

STUDIES ON NET BLOTCH OF RYEGRASS
CAUSED BY *DRECHSLERA DICTYOIDES*

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THESIS

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Verily, it is easier for a camel
to pass through the eye of a
needle than for a scientific man
to pass through a door.

Sir Arthur Eddington

The Nature of the Physical World

To my parents

ABSTRACT

Three species of *Drechslera* were isolated from ryegrass; *D. dictyoides* f. sp. *perenne*; *D. siccans* and *D. nobleae*. The species were distinguishable by taxonomic characters; conidiophore length, arrangement of conidia, conidial shape, the manner of germination and cultural characters; morphology and growth rate. *D. dictyoides* was characterised by tapered conidia produced at intervals on the conidiophore. Germination was predominantly from the basal cell of the conidium and growth in culture was slower than that of the other two species.

The life history of *D. dictyoides* on *L. perenne* was investigated using optical and electron microscopy. Changes in the ultrastructure of the host and pathogen were followed during infection. The vegetative fungal wall consisted of two layers but modifications, usually involving reductions in one or more of these layers, during infection were important in the successful establishment of a parasitic relationship with the host. Appressoria formed predominantly over the anticlinal wall of the host epidermis but germ tube length was variable. Penetration was usually into an epidermal cell but subcuticular infection was common. In the period of lesion development, hyphae spread intercellularly with limited host reaction until host senescence began, at which time intracellular hyphae were again found. *D. dictyoides* was interpreted as a hemibiotrophic pathogen and formed a close nutritional relationship with its host. Intrahyphal hyphae were occasionally found within lesions. Conidium development was interpreted as holoblastic and discussed in relation to theories concerning 'blastic' ontogeny.

A field trial indicated the presence of a wide variation in resistance of perennial ryegrass lines to net blotch.

Drought conditions were experienced during much of the field trial, but increases in disease intensity usually followed periods of rain, with a major increase occurring in March 1982.

The ryegrass epidermis was examined in relation to the effect on net blotch development. Epicuticular wax projections were present on the adaxial surface but absent on the abaxial surface and this resulted in greater spore accumulation on the latter. In contrast, the outer wall of the adaxial epidermis was approximately half as thick as that of the abaxial epidermis. Given sufficient moisture, penetration of the adaxial surface was more rapid than that of the abaxial surface. Host nuclei migrated towards the site of attempted penetration of the leaf and cytoplasm accumulated in this region.

Glasshouse and laboratory assessments of the effect of leaf age showed that infection was greater on older leaves. The interaction of exposure time and leaf senescence was responsible for the observed differences. Infection was possible over a wide temperature range but the optimum was close to 20°C. The *Lolium* endophyte was present in most perennial ryegrass plants but appeared to have no effect on net blotch disease even when hyphae of both fungi were closely associated within a leaf lesion.

Spore accumulation on the leaves of susceptible trap plants was greater than on those of more resistant lines, resulting in a higher density of infections on leaves of susceptible lines which was compounded over several disease cycles. This appeared to be largely responsible for the difference in disease intensity between lines in the field.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 General introduction and aims

Ryegrasses form the dominant grass species on nearly nine million hectares of sown pasture in New Zealand (Langer 1973) or about thirty-three percent of the land area of New Zealand. In 1980, eighty-eight percent of the machine-dressed grass seed produced in New Zealand was ryegrass (New Zealand Year Book 1981).

In spite of the importance of ryegrass in New Zealand and elsewhere, much remains to be determined about the nature of the diseases present. New cultivars are being bred with resistance to some ryegrass diseases and this provides an impetus for a study of these diseases. In New Zealand, crown rust is the most important foliar disease present on ryegrass, but recent work in England suggests that the *Drechslera* diseases of ryegrasses are more important than crown rust in Britain (Lam 1981). In 1966 *Drechslera dictyoides* (Drechsler) Shoemaker f. sp. *perenne* (Braverman & Graham) Shoemaker was the second most important foliar disease of perennial ryegrass in New Zealand, causing considerable damage under some conditions (Latch 1966). Since Latch's paper, little has been published on the disease in New Zealand apart from noting its presence in seed lines (Matthews 1971, McKenzie 1978).

The aim of this study was to examine the biology and life history of *D. dictyoides* in its association with *Lolium perenne* L. and to determine some aspects of the nature of the disease. Before this study was commenced, it was necessary to re-examine the species of *Drechslera* pathogenic to ryegrass as there has been some controversy concerning their taxonomy and misidentifications have marred some previous work.

The basis for the study was provided by an investigation of the fine structure of the disease process from the time a spore germinates on the leaf to the eventual sporulation of the pathogen. With this basic knowledge, some important aspects of the disease cycle were able to be examined more closely. The epidermis provides the initial barrier to infection and was worthy of a thorough investigation.

A field trial was set up to investigate changes in disease levels on a number of perennial ryegrass lines, all originating from the same long-term pasture. Changes in disease incidence and severity with time were recorded in an attempt to assess both the range in susceptibility to disease among the lines and the effect of environmental conditions on disease. Those lines consistently heavily diseased or consistently healthy were examined further to assess the nature of resistance to disease.

