial hyphae rather than appressoria. The region of browning round wounds was more extensive than in untreated.

Thus necrosis would seem to have arisen in three ways: from spore induced browning of heat damaged cells, from spore induced

Plates 70 & 71 Effect of heat treatment before inoculation of hypocotyls with an avirulent race



70) Colourless, granular, plasmolysed epidermal cells seen around a stoma in an uninoculated hypocotyl (c.v. Immuna) 4 days after hot water treatment (50°C for 90s) x 1260

71) Superficial growth of wide diameter germ tubes (race beta) in inoculation drops on c.v. Immuna hypocotyls 4 days after hot water treatment (50°C for 90s) and inoculation. Note darkening of granular epidermal cells. x 1260

browning around minute wounds and from enhanced hyphal growth in the tissue.

(ii) Effect on infection by a virulent race

In an experiment where Immuna hypocotyls were treated for 0, 15, 30, 60 and 240s at 50°C before inoculating with race delta more lesions developed on hypocotyls treated for 30 seconds than those receiving 15 or 0 seconds (47, 35 and 32% of the 100 inoculated sites respectively). Rotting was pronounced when longer treatments were given. Although poor spore germination made counts difficult, greater penetration (36%) was observed in the former than the latter (23%) and less reaction material was produced (0.3% of 6.5%).

b) Heat treatment after infection with an avirulent race

Post-infection heat treatment might have 2 effects: a) to affect the host's resistance sufficiently to allow hyphae to grow from brown cells, b) to cause necrosis preferentially in cells adjacent to those which had become brown in response to the avirulent race as was noticed in cells adjacent to necrotic TMV lesions by Ross and Israel (1970 - see Review of Literature).

Kievit hypocotyls were inoculated with race delta (AV - 10^6/ml) and incubated for 4 days by which time most cells affected by the avirulent race were brown. The Vaseline seal was then cut from the hypocotyls which were immersed (10/treatment) in water at 50°C for 0, 15, 30 45 and 60 s. After resealing, the hypocotyls were

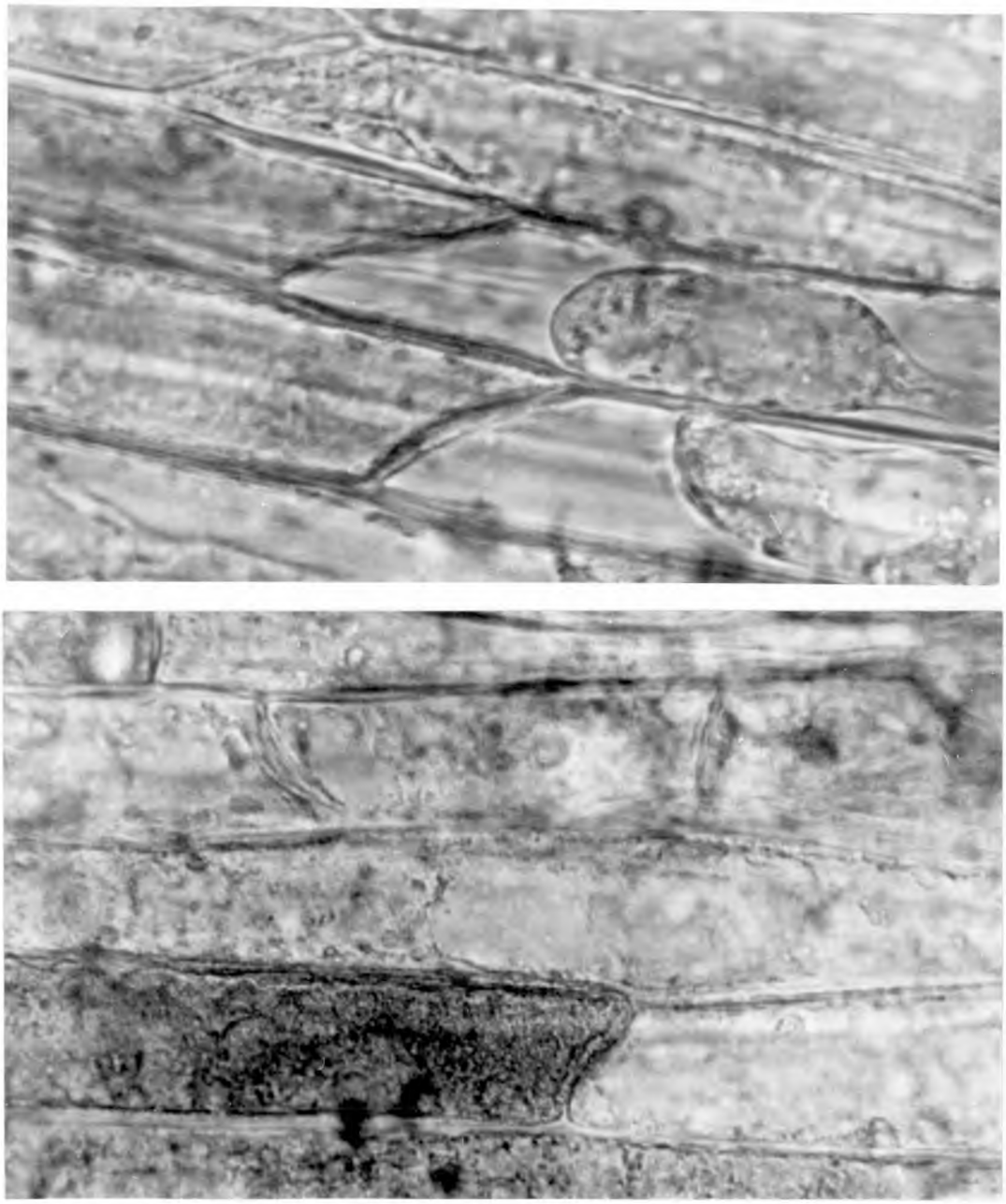
re-incubated for 24h. Strips (8/treatment) were removed from the inoculation sites and on each, 200 epidermal cells were examined. The percentages of cells which were dead and brown in the following treatments were: unheated (dipped in water at room temperature) 12.1%, heated 15s 34.6% heated 30s 97%, heated 45s 91.5%, heated 60s 40%.

Other strips were examined after 48h and at this time necrosis was even more marked. Inoculation sites from 30 and 45s treated hypocotyls were slightly sunken and water-soaked.

Cells outside the inoculated area (i.e. beyond the area containing brown cells) appeared to be completely unaffected. Cytoplasmic streaming was clearly visible in these cells and in those between the degenerating cells of 15 and 60s treated sites. The affected, inoculated and the unaffected, uninoculated areas were sharply delimited (Plate 72). The main effect noticed was the presence of numerous large particles or vacuoles in cells (Plate 73). Plate 74 shows cells of unheated tissue.

As no hyphae were found in these affected cells increased hyphal growth would not have been responsible. It was possible therefore that cells in the inoculated region were more sensitive to heat or that heating caused the movement of toxic products from the brown to adjacent cells.

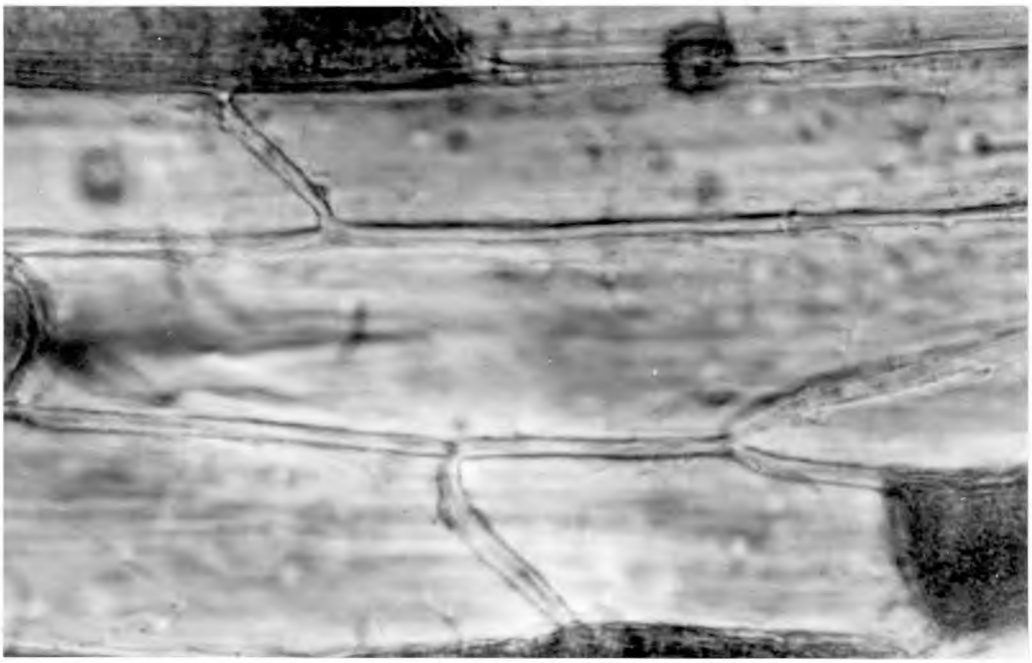
Plates 72 & 73 Effect of heat treatment on hypocotyls showing response to an avirulent race



72) Epidermal cells at edge of inoculated region 48h after heating hypocotyl at 50°C for 60s. Note granulation in cytoplasm (cells on ~~right~~ left) and unaffected cells as in uninoculated tissue (~~left~~ right) which plasmolysed in 0.5M sucrose. Hypocotyls (c.v. Kievit) treated 4 days after inoculation (race delta) x 1260.

73) As 72 but treatment 50°C for 45s; cells of inoculated region. Note marked granulation of cytoplasm of colourless cells as well as those showing typical response to an avirulent race (below) x 1260

Plate 74 Effect of heat treatment on hypocotyls showing response to an avirulent race (contd.)



74) As in Plate 72 but no heat treatment given, cells are of inoculated region and appear to be unaffected x 1260

3. Separation of epidermis from underlying cells

Tomiyama (1955) noticed that when strips of potato leaf midrib epidermis, in which a resistant response to an avirulent race of P. infestans was developing, were removed to the surface of a piece of agar and then incubated, the browning process was retarded compared with that of intact tissue. This effect was thought to be caused by the removal of a source of compounds involved in the browning reaction (i.e. which were produced in adjacent and then transported to the infected cells). The possible role of wounding was also acknowledged. Observations mentioned earlier (Page113) suggested that a similar effect might be operating in the C. lindemuthianum/bean interaction. If both a decrease in the intensity or speed of cell response and, an increase in hyphal growth occurred, then this would provide further evidence of a causal relationship between cell response and the inhibition of hyphal growth. The role of adjacent cells in a resistant response might also be suggested.

Hypocotyls of c.v. Immuna were inoculated with spores of race beta (AV) and incubated for 46h. To determine the stage of cell reaction at the onset of the experiment, strips from each of 3 replicate hypocotyls were examined. Others were washed in 4 changes of sterile distilled water before placing, cut surface downwards on 1% Ionagar No. 2 (Oxoid Ltd.) in sterile Petri dishes. Tissue from between the inoculation sites was removed at the same time and similarly treated. The Petri dishes and the remaining inoculated

hypocotyls were re-incubated for a further 42h after which 6 strips from each were examined for cell response and hyphal growth. Strips of the uninoculated tissue were tested for plasmolysis and deplasmolysis in 0.5M sucrose and water. The results are shown in Table 53.

Plasmolysis and deplasmolysis of all epidermal cells of the strips of uninoculated tissue were observed at the end of the incubation period indicating that cells were not severely damaged during removal of the strip. The results in Table 53 clearly show that removal from the hypocotyl retards the development of the resistant response to a considerable extent although the production of reaction material was not affected. Marked hyphal growth was associated with this effect, hyphae often being found in adjacent cell. Cells in which growth had taken place were unaffected.

The main effect of this treatment, therefore, was to cause epidermal cells to behave towards an avirulent race as if it were virulent (i.e. by failing to respond visibly and by allowing hyphal growth). Thus further indirect evidence of the close association between visible cell response and ability of the host to restrict growth of the avirulent race was obtained. The hypothesis that reduced cellular reaction was caused by; the removal of a source of compounds (derived from adjacent cells) utilized during the resistant response and/or, a decrease in the vitality and responsiveness of cells because of wounding during the removal of the epidermis, must in this case explain the failure of cells to die, as well as to undergo browning.

Table 53. Cell response and hyphal development in cells of tissue that had been removed from the hypocotyl or which remained intact

Grade	Description	% cells examined* assigned to grades with the following treatment		
		Intact: 42h	Intact: 88h	Strip: 88h
	Cell unaffected			
1.2	Hypha in penetrated cell	5.7	1.3	12.7
1.3	Hypha in second cell	0.0	0.0	7.0
	Cell affected			
5.2, 5.4	Cytoplasm granular, colourless	1.0	4.0	5.0
7.2,7.3,7.4	" " , very pale yellow brown	2.0	5.0	12.3
8.2, 8.4	" " , light brown	0.0	14.7	7.0
9.2, 9.4	" " , brown to dark brown	0.0	35.7	4.1
	Reaction material produced in cells			
2.1	Obvious particles streaming throughout cell	0.3	6.3	8.7
5.1,7.1,8.1,9.1	Cell affected or brown	0.0	2.0	0.3
	Total penetrated cells	6.3	7.7	22.3
	Total penetration of a second cell	0.0	0.0	8.3

Sakuma and Tomiyama (1967) found that, after applying solutions of chlorogenic acid to very thin potato slices which had lost their ability to resist an avirulent race of P. infestans, this ability was restored. Chlorogenic acid was thought to be transported from adjacent to infected cells and to be involved in the response of the latter. Hertzmann (1959) detected this compound in French bean. To obtain evidence of the possible role in cell response of compounds from outside the infected cell, the following experiment was undertaken in which inoculated strips of epidermis were incubated on agar containing chlorogenic acid.

One per cent Ionagar containing chlorogenic acid at 5000, 500, 50 and 5μM concentrations in 0.02M phosphate buffer pH5.6 was prepared. The acid buffer system was used to prevent autoxidation of chlorogenic acid. Strips from uninoculated hypocotyls and those which had been inoculated with race beta 48h previously were placed on the agar. This was in the form of a 1.5cm disc mounted on a glass slide in a plastic sandwich box. After 48h incubation on the agar 4 uninoculated strips (from duplicate hypocotyls) for each of the treatments and controls were placed in 0.5cm sucrose to test for ability to plasmolyse. Inoculated strips from duplicate hypocotyls were graded as were strips from intact hypocotyls. The results are shown in Table 54.