1.2 History of ryegrass and its cultivation.

Ryegrass, in particular *Lolium perenne*, has been cultivated in Britain longer than any other grass. The first published reference was in 1677, in Dr Plot's *Oxfordshire* (quoted by Morton 1855), where it was recorded that :

'... they have lately sown ray-grass, or the *Gramen loliaceum*, by which they improve any cold, sour, clay-weeping ground, for which it is best, but also good for drier upland grounds especially light, stony, or sandy land, which is unfit for Sainfoin.'

At that time the only other cultivated pasture plants were red clover, sainfoin, spurrey, trefoil and non-such (Sinclair 1869).

Most commercial seed mixtures for permanent pastures in

the nineteenth century consisted largely of perennial ryegrass (De Laune 1882a). Sinclair (1869), in his classic *Hortus Gramineus Woburnensis*, suggested the reason was that ryegrass :

'produces an abundance of seed, which is easily collected and readily vegetates on most kinds of soil, under circumstances of different management; it soon arrives at perfection and produces in its first years of growth a good supply of early herbage, which is much liked by cattle.'

Sinclair (1869)

He added that 'the plant impoverishes the soil to a high degree'. Ryegrass was also claimed to be markedly inferior in nutritive value in comparison with other grasses. De Laune (1882a) found newly formed pasture to deteriorate after the first two or three years and omitted ryegrass entirely when suggesting seed mixtures. Later De Laune (1882b) added :

' I do not wish to say that there may not be some value in ryegrass which is not contained in some other grass, although I have not perceived any.'

De Laune (1882b)

In New Zealand, the majority of grasslands are recently created communities, much of the country being covered in forest when the first Europeans arrived in New Zealand. By the end of the nineteenth century, most low-land and much hill country forest had been broken (Langer 1973) and by 1925 over 3.2 million hectares of podocarp-broadleaf forest had been converted to grassland. Today 9.33 million hectares of New Zealand's total land area of 27.13 million hectares, consists of improved grassland (N.Z. Official Yearbook 1981).

The most common mixture for a fertile primary bush

included perennial ryegrass, cocksfoot, crested dogstail, Kentucky bluegrass, brown top and clover, with perennial ryegrass predominating. (Levy 1951). While numerous species were originally sown on new grassland, perennial ryegrass soon gained supremacy in New Zealand. Wilkin (1877) noted Sinclair's reservations on the use of ryegrass for permanent pasture, but placed it 'top of the list' in New Zealand. As to Sinclair's statements, Wilkin suggested that 'no single grass will fatten stock so quickly as ryegrass'.

Sinclair (1869) considered perennial ryegrass to be a short-lived plant, seldom lasting longer than six years, but Wilkin (1877) found many fields in Canterbury, New Zealand, where it retained possession of the soil after double that time. McKay (1887) in *A Manual of Grasses and Forage-Plants Useful to New Zealand*, admitted ryegrass to be inferior in nutritive value to cocksfoot when green but suggested the reservations of Sinclair could be answered by grazing the crop and so preventing it from seeding. He also suggested the methods of Sinclair to be obsolete.

Today, New Zealand pastures are dominated by ryegrass and white clover, giving 'a uniform appearance to nearly nine million hectares of sown pasture' (Langer 1973). The standard perennial ryegrass cultivar in New Zealand is 'Grasslands Ruanui', a highly persistent strain which withstands continuous grazing (Langer 1973). Italian ryegrass is a more erect and slightly coarser plant, an outstanding property being its ability to make good growth in autumn and winter.

1.3 Diseases of ryegrass

The early accounts of ryegrass and its cultivation usually lack any mention of disease, although a number of diseases are often present, including some of economic import-

ance. Latch (1966) lists twelve fungal diseases infecting the foliage of ryegrasses in New Zealand. Crown rust, caused by *Puccinia coronata*, was found to be the most important foliar disease of ryegrass in New Zealand. The disease is present throughout the year, with severity highest from early summer to late autumn, coinciding with the main growth season of ryegrass. Plants heavily infected with crown rust show an increased amount of dead tissue, with tillering and root growth greatly reduced. Cruickshank (1957) found that perennial ryegrass infected with crown rust was unpalatable to sheep.

There appear to be several races of crown rust attacking *Lolium* species in New Zealand (Latch 1966). Considerable resistance to crown rust is available naturally and is now being incorporated into all new ryegrass cultivars (Latch *et al.* 1981).

Net blotch of ryegrass, caused by *Drechslera dictyoides* f. sp. *perenne*, was considered by Latch (1966) to be the second most important foliage disease of ryegrass in New Zealand, being widespread and found on all ryegrass cultivars. It is particularly severe when conditions are favourable for the spread of the fungus - in long dense swards which allow rapid sporulation and in short pastures over the winter months, when badly infected pastures have a yellow appearance.