All cells of 5000μM treated tissue were granular but not brown at the time of examination as were the spores thus indicating a toxic effect at this concentration. Plasmolysis was readily observed

Table 54. Effect of different levels of chlorogenic acid (applied for 48h) on the response of epidermal cells of c.v. Immuna in epidermal strips detached 48h after inoculation

		% cells examined* assigned to grades at 96h in strips incubated on:-					
				Agar + Chlorogenic acid(μM)			
Cell grade	Description	Hypocotyl	Agar + buffer	500	50	5	Intact 48h
	Cell unaffected						
1.2, 1.3	Hypha	1.0	27.5	34.0	15.0	37.5	1.5
	Cell affected						
5, 7	Colourless or v.pale brown	7.0	4.5	4.0	5.0	0.5	0.0
8, 9	Light-dark brown	29.5	2.5	5.0	1.5	2.5	0.0
	Reaction material						
2.1,4.1,7.1		5.5	6.0	5.0	3.5	4.5	0.0

* Two replicate hypocotyls, giving a total of 200 cells, examined for each treatment

in cells of other strips receiving other concentrations. Table 54 shows that none of the treatments overcame the inhibition of the resistant response.

Thus no evidence was obtained to indicate that exogenously supplied chlorogenic acid restored the ability of cells in thin strips to respond to an avirulent race.

Conclusions (Section IV)

Bean tissues became more susceptible to an avirulent race after treatments designed to decrease the host's ability to respond without killing host cells. The following treatments were found effective:-

1 Chloroform vapour (170ppm) in air for 1h before inoculation (stripped Immuna hypocotyls)

2 Immersion in hot water (44-50°C) for various times before inoculation (unstripped Immuna hypocotyls)

3 Removal of infected cells in a thin strip taken from Immuna hypocotyls about 48h after inoculation.

The effectiveness of the third treatment was particularly interesting because it suggested a role for neighbouring cells in the limitation of infection hyphae to resistant cells. It will be recalled that interactions and 'communication' between cells were suggested to be involved in the process of cross protection in Section III.

That uninfected cells near those showing a visible response to an avirulent race were affected was further indicated by their greater heat sensitivity than cells of uninoculated tissue.

DISCUSSION

1. Microscopic events in cells of French bean infected with C. lindemuthianum

a) Use of a cell grading system

As mentioned earlier, the object of the light microscope studies in Section I was to give a complete picture of the visible changes in infected host tissue during the course of resistant and susceptible reactions. For this purpose a cell grading system was devised and used to study two aspects of the French bean/C. lindemuthianum interaction:

(i) the "pattern" of response shown by populations of cells beneath infection drops (i.e. the relative proportions of cells showing different degrees and types of response at a particular time)

(ii) the events taking place in individual epidermal cells during and after penetration (this was shown by changes in the pattern of response during incubation). Information on the pattern of response was considered essential for an examination of the relationship between histological and biochemical changes in host tissue (see Page313). As it was not possible to make continuous observations on individual cells using this method, the course of cellular response could only be inferred. However the examination of large numbers of cells ensured that the suggested course of reaction did not represent a chance occurrence in a few cells. The question remained, was the cell grading system reliable? Reliability seemed to depend on two

main factors; the objectivity of grading and the variation between inoculation sites.

As was inevitable, personal judgement was used to some extent, for instance in deciding the degree of cell browning, the visibility of cytoplasmic particles and the extent of streaming activity. The last two features were particularly important because they seemed to be related to the initial processes of cell response to infection. However the rapid movement of particles loosely aggregated in the vicinity of the appressorium (grade 3) was usually clearly distinct from the highly restricted or absence of motion used for grade 4.

The problem of variation between inoculation sites was examined in Section II 6, where it was shown that affected cells could generally be classified within a few major grades which, at any one time, were the same for most inoculation sites. Thus the six or eight strips normally examined appeared to be adequate to indicate the grades which were most common, and their relative frequency. It will be recalled that it was this relative frequency which indicated the "pattern" of response and course of cellular reaction. However when the total proportion of examined cells showing any response was considered, marked variation between inoculation sites was evident. For instance, standard deviations from the mean % affected cells (from three or four replicates) in Figs. 3 and 4 , ranged from 10-65% of that mean. It seems likely that inherent variation between spores, augmented during the rather slow development before penetration and coupled with differences between hypocotyls in resistance to cuticular penetration, were factors which caused this marked variation.

Therefore, if a numerical estimate (e.g. of the number of penetrated cells) were required, accurate results could only be obtained by the use of a large number of replicates; fewer were needed when relative frequencies of cell grades were considered.

b) Infection and host changes in different tissues

The formation of appressoria took place as described by Dey(1919). Neither qualitative nor quantitative differences could be found between the germination of spores of a virulent and an avirulent race on a particular variety. Resistant and susceptible varieties were penetrated by C. lindemuthianum. Often, however, cells of resistant varieties appeared not to have been penetrated, but were seen to be affected. It seemed probable that in these cases an infection hypha was present but was obscured by the opaque appressorium. Higgins and Millar (1968) suggested that the presence of a transparent area at the base of the appressorium of C. phomoides indicated that penetration was in progress. "Light spots" were seen in appressoria in the present study (Plate 1) but as they were often absent when a hypha was clearly visible within the cell, they were not considered a reliable indication of penetration. Marks, Burbee and Riker (1965) and Mercer (1970) noticed a funnel-shaped ring at the base of mature appressoria of Colletotrichum spp; this structure might have produced the effects of a light spot.

The infection of susceptible hypocotyls was similar to that reported by Leach (1923) and Sinden (1937), summarized in Review of Literature (Page 14). Sinden (1937) observed that in one cell a

primary hypha grew completely through the host protoplast. As it did so the plasmalemma fused, causing the protoplast to form a collar round the hypha. Such an event was not seen in the present study where hyphae were found between cell wall and plasmalemma; it may have been a rare occurrence in the tissue examined by Sinden.

The resistant reaction reported by Sinden (1937 - summarized on Page 16) was quite different from Resistant Type 1 (Page 97) but similar to Type 2 (Page 112) in that streaming activity increased, particles accumulated beneath the appressorium and the cytoplasm coagulated and became brown from this point outwards. However, in the present study the particles did not become brown before movement stopped, as was reported by Sinden. Tomiyama (1967) found that particles appeared in the cytoplasm of potato epidermal cells responding to an avirulent race of P. infestans. These stained in Janus green and were thought to be mitochondria; no positive identification of mitochondria was obtained when the same test was used in this study. The appearance of particles in cytoplasm in all three studies could mean that they were newly produced or had become obvious because of an increase in size.

Cruickshank (1963) suggested that resistance and susceptibility of plants to pathogenic organisms could be determined by their ability or failure to produce inhibitory concentrations of fungitoxic compounds in response to infection. The seed cavities of legume pods have been widely used in studies on host produced fungitoxic compounds and such a system was used by Smith (1967) who found that similar concentrations of inhibitor were produced in infection droplets

of C. lindemuthianum spores in pod cavities of resistant and susceptible French bean varieties. Thus it appeared that differences in the ability of varieties to produce inhibitor was not the cause of their resistance or susceptibility. However it was also possible that pod tissue had not given a differential response similar to that of hypocotyls, normally used for pathogenicity tests.

There is some evidence to suggest that infection and response of hypocotyls and pods do in fact differ. It will be recalled that cellular events in the resistant and susceptible responses of hypocotyls were quite different for at least two days after penetration. However, in the present study (Page 159) it was found that reaction material was produced at the site of penetration in infected pod endocarp cells, regardless of race or variety. Smith (1967) also suggested that during the early stages of incubation (24-48h) there was little difference between infection events in resistant and susceptible varieties. It was at this stage that inhibitors accumulated in infection drops.

At a later stage, Smith was able to distinguish susceptible and resistant reactions in pod cavities and found that these agreed with classifications made in hypocotyl tests. Drops on susceptible pods were darker brown after six days than those on resistant, which suggested that more cell damage had occurred in susceptible tissue. However, when tissue necrosis was examined in the present study (Table 25), it was found that pods of varieties shown to be resistant in hypocotyl tests often developed extensive areas of necrosis; thus pod and hypocotyl responses differed. Therefore because of the poor differentiation of resistant and susceptible responses, pod tissue

seems to be unsuitable for critical studies concerning the differential susceptibility of bean varieties to races of C. lindemuthianum.

Observations in Section I suggested that the production of inhibitors and brown substances might be stimulated by substances diffusing from spores of all races. Therefore, the lack of correlation between infection events and inhibitor production on pods might be explained in the following way :-

Inhibitor production was initiated by substances produced by spores of all races but did not normally reach fungitoxic levels until after host cells were infected. Infection hyphae remained encapsulated within reaction material for some time but did not die. Eventually some emerged, resumed tissue colonization and caused necrosis. This colonization took place more readily in susceptible than resistant pods, but the reason was not known.

c) Observations on reaction material

The dense deposit, termed "reaction material", was often found in cells of pod endocarp, hypocotyl epidermis and cortex, infected by both virulent and avirulent races of C. lindemuthianum. Hyphae rarely grew from this material and it seemed likely that its production constituted a means by which the host resisted fungal infection.

The constituent substances and origin of reaction material were investigated by Mercer (1970) who detected callose in reaction material in infected endocarp cells of Kievit pods. A positive reaction for this substance could only be obtained if the test was made during the first 12h after inoculation. It was shown in Section I that reaction

material became increasingly yellow as incubation proceeded; thus it seemed likely that the callose became impregnated with phenol oxidation products which prevented its detection in histochemical tests. Mercer's electron microscope studies indicated that reaction material was produced by the host, constituent substances passing through the host plasmalemma.

The production of reaction material has not previously been reported for cells of French bean infected with C. lindemuthianum, but similar deposits were found in other diseases. De Bary (1863) described similar material around haustoria in plants infected with downy mildews. Rice (1927) termed this type of deposit the "sheath" of the haustorium. Hardwick (1969) concluded that the sheath around haustoria of Uromyces appendiculatus in French bean contained callose, was mainly of host origin and lay outside the plasmalemma of the host cell; the site of reaction material found in the present study. The sheath described by Hardwick normally formed a ring around the base of the haustorium but Fraymouth (1956) found that haustoria of the Peronosporales were sometimes completely "encapsulated" by the sheath. Fraymouth considered that the sheath was a gel at first and became solid later; if the haustorium grew slowly it would be encapsulated, if rapidly it would break free and the sheath would remain as a collar at the base. The "escape" of a hypha from its surrounding reaction material was shown on Page 146 of this thesis. Thickenings and deposits have commonly been observed in plants infected with facultative parasites. Descriptive terms such as "callosity" (Young, 1926) and "lignituber" (Fellows, 1928) were applied when either callose or lignin, respectively, were thought to be the major

constituents. Hardwick (1969) thought that sheaths, callosities and lignitubers were similar phenomena; it might be useful to term all three "reaction material". In the present study the reaction material could probably be considered as a "callosity".

Cells containing reaction material were usually affected in other ways, for instance they often possessed large particles which accumulated, together with cytoplasm, beneath the appressorium (cell grades 2, 3 and 4). Effects such as this were not seen exclusively in cells producing reaction material,but were also found in cells showing a specific response to an avirulent race of the pathogen (Resistant Type 2 Page 112). It will be recalled that the production of reaction material was a non-specific response to both avirulent and virulent races. What was the relationship between cytoplasmic movements, the production of reaction material and the specific response to an avirulent race?

It is well known that nuclear and cytoplasmic movements take place towards the site of "chemical irritation" and mechanical injury (Allen, 1923). Such effects were termed "chemotaxis" and "traumotaxis" by Ritter (1911). Fellows (1928), Ito (1949) and Stanbridge (1970 pers. comm.) all suggested that callosities or reaction material might form before infection hypha and host protoplast met and thus might be "induced" chemically. In the present study, cell wall thickenings resembling reaction material were found in tissue treated with culture filtrate when examined by light microscopy (Plate 62) and electron microscopy (Mercer, 1970). Ito also reported that callosities appeared in mechanically wounded cells. Mercer (1970)

could not detect any reaction material in French bean cells punctured with a fine needle. Despite this latter observation it seemed probable that the production of reaction material and associated cytoplasmic movements were non-specific responses to irritation of the host protoplast during penetration of the cell wall by virulent and avirulent races.

Could the cytoplasmic movements in cells responding to an avirulent race (Resistant Type 2, Page 112) be associated with a reaction material response? Clearly this question was important in deciding whether the Type 2 reaction was completely different from, or was a modified, Type 1 reaction (reaction material might have protected the cytoplasm and caused a relatively slow, local coagulation/browning reaction typical of Type 2). In the Kievit(R)/delta interaction (Type 2) those few hyphae which were visible often seemed to be embedded in a deposit, possibly reaction material, beneath the appressorium (Plates 33 and 34); cells containing this deposit were found showing all intermediate stages of Resistant Type 2. However cells containing unthickened hyphae (Plate 7) were occasionally found and also showed these intermediate stages. Thus it appeared that cytoplasmic movements also took place in the specific response to an avirulent race. Similar movements were reported by Sinden (1937) for the response of beans to avirulent races of C. lindemuthianum, Allen (1923) for a resistant response to P. graminis on wheat and Tomiyama (1954 and 1956) for Phytophthora infestans on potato.

It seems likely that cytoplasmic movements were involved in both a non-specific response to cell penetration and a specific response to an

avirulent race. A Resistant Type 2 response was probably completely different from that of Type 1, not a Type 1 reaction modified by the presence of reaction material.

Conclusions (Sub-section 1)

Using a cell grading method, the course of cellular events in resistant and susceptible reactions was reliably indicated by changes in the relative proportions of different grades of cells during incubation. Pre-penetration events and the course of the susceptible reaction were as described previously by Leach (1923) and Sinden (1937). However in the present study two types of resistant response were found, one somewhat similar to that described by Sinden and the other completely different. The production of a dense deposit (reaction material) at the site of penetration, which has not previously been reported for this disease, was considered to be a host response to penetration of the cell wall and was not specific to virulent or avirulent races. Changes in the appearance of cells containing reaction material were similar to those thought to be intermediate in the Resistant Type 2 response (Page 112).