Two other species of *Drechslera* are recorded on ryegrasses in New Zealand. Brown blight, caused by *D. siccans*, is found in New Zealand throughout the year, in particular on *L. multiflorum*. It can cause more damage to individual plants than *D. dictyoides* but is less common and so of less importance (Latch 1966). *D. nobleae*, first recorded on ryegrass seed (Matthews 1971), has been isolated occasionally from leaf

lesions on *L. multiflorum* in New Zealand (Mckenzie & Matthews 1977) but its importance has not been assessed. Species of *Drechslera* have been reported causing damage to ryegrass foliage in various parts of the world and these will be discussed in chapter three.

Scald caused by *Rhynchosporium orthosporium* is the only other disease of ryegrass considered by Latch (1966) to be of any economic importance in New Zealand. Heavily infected plants were reported to be unpalatable to sheep.

Stem rust, caused by *Puccinia graminis*, is seldom of any importance in New Zealand (Latch 1966). It has, however, been reported to be the most serious disease of seed production fields of perennial ryegrass in Western Oregon, where fungicide applications are required to prevent seed yield losses of up to 93% (Meyer 1982).

1.4 Major epiphytotics caused by species of *Drechslera*.

Two major disease outbreaks attributed to species of *Drechslera* serve to illustrate the destructive potential of this widespread genus.

Padmanabham (1973) reviewed the Bengal famine of 1943, caused by an epiphytotic of *D. oryzae* on the rice crop that year, where it was estimated that two million people died of starvation. Environmental conditions favourable to the fungus appear to have caused losses in yield of forty to ninety percent among all cultivars. The only other incident of comparable magnitude is the Irish potato famine of 1845.

In 1970 the corn crop of the United States was threatened by an epiphytotic of southern corn leaf blight caused by *D. maydis* (Ullstrup 1972). Yield losses of fifty to seventy-five percent were prevalent in the southern states,

with total losses common. The outbreak was due to an extreme reduction in genetic diversity of the host crop together with the appearance of a new physiological race of the pathogen. A moist growing season in 1970 initiated an epiphytotic disastrous to the farming community and one that would have been a major disaster in a less developed country.

1.5 Taxonomy of the genus *Drechslera*

When the net blotch fungus was first described by Drechsler in 1923 it was placed in the genus *Helminthosporium*, as were all the graminicolous species which are presently within the genera *Drechslera* and *Bipolaris*. The discovery that the spores of the type species of *Helminthosporium*, *H. velutinum* Link ex Fries, were produced apically and laterally on erect conidiophores, whilst the spores of the graminicolous species were produced on sympodullae (Subramanian & Jain 1966) prompted a revision of the genus.

Shoemaker (1959) transferred the graminicolous species of *Helminthosporium* into two genera, *Drechslera* Ito and *Bipolaris*, a new genus he established. The two genera could be distinguished by the mode of germination of the conidia. Luttrell (1964) suggested, alternatively, that in deference to long-established usage, the name *Helminthosporium* should be retained for the graminicolous species and the genus *Spondylocladium* be used for the others.

Ibrahim & Threlfall (1966), in a numerical taxonomic study, confirmed *Drechslera* and *Bipolaris* as separate genera. Subramanian & Jain (1966), on the other hand, considered they should be contained within a single genus - *Drechslera*. While they agreed that the two groups could be distinguished it was felt that the differences were not of sufficient

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magnitude to justify their separation.

Today all three genera - *Helminthosporium*, *Drechslera* and *Bipolaris* - are found in the literature. In New Zealand and Great Britain, it is standard to use the generic name *Drechslera* where the imperfect state is referred to. *D. dictyoides*, *D. siccans* and *D. nobleae* all conform to Shoemaker's (1959) criteria for the genus *Drechslera* which, where known, have perfect states within the ascomycete genus *Pyrenophora*.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Isolation and Maintenance

2.1.1 Isolation of fungal pathogens

Naturally infected ryegrass leaves, showing disease symptoms, were collected. They were placed on moist filter paper in plastic petri dishes and incubated at room temperature with a cycle of twelve hours near ultra-violet light (Phillips black light, model number TL 10W/08 peak output 350 nm) plus white light (cool white fluorescent tube) and twelve hours dark, to induce sporulation. When lesions had sporulated the identity of the fungus was confirmed using a stereoscopic microscope. Conidia were picked from the lesions with a sterile needle and mixed with a few drops of sterile distilled water on a sterile microscope slide. The suspension was streaked over the surface of Malt Extract Agar (MEA), solidified on sterile microscope slides and then incubated at 25°C in sterile petri dishes. After about three hours, single germinated conidia were transferred to pure culture using a single spore isolator constructed to fit the low power (X 10) objective of a Bausch and Lomb compound microscope. The culture medium contained 10 µg ml⁻¹ benomyl to restrict growth of unwanted saprophytes such as *Penicillium* and 20 µg ml⁻¹ streptomycin to inhibit bacterial growth.

2.1.2 Maintenance of cultures

Monoconidial isolates were cultured on slopes of MEA in McCartney bottles. One set was kept under sterile mineral oil for long term storage and at 5°C for periodic subculturing.

Some isolates were also stored on dried, reinfected ryegrass leaves. Spore suspensions were periodically inoculated onto living leaf tissue and reisolated when sporulation commenced, to maintain virulence and cultural characteristics at their initial level.

2.1.3 Growth media

Details of the culture media used are given in appendix one.