2. The cause of inhibition of the growth of C. lindemuthianum hyphae in bean tissue

There are three hypotheses that could provide an explanation of the inhibition of hyphal growth:-

a) Resistance of cell wall polysaccharides to degradation by C. lindemuthianum enzymes (Albersheim et al, 1969)

b) Production of fungitoxic compounds during and after cellular necrosis (Rahe et al,1969a; Bailey, 1970)

c) Production of fungitoxic compounds by living cells.

These hypotheses are discussed below in the light of findings of the present study.

a) Mechanism based on cell wall polysaccharides

With regard to resistance to an avirulent race, Albersheim et al (1969, see Review of Literature, Page 24) suggested that the necrotic response may have been of secondary importance in resistance, the ability to initiate an infection being decided when the fungus first came in contact with the host cell wall. It was suggested earlier that if resistance were caused by the mechanism suggested by Albersheim, penetration would be infrequent, treatments reducing visible host response would not increase tissue susceptibility and there would be a poor correlation between host response and in vivo inhibition of hyphal growth. Although in some resistant responses few cells were seen to be penetrated, in that of c.v. Immuna to race beta (Table 13)

the % penetrated cells was as great as was obtained in any susceptible reaction. Acquired susceptibility following treatment of resistant tissue and a close correlation between the time of cellular necrosis and inhibition of hyphal growth (discussed in b below) were also demonstrated in the present study. Thus it would appear unlikely that resistance to an avirulent race was determined by factors associated with host cell walls.

The increase in resistance of tissue on ageing could, however, be explained more readily in terms of resistance to fungal cell wall degrading enzymes. English and Albersheim (1969) showed that very marked changes in cell wall polysaccharides took place during hypocotyl extension, and that cell wall material of 18 day bean hypocotyls was more resistant to extracellular enzymes of C. lindemuthianum than young (5 day) tissue. A similar effect was found by Bateman, Van Etten, English, Nevins and Albersheim (1969) with enzymes from Rhizoctonia solani; old hypocotyls were resistant to this organism. It was pointed out that the production of cell wall degrading enzymes was one of the first detected events in R. solani infected hypocotyls and occurred sooner than phytoalexin accumulation. In Section II 3 of this study, it was shown that penetration and growth to a second cell occurred less readily in older than in young tissue suggesting that cell walls of this older tissue were more resistant to hyphal penetration. These observations were consistent with, but provided no direct evidence for, a hypothesis that age resistance was based on the contents of cell walls.

b) Association between necrosis and inhibition of hyphal growth

Rahe et al (1969a) and Bailey (1970) showed that during the development of necrosis in French bean infected with virulent and avirulent races of C. lindemuthianum, fungitoxic compounds accumulated. Furthermore, Stholasuta (1970) detected phaseollin in bean leaves showing a hypersensitive response to an avirulent race of P. phaseolicola and Bailey (1970, pers. comm.) found this substance in leaves with local lesions caused by Tobacco Necrosis Virus. The observational studies of Leach (1923) and Sinden (1937) indicated that in susceptible varieties necrosis occurred later than in resistant varieties and after extensive colonization of host tissue had taken place. Thus if the fungitoxic compounds (produced during and after infected cells became necrotic) inhibited hyphal growth in vivo, then resistance or susceptibility would be explained by the fact that necrosis occurred soon after cells of resistant varieties were penetrated, but was delayed in susceptible varieties, for a time sufficient to allow considerable intracellular hyphal growth.

That inhibition of hyphal growth of an avirulent race was related to the onset of necrosis was indicated by results obtained in the present study. In Section I 6 it was shown that hyphae of race beta (AV) grew more slowly in necrotic cells of c.v. Immuna than in those which were apparently unaffected. Hyphal growth appeared to be inhibited in cells showing the earliest stage of resistant response (cell grade 5). In Section IV 3 it was found that hyphae of an avirulent race grew freely in cells in which the necrotic response had been inhibited. Thus there was strong evidence that in resistant

varieties, growth of hyphae of an avirulent race was inhibited during the process of cell death and browning.

The intermediate response of c.v. Pinto to race beta could also be explained on the basis of a "time of necrosis" hypothesis. In Section I 8 it was shown that necrosis of infected Pinto cells occurred earlier in response to race beta than to a race inciting a susceptible reaction on this variety. The number of cells colonized from individual infections did not increase much, if at all, after these cells had become necrotic. English and Albersheim (1969) were able to explain the Pinto/beta intermediate reaction by the fact that amounts of alpha galactosidase produced by race beta on isolated cell walls of this variety were intermediate between the amounts produced on walls of resistant and susceptible varieties.

Although it seemed likely that resistance of cell walls to fungal penetration played an important part in slowing the growth of hyphae in old tissue it was possible that processes associated with cellular necrosis were also involved in lesion limitation in older tissue of susceptible varieties. Pierre and Bateman (1967) have provided strong evidence to indicate that the failure of R. solani lesions on bean hypocotyls to increase in size after the tissue had become watersoaked and started to go brown, was due to the accumulation of phaseollin and another fungitoxic substance in the lesions. It seems quite possible that a similar lesion limitation mechanism, associated with the necrosis of host tissue, could be in operation in tissue infected with C. lindemuthianum. If resistance of cell walls to penetration were the only mechanism of lesion limitation there is no reason why eventually hyphae could not have colonized most of the infected hypocotyl.

Protection of hypocotyl tissue from a virulent race by treatment with an avirulent certainly seemed to be associated with necrosis in infected cells (Section III 5). Cells infected with hyphae of a virulent race became necrotic earlier in protected than in non-protected tissue. Only limited symptoms of infection developed at protected sites suggesting that little colonization took place after the onset of necrosis.

Results of experiments on cross protection were particularly interesting because they suggested that cells near those infected by, and responding to, an avirulent race developed an increased sensitivity and capacity to respond to a virulent race, thus resulting in a restriction in its growth. Increased sensitivity was also suggested by the observation that uninfected cells of tissue inoculated with an avirulent race became necrotic when subjected to mild heat treatment; no necrosis occurred in uninoculated tissue (Section IV 2). In another experiment (Page 249) an increased capacity of tissue to respond to a virulent race was indicated. Tissue developing non-limited lesions in response to a virulent race not only became necrotic earlier, but showed a greater degree of browning when an avirulent race was also present at the same inoculation site.

Increased sensitivity of host cells to a fungal pathogen which led to a necrotic response was shown by other workers. Rahe and Kuc (1970) found that bean hypocotyls infected by a virulent race of C. lindemuthianum became necrotic after a heat treatment which did not itself cause necrosis; tissue colonization was limited. Hardwick (1969) found that in French bean plants treated with the systemic

fungicide "Plantvax", cells of leaves infected with U. appendiculatus underwent a necrotic response resembling a hypersensitive reaction; untreated infected tissue did not become necrotic. King et al (1964) found that clover systemically infected with bean yellow mosaic virus was less extensively colonized by E. polygoni than uninfected clover and host cells at the site of mildew infection became necrotic. Electron microscope studies of Mercer (1970) revealed cells neighbouring those responding to an avirulent race of C. lindemuthianum had a much greater content of cytoplasm than those of uninoculated tissue.