2.1.4 Growth of ryegrass

Seeds were planted in John Innes potting mix (two parts loam, one part sand, one part peat, plus dolomite) and plants were grown in a glasshouse. Tillers of perennial ryegrass lines selected from the field trial were grown in a glasshouse on John Innes potting mix and outdoors.

2.2 Experimental methods

2.2.1 Preparation of spore suspension

For the production of uniform conidia for spore suspensions, the fungus was cultured on sterile leaf pieces placed on the surface of Tap Water Agar (TWA) plates. Plates were incubated at room temperature with a cycle of twelve hours white light (cool white fluorescent tube) supplemented with near ultraviolet light (Phillips black light tube, model TL 20W/08, peak output 350 nm) followed by twelve hours dark. This encouraged production of a dense cover of conidia on the leaf surface, with dimensions similar to those found in nature.

One to two weeks after inoculation, sporulating leaf pieces were added to sterile double glass distilled water with $0.5 \mu\text{l ml}^{-1}$ 'Tween 80' (a wetting agent) in sterile

McCartney bottles. They were shaken with a mechanical vibrator to dislodge the conidia and then the leaf pieces were removed. The resulting spore suspension was centrifuged and resuspended to give a final concentration of 30,000 to 40,000 spores ml^{-1} with sterile double glass distilled water plus $0.5 \mu\text{l ml}^{-1}$ 'tween 80'. Spore suspensions were made up just prior to use.

2.2.2 Spore germination on glass slides

To assess spore germination on glass slides, 20 μl drops of spore suspension were placed on glass microscope slides which had been cleaned in chromic acid or ethanol and rinsed in sterile double glass distilled water. When sampling, three replicate sets of 100 conidia per treatment were assessed microscopically for evidence of germination. Germination was defined as having occurred when a germ tube at least $10^{-1} 15 \mu\text{m}$ in length had been produced.

2.2.3 Inoculation methods

Detached leaves

Unless effects of leaf age were being investigated, leaf three (the youngest fully expanded leaf) was inoculated to reduce variability. Detached leaf pieces approximately 3 cm in length were placed in petri dishes on filter paper moistened with $0.2 \mu\text{g ml}^{-1}$ kinetin. The abaxial surface was inoculated with a 10 μl drop of spore suspension which usually spread about 5 mm along the surface of the leaf, dispersing the conidia sufficiently to enable observation of individual conidia. Control leaves were inoculated with distilled water with the usual concentration of 'Tween 80'. Where the adaxial surface was inoculated, the spore suspension

was more dilute to prevent clumping of conidia and the concentration of 'Tween 80' was increased to enable the inoculation drop to stay on the waxy leaf surface. Appendix two outlines the reasons for the use of 'Tween 80', kinetin and detached leaves. Inoculated leaves were incubated at 21°C with a sixteen hour photoperiod (approximately 200 μ E light intensity).

Whole plants

Where whole plants were inoculated they were sprayed to run-off point using a chromatographic visualisation sprayer with a spore suspension adjusted to 10,000 spores ml^{-1} and placed in a humid chamber (a perspex box with limited ventilation for at least twenty-four hours).

2.2.4 Leaf clearing

To enable microscopic examination of the infection process, it was first necessary to clear the leaves of chlorophyll and two methods were found to be satisfactory. The first technique is a modification of that used by Ryan & Clare (1974). A sheet of chromatography paper (Whatman No. 1) was placed in a chromatography tank with one end in the upper solvent trough and the other draped over a glass rod 5 cm from the trough and level with it. Leaf pieces were placed on the horizontal surface, the inoculated side uppermost. Carnoy's fluid, consisting of ethanol, chloroform and acetic acid at the rate of 60:30:10, was added to the trough and allowed to flow down the sheet until the leaves were completely decolorised (about twenty-four hours). Leaf pieces were then stained with cotton-blue and mounted in lactophenol. This technique allowed leaf pieces to be cleared without dislodging the conidia on the surface, enabling a calculation of percent germination, germ tube production etc. when studying

prepenetration stages.

The second technique was more rapid, but caused dislodging of many unpenetrated and most ungerminated conidia. Leaf pieces were cleared by boiling in alcoholic lactophenol (equal parts lactophenol and ethanol) for eight minutes, then stained with cotton-blue and mounted in lactophenol.

2.2.5 Assessment of infection

Mounted leaf pieces were examined microscopically, to assess the stages in pre-penetration to penetration. Conidia in clumps were disregarded as it was often impossible to determine the origins of individual germ tubes. Spore germination was assessed for two hundred conidia per leaf piece and germ tube number and numbers of appressoria and penetrations were recorded for fifty random conidia.