The work of Ross and Israel (see Review of Literature, Page 36) on the necrotic local lesion response of a resistant variety of tobacco to TMV indicated that effects very similar to those reported above occurred in cells near the site of necrosis; a) increased resistance to TMV applied in a subsequent inoculation (Ross and Israel, 1961b), b) increased heat sensitivity (Ross and Israel, 1970) and c) increased cytoplasmic content (Israel and Ross, 1970). Increased cytoplasm was considered to be an indication of increased cellular activity.

Thus it appears that treatments (e.g. those which cause a local necrotic response, virus or fungicide) which make plant tissue more sensitive to the effects of fungi, viruses and other agents, cause the tissue to become more resistant to the pathogens because necrosis occurs more readily, and its associated resistance processes are brought into action sooner. This increased sensitivity of these adjacent cells might represent the results of "stress" caused by the damaging effect of treatments.

c) Production of fungitoxic compounds by living cells

Fungitoxic compounds were known to be produced in necrotic tissue but there was evidence in the present study to indicate that inhibitors were also produced by living cells.

Smith (1967) and Bailey (1970 pers. comm.) found that drops of water containing spores of C. lindemuthianum became inhibitory when incubated in French bean pods. In Tables 26 & 27 it was shown that few cells in inoculated pod cavities became necrotic; the bean varieties, races and spore concentrations used were the same as those of Smith and Bailey. Spore drops, but not drops of water, became brown during incubation; drop-browning normally accompanied inhibitor production in the studies of Smith (1967). In Table 26 it was shown that although drops of spores in cavities of c.v. Dubbele Witte became brown during incubation, spores failed to germinate and no endocarp necrosis was detected. This observation suggested that drop browning and inhibition of germination were caused by substances produced by living endocarp cells. Smith showed that drops of water in pods of this variety became inhibitory thus the germination inhibitor may have been pre-formed.

Further evidence was provided by observations on stripped hypocotyls. Necrosis was rarely observed in cells of stripped tissue beneath drops of culture filtrate or spore suspension of an avirulent race, but drop-browning occurred in both cases (and in response to spores of the virulent race). Avirulent spores, and spores of a virulent race applied after the tissue had been treated with culture filtrate, showed poor germination on stripped hypocotyls. Tissue treated with medium

alone was not protected and did not become brown. Often spores applied to intact hypocotyls and which came to lie in small wounds failed to germinate; they were usually embedded in a brown film. Cells around these small wounds were rarely necrotic. It was concluded that when, in the absence of host cuticle, free diffusion could occur between host cell and fungal spore, an inhibitor of germination and germ tube growth produced by some process not associated with necrosis of host cells, diffused into the spore drop. The nature of these inhibitors was not known but drop-browning suggested the production of phenolic compounds; phaseollin was found by other workers in spore drops in pod cavities.

The results of other work have suggested that inhibitor production does not necessarily accompany necrosis. Bailey (1968) found that pea leaves produced pisatin in response to culture filtrates of P. expansum without any accompanying necrosis. Cruickshank and Perrin (1963b) noticed that no colour appeared in droplets where the production of pisatin had been stimulated by chemicals. Monilicolin A, a peptide extracted from Monilinia fructicola, stimulated phaseollin production but failed to cause necrosis (Cruickshank and Perrin, 1968). Muller (1958) suggested that the non-brown cells probably contributed to inhibitor production in bean pods in response to P. infestans. →

Was it possible that inhibition of intracellular hyphal growth was caused by fungitoxic substances produced by living, apparently unaffected cells? It will be recalled that the production of reaction material was the most frequently seen response of cells of pod and stripped hypocotyls and this material was probably impregnated with

oxidised phenolic compounds. The processes involved in synthesis and transport through the plasmalemma of substances causing browning of reaction material and spore drops may well be the same. As the appearance of inhibitory activity in spore drops was normally accompanied by browning, inhibitors, as well as compounds responsible for browning (these might themselves be inhibitory) might be produced locally at the site of reaction material accumulation, where browning was seen to occur. It was possible, therefore, that the inhibition of infection hyphae in cells producing reaction material might not be an effect of the material itself, but be caused by the associated production of fungitoxic substances. With regard to the Kievit(R)/delta situation (Resistant Type 2), inhibition of hyphal growth before the onset of necrosis might have been the primary expression of resistance in this combination. As yet there is no direct evidence for the role of fungitoxic compounds in the inhibition of hyphal growth within living cells of French bean. One possibility which should not be overlooked, is that the production of phaseollin(and other fungitoxic compounds) in tissue where some cells are undergoing necrosis, occurs in adjacent living cells in response to the stimulus of substances released from dying cells.

There have been a number of other reports that resistance might be due to processes not related to visible host responses. Many reports concerning resistance to obligate parasites suggest, as in this study, that the expression of resistance is associated with the development of necrosis in host cells at an early stage of infection (e.g. Ward, 1902., Stakman, 1915., White and Baker, 1954. and Staveley and Hanson, 1966). However Leath and Rowell (1966) found that when

corn (Zea mays) was inoculated with Puccinia graminis to which this host is immune, chlorotic spots, but no necrosis, developed on leaves. Fungal growth ceased and a diffusible inhibitor produced by the affected chlorotic cells was thought responsible. Cell free germination fluids of the fungus, when injected into the leaf, caused similar chlorosis. Both the fungus and germination fluids caused marked depletion of chlorophyll, granulation of cytoplasm (but not cell collapse) and later a marked thickening of mesophyll cell walls in the affected area. In other cases cell death occurred but often rather slowly (Zimmer, 1965) and sometimes after movement of cytoplasm and chloroplast disintegration (Allen, 1923); inhibitor production before cell death could not be ruled out. Brown, Shipton and White (1966) found little correlation between amount of necrosis and hyphal growth of P. graminis in wheat varieties of differing susceptibility to this fungus.

Conclusions (Sub-section 2)

When cells of French bean containing hyphae of C. lindemuthianum became granular and brown, fungal growth was inhibited; fungitoxic compounds produced at this time were probably the cause of inhibition. If the amount of tissue colonized at the time necrosis occurred is considered, then an explanation of inhibition of hyphal growth can be provided for the following situations:-

1) an avirulent race in a resistant variety

2) the final inhibition of growth of a virulent race in old tissue

3) a virulent race in tissue protected by an avirulent race.

Necrosis of host cells does not always precede hyphal inhibition. In older tissue colonization is slower, probably because cell walls are resistant to hyphal penetration. Furthermore, diffusible inhibitors of spore germination and germ tube growth were probably produced by living cells in response to spores and culture filtrates of C. lindemuthianum. Although no evidence was available from the present study, the possibility that the production of fungitoxic substances by living cells might result in the inhibition of an infection hypha soon after its penetration through the cuticle, could not be ruled out.

3. Specificity of race/variety interactions in Bean Anthracnose

If, as was considered likely, the differential virulence of races was related to the extent of fungal growth before host cells died, then race/variety specificity could be explained in one of the following ways:-

(1) the avirulent race alone produces a substance which kills host cells soon after their penetration. Toxin hypothesis.

(2) the avirulent race alone produces a substance which does not kill the host cell but initiates a host reaction leading to cell death (autolysis).

a) Induction hypothesis - a specific fungal product induces the action of a particular host gene.

b) Recognition hypothesis - the host is capable of distinguishing between compatible (host) or incompatible (foreign) substances; when the latter are recognised the host cell responds.

c) Irritant hypothesis - a fungal product damages host cells without directly killing them but causing a non-specific "injury response".