Visual symptoms were recorded after four days using a scale of 0 - 4 where:

- 0 = no visible symptoms
- 1 = small spot or fleck lesions
- 2 = small net lesions
- 3 = net lesions + chlorosis
- 4 = complete chlorosis and necrosis

2.2.6 Assessment of sporulation on the leaf

Sporulation was assessed after seven days on a visual scale of 0 - 4 where:

- 0 = no sporulation
- 1 = limited sporulation over a limited area
- 2 = limited sporulation over a wider area or moderate sporulation over a limited area
- 3 = marked sporulation over parts of the leaf
- 4 = marked sporulation over most of the leaf

2.2.7 Photography and drawing

All light microscope photographs were taken on a Leitz Orthomat microscope. Stereomicroscope photographs were taken on a Wild stereomicroscope. Pan F was used for black and white photographs and developed in Rodinal followed by Amfix. Agfachrome 50L was used for colour slides. Drawings were made with the use of a Reichert microscope with a drawing arm attached.

2.2.8 Microscopic examination of living material

Time course studies using living host material require the use of tissues which allow the transmission of light for optical microscopy. The chlorophyll present in the mesophyll cells of the ryegrass leaf precludes the use of entire leaves in the microscopic examination of the host epidermis. Two simple systems were developed to avoid this problem.

(i) The epidermal peel

The abaxial epidermis is peeled from a ryegrass leaf and placed on a small amount of distilled water on a microscope slide. A drop of spore suspension is placed on the epidermis and a coverslip is placed on top. The slide is then placed in a humidity chamber and removed at intervals for observation under a compound microscope.

(ii) Leaf sheaths

The ryegrass leaf sheath is largely free of chlorophyll and so can be mounted intact for microscopic examination of the infection process. The active process of cytoplasmic streaming can be followed and the reaction of the host epidermal cell to the pathogen visualised. Two to three centimetre leaf sheath pieces are cut and treated in the same manner as the epidermal peels.

The advantage of leaf sheaths over epidermal peels is that the cytoplasm of the cells continues to react normally for several days after excision. Cytoplasmic streaming and nuclear migration can be followed. The disadvantage of using leaf sheaths is that fewer appressoria are formed than on the leaf. This appears to be due to the slight differences in surface morphology between leaves and leaf sheaths.

The advantage of using epidermal peels is that the morphology of spore germination, germ tube production and appressoria formation is essentially the same as that on entire leaves. The disadvantage is that host cells only live for a few hours and show little internal reaction to the invading hyphae.

In view of the stated advantages and disadvantages, epidermal peels were used to determine the sequence of fungal events, especially up to penetration, while the leaf sheath was used to investigate the host reaction to invasion.

2.2.9 *Lolium* endophyte

To visualise the *Lolium* endophyte by light microscopy, a method similar to that of Lloyd (1959) was used. Leaf sheaths were boiled in alcoholic lactophenol cotton blue and examined for the typical blue-stained endophytic fungus.

2.2.10 Assessment of *Drechslera* species in ryegrass seed samples.

Ryegrass seed samples were placed on moist filter paper in petri dishes and incubated at room temperature with twelve hours near ultraviolet plus white light followed by twelve hours dark. Fructifications were identified under a stereo-microscope.

2.2.11 Statistical analyses

Results of multivariate experiments were analysed with an analysis of variance in conjunction with Duncan's New Multiple Range Test. Data from single variate experiments were submitted to an analysis of variance and means compared with the students T- distribution or by calculation of the least significant difference (LSD).

2.3 Techniques for electron microscopy

2.3.1 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the ryegrass epidermis and its interaction with conidia of *D. dictyoides* as well as the processes of conidium ontogeny. Fresh fungal material is easily damaged by the high vacuum of the SEM and this necessitated fixation and dehydration by critical point drying.

2.3.1.1 Critical point drying

The technique chosen was similar to that of Samson *et al.* (1979). Inoculated leaf samples were fixed overnight in six percent gluteraldehyde at 4°C. The material was then rinsed twice in distilled water (10 minutes each) and dehydrated in 2-methoxyethanol for ten minutes, followed by two washings in 100% acetone (ten minutes each). The samples were transferred to a critical point dryer and dried in CO₂. The dried specimens were mounted onto stubs with double-sided 'sellotape', coated with gold in a sputter coater (Polaron Equipment Ltd, Coating Unit E 5000) and examined in a SEM (Cambridge Stereoscan 600) at 15 kv. Photography was with a Nikon F2 35 mm camera on Ilford FP4 developed in Microphen and fixed in amfix.

2.3.1.2 Fresh coating

The solvents used in critical point drying remove epicuticular leaf waxes, therefore when these were studied, it was necessary to coat fresh material. Specimens were attached to stubs and coated as before.

2.3.2 Transmission electron microscopy

The ultrastructure of *D. dictyoides* and ryegrass leaves and their interaction, was studied using the transmission electron microscope (TEM). Leaf material, infected and healthy, was prepared using the following technique.

2.3.2.1 Standard TEM procedure

- (i) Leaf material was flooded with 0.025 M phosphate buffer, pH 7.2 and cut into small segments. A stereomicroscope was used to identify suitable sites in the case of inoculated material.
- (ii) segments were plunged into four percent glutaldehyde plus one percent acrolein (although this was sometimes omitted) in phosphate buffer and fixed for four hours or overnight, at 4°C.
- (iii) Specimens were washed in two changes of fresh buffer (five minutes each) and then post-fixed in two percent osmium tetroxide in phosphate buffer at 4°C for four hours.
- (iv) Specimens were washed in two more changes of buffer and dehydrated in an acetone series at twenty percent steps (15 minutes each except for 30 minutes for 80% and 100%).
- (v) Dehydrated specimens were then placed in a vacuum for ten minutes.
- (vi) Dehydrated specimens were infiltrated with Spurr's resin (Spurr 1969) in an acetone/Spurr's mixture 50:50 v/v on a rotary mixer for 24 hours.
- (vii) The mixture was replaced with straight Spurr's resin and left for one hour.
- (viii) Specimens were embedded in fresh Spurr's resin and polymerised for twelve hours at 60°C.

Araldite was tried as an alternative to Spurr's but appeared to be too viscous for the grass material, giving poor infiltration.

Optical sections, 4 μm thick, were cut on an LKB Pyramitome, placed in a drop of water on a clean microscope slide and heat fixed to the slide. Where it was necessary to increase definition of structures, sections were stained with an equal mixture of azure-blue and toluidine-blue, but this was not normally necessary.

Where possible, a mesa was cut from the original block incorporating the desired structure and ultrathin sections showing silver to grey interference colours, were cut on an LKB Ultratome II. Sections were flattened with chloroform, mounted on formvar-coated 100 mesh copper grids and stained with uranyl acetate in 50% ethanol (15 - 20 minutes) followed by lead citrate (5 minutes) (Sato 1967). They were then examined in a Hitachi HS-7S electron microscope.

2.3.2.2 Method for remounting thick sections for ultrathin sectioning.

It is frequently advantageous to be able to re-mount thick (3 - 8 μm) sections for further ultrathin sectioning. In studies of small structures, such as fungal spores or penetration pegs of hyphae, only one thick section in a hundred may contain the required stage in the correct plane, making location of desired structures difficult. An added advantage of remounting thick sections, is that the morphology of material can be observed by optical microscopy prior to ultrathin sectioning. This often enables problems of interpretation due to sections which are not median or not in the same plane as the structure, to be overcome. Several techniques for remounting thick sections have been published

but most are time-consuming or prone to failure. The methods of Grimley (1965) and Woodcock & Bell (1967) require the use of special equipment. The technique of Singh (1980) uses adhesive tape and that of Maunsbach (1979) uses sections that are only 0.5 - 1 μm thick. During the course of this study, a simple and rapid method of re-mounting thick sections was developed, which had an adequate success rate.

Sections 5 - 8 μm thick were cut from Spurr's-embedded material and heat-fixed onto glass slides which had been cleaned in ethanol. Sections containing objects of interest were photographed or drawn under a compound microscope. The rounded ends of empty gelatine capsules were cut off, leaving hollow cylinders and the flat end was dipped into a small amount of unpolymerised araldite. The cylinder was then placed over the section of the glass slide and incubated at 70°C for about six hours to seal the cylinder to the slide. Once sealed, the cylinder was filled with fresh araldite and incubated at 70°C. When polymerised the araldite cylinder could easily be snapped off the glass slide while still warm.

The remounted section could be viewed under a compound or stereo-microscope, the desired area chosen and a mesa cut. Ultrathin sectioning could then proceed as normal. At any stage, the mesa could be viewed under a compound microscope and it was simple to determine when the thick section had been completely re-sectioned.

When thick sections were sealed to a glass slide, it was important that they lay flat as any buckling may result in those parts floating up a few micrometres. Otherwise, there was usually little lifting of the section during re-embedding. Spurr's resin was tried in place of Araldite but was not as successful because it bound too strongly to the glass.

2.3.2.3 Preparation of a spore suspension for the TEM.

Spore suspensions were added to centrifuge tubes and centrifuged at each solution change to avoid losing spores. After post-fixing in osmium tetroxide and washing, spores were centrifuged to a pellet and then solidified in a little molten agar. Agar blocks containing spores were cut and treated according to the standard technique.

2.3.2.4 The PA-TCH-AgPr test for polysaccharides.

- 1) Ultrathin sections were mounted on gold, formvar-coated grids.
- 2) Grids were placed in 1% periodic acid for thirty minutes. Control grids were placed in distilled water.
- 3) Grids were washed briefly in two changes of distilled water and then two longer changes of about ten minutes each.
- 4) Grids were placed in 0.2% thiocarbohydrazide in 20% glacial acetic acid for twenty to twenty-four hours.
- 5) Grids were passed through decreasing glacial acetic acid concentrations: 20%, 15% then three changes of 10%. They were left in each stage for twenty minutes.
- 6) Grids were placed briefly in 5% glacial acetic acid, followed by 2% and then distilled water. Three further changes of distilled water for twenty minutes each, were given.
- 7) The grids were put into 1% silver proteinate in distilled water. The solution was made up in a darkened room just before use. Grids were left thirty minutes in the dark.
- 8) Grids were rinsed two or three times briefly in distilled water, then dried and stored.

CHAPTER THREE

DRECHSLERA DISEASES OF RYEGRASSES

3.1 Introduction

In the following chapter and throughout the remainder of this thesis, the generic name *Drechslera* will be used in preference to *Helminthosporium* or *Bipolaris*. This conforms to the re-interpretation by Subramanian & Jain (1966) of Shoemaker's (1959) taxonomic revision of the genus *Helminthosporium* outlined in chapter 1.5 (p. 7).