(3) The host makes an active response to any fungal invasion but this response is subdued by the virulent race. Suppression hypothesis.

When a race which is avirulent on one variety is transferred to another susceptible variety, the reverse of these events would occur. Thus properties of the host variety must be equally important in the specificity of interactions. For instance, if a toxin hypothesis

were correct, a race would be virulent on a second variety because: a) the fungus was unable to produce the toxin, or b) the host was not affected by this substance.

In the present study no conclusive evidence was obtained to show why races of C. lindemuthianum attacked some bean varieties but not others. However there was some information that seemed worth considering:-

With regard to the direct action of a fungal toxin, it was shown in Section IV 3 that when strips of tissue cut from hypocotyls infected with an avirulent race were incubated further on pieces of agar, the visible necrotic response of infected cells in intact tissue did not take place and the growth of hyphae was not inhibited. If necrosis were caused purely by the action of a fungal toxin, then the death of infected cells should have taken place in both the strips of tissue and the intact hypocotyls. That streaming activity increased in cells showing a Resistant Type 2 reaction also suggested that cell death, and associated inhibition of hyphal growth, was the result of an active host reponse.

Raa and Kaars Sijpesteijn (1968) found that symptoms similar to those of a hypersensitive response to an avirulent race occurred on resistant, but not susceptible, apple varieties treated with low concentrations of culture filtrates of Venturia inaequalis. Culture filtrates were used in the present study to determine whether those from avirulent races specifically initiated a resistant response. The subsequent resistance of treated tissue was indicated by its failure to allow the growth of a virulent race of C. lindemuthianum. In

Section III 4, filtrates from all races caused protection regardless of the reaction of the variety to spores of the particular race. Therefore no evidence was found to suggest that fungal products from only an avirulent race might initiate a host's resistance response.

Cruickshank and Perrin (1968) isolated a polypeptide from a non-pathogen of bean, Monilinia fructicola, which stimulated phaseollin production in bean pods. Hadwiger and Schwochau (1969) suggested that microbial products induced inhibitor production and thereby specifically initiated host resistance responses. Could induced inhibitor production explain differential resistance and susceptibility to races of C. lindemuthianum?

The activity of culture filtrates, in their stimulation of inhibitor production, might be due to the induction of a particular host gene; culture filtrates were not specific in their activity. Furthermore, although it seemed likely that substances diffusing from spores stimulated inhibitor production in pod cavities and stripped hypocotyls, it appeared that such a stimulation was also non-specific. Certainly drop browning occurred in response to both virulent and avirulent races and it will be recalled that Smith (1967) found that inhibitor production was not specifically stimulated by an avirulent race. Therefore, if specificity is determined by a mechanism of gene induction, it is more probable that these genes control processes leading to the rapid death of host cells, rather than the production of specific inhibitors. The initiation of a necrotic response seemed to be very closely related to race/variety specificity.

Recognition of foreign or compatible substances might also provide a basis for specificity. There have been recent reports

(Doubly, Flor and Claggett, 1960 and De Vay, Schnathorst and Foda, 1967) that virulent races and susceptible host plants may be "antigenically similar". De Vay et al (1967) further found that injection of sonicated Xanthomonas malvacearum cells (the antigen) into resistant cotton varieties caused a hypersensitive response; no such response occurred in susceptible varieties. It was possible, therefore, that a race was virulent because it was not "recognised" as being foreign and thus elicited no host response. Such a phenomenon might well occur in bean anthracnose.

If host response were initiated in one of the above mentioned ways, the possibility remained that a virulent race, although causing a response, would also be able to subdue it. Pringle and Scheffer (1964) indicated that the ability to produce a fungal toxin, "victorin", was essential for the virulence of Helminthosporium victoriae on certain varieties of oat. Susceptibility to H. victoriae (and to the action of purified toxin) and resistance to Puccinia coronata were controlled by the same single dominant gene. Infection of oat leaves of a susceptible variety (Paddock, 1953) was followed by a period of fungal growth during which infected host cells were apparently unaffected by the fungus; necrosis occurred later. This was somewhat similar to a susceptible reaction of bean hypocotyls to C. lindemuthianum. In resistant varieties, inhibition of fungal growth was associated with either disintegration of the hypha or browning around the point of penetration. Scheffer and Pringle (1967) noted that spores of H. victoriae contained toxin and concluded that this compound was involved in suppressing host response during infection. Later the

toxin concentration may have become sufficiently great to cause necrosis. (Purified toxin was known to cause all the symptoms of Victoria blight - the disease caused by H. victoriae). Further evidence that these suggestions might be true was obtained by Yoder and Scheffer (1969).

There was some evidence to suggest that C. lindemuthianum produced a "toxin" in susceptible varieties of French bean. The collapse and browning of cells uninfected by the fungus such as those found in the streaks between lesions (Plates 24 and 25), and the collapse of leaf lamina adjacent to infected veins reported by Sinden (1937), may have suggested "toxin" action. Perhaps, as suggested by Scheffer and Pringle (1967 - for H. victoriae on oats), necrosis occurred in a susceptible reaction when very high concentrations of toxin accumulated in the tissue. However, the fact that secondary hyphae caused marked effects on membranes (Plate 21) and easily penetrated cell walls (Plates 19 and 21), suggested that necrosis might have occurred after developmental changes had taken place in the fungus, which led to the formation of these hyphae and associated degradative enzymes. No secondary hyphae were found in old tissue of susceptible varieties but necrosis did occur. Therefore effects caused by primary hyphae seemed to be responsible for necrosis in susceptible varieties and indeed the development of secondary hyphae might result from a stimulus provided by the release of substances from necrotic cells. Thus the possibility that necrosis, in a susceptible reaction, was the result of the slow accumulation of a toxin to a finally lethal level, could not be ruled out.

In order to determine specificity in bean anthracnose:-

a) different races would produce different "subduing substances" or toxin production would only occur on susceptible varieties.

b) sufficient "subduing substance" must be produced by the minute infection hypha to counteract the response of the whole cell, which is of much greater volume.

Apart from the production of reaction material, no visible response could be detected in infected cells of susceptible varieties, nor was there any evidence of decreased activity in these cells; streaming occurred normally.

From the limited information available, it seemed unlikely that race/variety specificity was determined by the production of a substance by the avirulent race alone, which either directly caused cellular necrosis, or stimulated the host's production of inhibitory compounds. Although it was possible that a virulent race subdued the response of infected host cells, it seemed more likely that avirulent races incited cellular resppnse while virulent failed to do so. Of the three hypotheses mentioned on Page 303 (2), that of recognition of foreign organisms or substances would seem most readily to explain the different events occurring in cells of resistant and susceptible hypocotyls described in Section I.

4. Implications of the results of this study for biochemical work

It seemed likely that hyphae of avirulent races of C. lindemuthianum failed to grow from the first-penetrated cells of resistant bean varieties because of the action of fungitoxic compounds produced at the time when marked changes in cell appearance took place. As suggested earlier, an important test of the causal role of a particular compound in such inhibition would be to establish whether retardation of hyphal growth occurred at a time when the substance reached inhibitory concentrations in the infected host cell. For such a test to be made it would appear to be necessary to consider three aspects of the host fungus interaction:-

a) The exact course of inhibitor production in infected tissue

b) The relationship between biochemical changes in host tissue and those in the infected cells where inhibition was taking place

c) The exact course of hyphal inhibition in infected cells.