Three species of *Drechslera* have been recorded causing leaf diseases on ryegrasses. These are *D. dictyoides* f. sp. *perenne*, *D. siccanis* and *D. nobleae*. Drechsler (1923) recorded a disease on meadow fescue with symptoms very similar to those induced by *D. teres* on barley. The lesion consisted of longitudinal and transverse linear streaks, forming a minute reticulate design. Because of the similarity in form to net blotch of barley, caused by *D. teres*, Drechsler (1923) used the same term for the fescue disease. The specific name *dictyoides* refers to the reticulate lesion. Dennis & Wakefield (1946) recorded a species of *Drechslera* on *Lolium perenne* which conformed to Drechsler's description of *D. dictyoides*.

Braverman & Graham (1960) separated the forms of net blotch on *Festuca* and *Lolium* into two formae speciales, *D. dictyoides* f. sp. *dictyoides* causing a net blotch and blotch on leaves of *Festuca* and *D. dictyoides* f. sp. *perenne* causing a net blotch on leaves of *Lolium*. Scharif (1963) elevated the two formae speciales of *D. dictyoides* to specific rank, naming the species on *Lolium* *D. andersenii*,

but neglected to give a Latin description. Two forms of *D. andersenii* were also described (Scharif 1963), differing mainly in the frequency with which secondary conidia were produced in moist chambers.

Numerical taxonomic techniques were applied to a number of *Drechslera* species by Ibrahim & Threlfall (1966) who confirmed the two forms of *D. dictyoides* but did not consider them worthy of specific rank. Again using numerical taxonomy, Lam (1981) found the differences between the two forms of *D. dictyoides* to be as great as those between *D. nobleae* and *D. siccans* and considered them worthy of specific rank in accordance with Scharif (1963). However in the absence of a Latin description *D. dictyoides* and *D. dictyoides* f. sp. *perenne* remain the only valid names.

D. siccans Drechsler is the other species of *Drechslera* commonly found on ryegrass and was first observed in June 1922 in the United States (Drechsler 1923). The symptoms consisted of minute, longitudinally elongated, dark brown spots. Severely affected leaf blades often had more than a hundred of these spots, sometimes giving the appearance of a net blotch, but without the transverse flecks. Sampson & Western (1940) reported finding *D. siccans* as early as 1921 and Dennis & Foister (1942) first reported the disease in Scotland. Ibrahim & Threlfall (1966) considered *D. siccans* to be identical to *D. avenae*.

The most recently recorded *Drechslera* disease of ryegrass is *D. nobleae* McKenzie & Matthews. Matthews (1971) reported the common occurrence of an undescribed species of *Drechslera* on seeds of New Zealand ryegrass cultivars and commented that this species had also been found in Scotland and West Germany. Small scale trials with the cultivars Manawa and Paroa showed the fungus to be pathogenic to those

Taxonomic details of the three species will not be given here but referred to as required in the discussion.

The perfect state of *D. dictyoides*, *Pyrenophora dictyoides*, has not been found in nature (Latch *et al.* 1981) but has been produced in culture by pairing isolates from *L. perenne* and *Vulpia myurus* (Paul & Parbery 1968). The absence of *P. dictyoides* in nature indicates that the asexual cycle of the fungus is capable of effectively maintaining the disease in ryegrass stands (Latch *et al.* 1981).

Dovaston (1948) reported the production of a species of *Pyrenophora* in culture with an imperfect state corresponding to *D. siccans*. He named it *P. lolii*, recorded its similarity to *P. avenae* and noted it was slightly pathogenic to oats as well as causing a net blotch on ryegrass. This report has not been confirmed (Ellis & Holliday 1976).

The *Drechslera* diseases of ryegrasses have been reported widely around the world from both seed and infected leaves. Matthews (1971) recorded levels of seed-borne inoculum in four ryegrass cultivars in New Zealand and found *D. dictyoides* was the predominant species on the cultivar 'Ruanui' with *D. siccans* and a trace of *D. nobleae* also present. In the Italian ryegrass cultivar 'Tama', the situation was reversed with *D. siccans* and *D. nobleae* most common. The cultivar 'Manawa' (*L. multiflorum* X *L. perenne*) had similar levels of all three species, whilst the cultivar 'Ariki' (*(L. multiflorum* X *L. perenne)* X *L. perenne*) showed a similar species distribution to that on 'Ruanui'. This was confirmed by McKenzie (1978). Tribe & Herriot (1968), in Scotland, found perennial ryegrass seed had 67% *D. dictyoides* infection and four percent *D. siccans* infection. Lam (1982) surveyed certified seed lots in the United Kingdom for

presence of *Drechslera* species, finding that *D. dictyoides* was the predominant species on perennial ryegrass with *D. siccans* present in a few samples. On Italian ryegrass *D. dictyoides* and *D. siccans* occurred with similar frequency and *D. nobleae* was present in a few samples.