By chemically extracting infected bean tissue at intervals after inoculation, Rahe et al (1969a) and Bailey (1970) have sought to obtain information concerning the course of production of fungitoxic compounds in response to infection by C. lindemuthianum. Bailey (1970) has obtained quantitative data concerning the changes in phaseollin content of tissue infected with an avirulent race of C. lindemuthianum and found that phaseollin did not appear until after some cells of the extracted tissue had become necrotic. Cellular necrosis occurred at the time of or after penetration (i.e. 48h after inoculation).

Thus it seemed likely that the data on course of phaseollin production obtained by Bailey represented that occurring in host cells responding to infection.

It was found in the present study that where free diffusion could occur between spores and host cells (i.e. where the cuticle did not provide a complete barrier), the production of inhibitors occurred fairly early after inoculation and without penetration or necrosis taking place. In such an event uninfected cells would be expected to produce inhibitors. Thus the inhibitor content of extracts of such tissue would not represent that of infected cells, and changes in concentration would not indicate the course of inhibitor production in infected cells. It was clear that where the study of biochemical changes in host cells responding after penetration by C. lindemuthianum was involved it was necessary to use tissue where such diffusion effects did not occur. The fact that appressorium formation increased continuously for 48h or more on resistant varieties and that drop browning did not normally occur, suggested that diffusion effects were not important in hypocotyl tissue used by the author or by Bailey (1970). Spore drops on hypocotyls did not become inhibitory (Page 245).

Rahe et al (1969b) reported the detection of inhibitors in tissue extracted before penetration or necrosis had occurred. This might have indicated that inhibitor production associated with necrosis was not the major cause of resistance to an avirulent race, or that this response was not responsible for the cross protection they obtained. However etiolated tissue was used by Rahe et al and in the present study diffusion between inoculation drops and host epidermal cells

was suggested by drop-browning and the formation of distorted superficial germ tubes by the fungus (Plate 55). Thus the early appearance of inhibitors in the tissue used by Rahe et al might have been caused by substances from spores which initiated a host response.

In a situation where the major biochemical changes were probably taking place in infected cells, how were these changes related to events in the population of cells beneath the infection drops? From Tables 12 - 14 it was evident that two factors could contribute to increases in the content of a fungitoxic compound in tissue extracts during a resistant response; a) an increase in the number of affected cells (by penetration from more appressoria), b) an increase in the degree of response of such cells (by the continued synthesis of the compound). It was also clear that apart from the period when effects on host cells were first observed, and when response was completed, a whole range of cellular changes were observed in the inoculation sites; presumably the inhibitor content of individual cells varied considerably.

It was suggested earlier that the localization of toxic compound and the site of its production were important in considering its role. There were indications that in tissue where a necrotic response was developing, apparently unaffected cells had increased metabolic activity and were in fact contributing to the processes of the necrotic response. The contribution of uninfected cells was probably indicated by the fact that response to an avirulent race was much less intense when a thin strip of infected cells was removed from the remainder of hypocotyl tissue (Section IV 3). It was possible that the supporting role of these other cells was in producing phenolic compounds involved as

intermediates and products of the "browning reaction" or in the production of phytoalexins such as phaseollin. Stholasuta (1970) found that hair cells of tissue showing a hypersensitive response to Pseudomonas phaseolicola gave a positive reaction for catechol tannins. Uninoculated tissue or tissue inoculated with a virulent race failed to give this reaction. This suggested apparently unaffected cells might undergo changes in phenolic metabolism. In the present study, although uninoculated tissue was not specifically tested, a positive reaction was obtained in hair cells of tissue inoculated with an avirulent race of C. lindemuthianum. The increase in cytoplasm noticed by Mercer (1970) also suggested that adjacent cells were at least affected if not actively contributing to the response of infected cells.

The contribution of neighbouring cells to the resistant response of an infected cell was thought important by Sakai, Tomiyama, Ishizaka and Sato, 1967) and more active production of phenolic compounds occurred in uninfected tissue adjacent to cells responding to P. infestans. Imaseki and Uritani (1964) suggested a similar role for neighbouring tissue in the production of intermediates in the pathway of ipomeamerone synthesis in sweet potato. Tomiyama (1955) demonstrated the same increased susceptibility of strips of inoculated tissue after removal from the plant that was described in Section IV 3.

It is possible that the very high levels of phaseollin calculated by Bailey (1970) to be present in cells responding to an avirulent race of C. lindemuthianum could be explained in three ways; a) by its synthesis in the infected cell alone, b) with the aid of adjacent cells not themselves producing phaseollin, or c) in both adjacent and infected

cells (thus infected cells would contain less than the calculated amount).

In the present study (Section I 6) it was shown that growth of hyphae of race beta (AV) in cells of c.v. Immuna appeared to be inhibited soon after the host cell had become granular (grade 5). It was also shown that the major cell grades found at any one time in inoculation sites did not vary much between different hypocotyls or at different positions on the hypocotyl. The results in Table 12 indicate that for a short period after the first effects on cells were noticed, all affected cells were of grade 5. Thus the Immuna/beta experimental system could provide the basis for a critical test of the role of phaseollin in preventing further growth of hyphae in epidermal cells of resistant bean hypocotyls infected with an avirulent race of C. lindemuthianum. This experiment could be conducted in the following way:-

1) Confirm, by the use of the cell grading system that tissue used for extraction was homogeneous in its response and that all affected cells were of grade 5 (granular but colourless).

2) Extract the tissue and determine the phaseollin content per inoculation site

3) By microscopic examination determine the mean number of cells of grade 5 per site and thus the phaseollin content per cell (Bailey, 1970)

4) Compare these concentrations with those found in in vitro tests (Bailey, 1970) to cause inhibition of hyphal growth. If greater than, or similar to, the values for complete inhibition of hyphal growth then

a role of phaseollin in resistance would be indicated.

In the present study, quantitative determinations of phaseollin content and use of the cell grading system were only applied together in one experiment (Section I 7) where the Kievit (R)/delta combination was used. Few cells were normally found to be penetrated, thus the stage of inhibition of hyphal growth could not be determined, and a similar test to that described above could not be attempted.

Clearly the problem of localization of phaseollin production is of critical importance in finally establishing its role in resistance of French bean to an avirulent race of C. lindemuthianum.

Conclusions (Sub-section 4)

Biochemical changes in host tissue leading to the production of fungitoxic compounds can probably be initiated by an avirulent race at either of two stages of infection:-

a) before penetration by the action of substances diffusing from spores or germ tubes in the inoculation drop. This effect is most marked in tissue where host cuticle does not provide a barrier to free diffusion.

b) after penetration, i.e. when the infection hypha has passed through the cuticular barrier.

When attempting to determine the course of biochemical changes in host cells following infection by C. lindemuthianum it appeared to be necessary to use tissue where free diffusion was prevented. A study of both situations would be of value in the further elucidation of mechanisms by which French bean resists fungal pathogens.

In tissue inoculated with an avirulent race, cells adjacent to those in which visible changes had occurred were probably affected and might contribute to the production of inhibitors. The number of affected cells and the degree of host response in individual cells of particular sites varied at any one time and during incubation.

By combining observational and biochemical studies the role in resistance of a particular compound might be ascertained.

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