In summary, *D. dictyoides* is normally the predominant *Drechslera* species on perennial ryegrass seed, whilst *D. siccans* and *D. nobleae* are found on Italian ryegrass seed.

Levels of seed infestation are mirrored by the leaf infection observed in the field. A number of workers have commented on the relative abundance of the three *Drechslera* species on ryegrass. Drechsler (1923) initially found *D. siccans* on Italian ryegrass, although he later found it on perennial ryegrass. In New Zealand *D. dictyoides* is widespread on all cultivars, but is especially severe on *L. perenne* (Latch 1966). Scharif (1963), in England, found *D. dictyoides* only on *L. perenne* and *D. siccans* predominantly on *L. multiflorum* whilst Lam (1981) found *D. dictyoides* on *L. perenne* but also on *L. multiflorum*. Morrison (1982), in the United States, recorded *D. nobleae* on *L. multiflorum* whilst Lam (1981) found it predominantly on *L. multiflorum* and occasionally on *L. perenne*.

Some workers: Teuteberg (1977) in Germany, Makela (1971) in Finland and Wilkins (1973) in Wales, have claimed that *D. siccans* was more common than *D. dictyoides* on *L. perenne* but Lam (1981) suggested the differences may have been due to misidentification. She examined fructifications under the stereo-microscope and claimed it was difficult, if not impossible, to identify these species accurately by symptoms alone.

The natural host range of *D. dictyoides* f. sp. *perenne* is confined to the ryegrasses (Latch 1966) and *D. nobleae*, likewise, has only been reported on ryegrass (Mckenzie & Matthews 1977, Lam 1981, Morrison 1982). *D. siccans* is confined to the ryegrasses, except for one report of the fungus on haricot beans (Meyer 1962). Ibrahim & Threlfall (1966) believed *D. siccans* to be identical to *D. avenae*, resolving the problem of alternative hosts to *D. avenae*. Lam (1981) found isolates of *D. avenae* to be non-pathogenic to ryegrass but *D. siccans* to be pathogenic to oats and this does not verify the claim of Ibrahim & Threlfall (1966).

While the natural host ranges of these three *Drechslera* species are limited almost entirely to ryegrasses, a number of other hosts have been artificially infected. Artificial hosts of *D. dictyoides* f. sp. *perenne* reported in the literature are:

Bromus mollis, *B. willdenowii*, *Festuca arundinacea* (Latch 1966) *F. pratensis* (Latch 1966, Ibrahim & Threlfall 1966, Makela 1971), *F. rubra* (Latch 1966, Makela 1971) and *Arrhenatherum elatius* (Latch 1966).

Also on seed of: *Dactylis glomerata*, *Cynosurus cristatus* and *Phleum pratense* (Mckenzie 1978).

Artificial hosts of *D. siccans* are:

B. mollis, *B. willdenowii*, *F. arundinacea*, *Holcus lanatus* (Latch 1966), *A. elatius*, *F. pratensis*, *D. glomerata* (Latch 1966, Ibrahim & Threlfall 1966), *Hordeum vulgare*, *Anthoxanthum odoratum* (Ibrahim & Threlfall 1966) and *Avena sativa* (Ibrahim & Threlfall 1966, Lam 1981).

Artificial hosts of *D. nobleae* are:

F. arundinacea and *F. rubra* (Morrison 1982).

3.2 Taxonomy

3.2.1 Taxonomic descriptions

During the course of this study, leaves with lesions were collected and incubated in moist chambers. Taxonomic characters were then assessed and the results summarised with taxonomic descriptions based on these lesions. Dimensions were measured using a Bausch and Lomb microscope with a calibrated graticule eyepiece.

Drechslera dictyoides (Drechsler) Shoemaker f. sp. *perenne* (Braverman & Graham) Shoemaker. (based on twenty lesions)

Conidiophores: mainly single but sometimes in groups of two to six; sometimes from stomata but usually from or between epidermal cells; light brown; septate; length to first conidium 20 - 110 μm (usually 30 - 90 μm); width uniform (6 - 11 μm) but enlarged at points of conidium attachment; often globose at base (9 - 15 μm); geniculate at points of conidium attachment; conidia produced at intervals on conidiophore.

Conidia: solitary; sometimes with secondary conidia present in damp chamber or culture; very pale straw coloured; straight; maximum diameter (15 - 23 μm , usually 17 - 19 μm) usually near basal septum, tapering to apical segment (9 - 20 μm , usually 10 - 15 μm); more rarely approximately cylindrical; length 45 - 220 μm (usually 70 - 120 μm); number of septa 2 - 10 (usually 3 - 8); hilum 4.5 - 8.5 μm (usually 4.5 - 6.5); germination from basal cell and often apical cell.

Lesion types: most commonly 'net' or 'net blotch' lesion although occasionally isolated from 'spot' lesions. Also isolated from yellowing tips of leaves.

Forms: two forms are present. In form one, secondary conidia are rarely formed, even in a moist chamber. Maximum length of conidium is 170 μm . In form two, secondary conidia are frequently formed in a damp chamber. Maximum length of primary conidium is 220 μm , with secondary conidia usually shorter (60 - 95 μm) and less tapered.

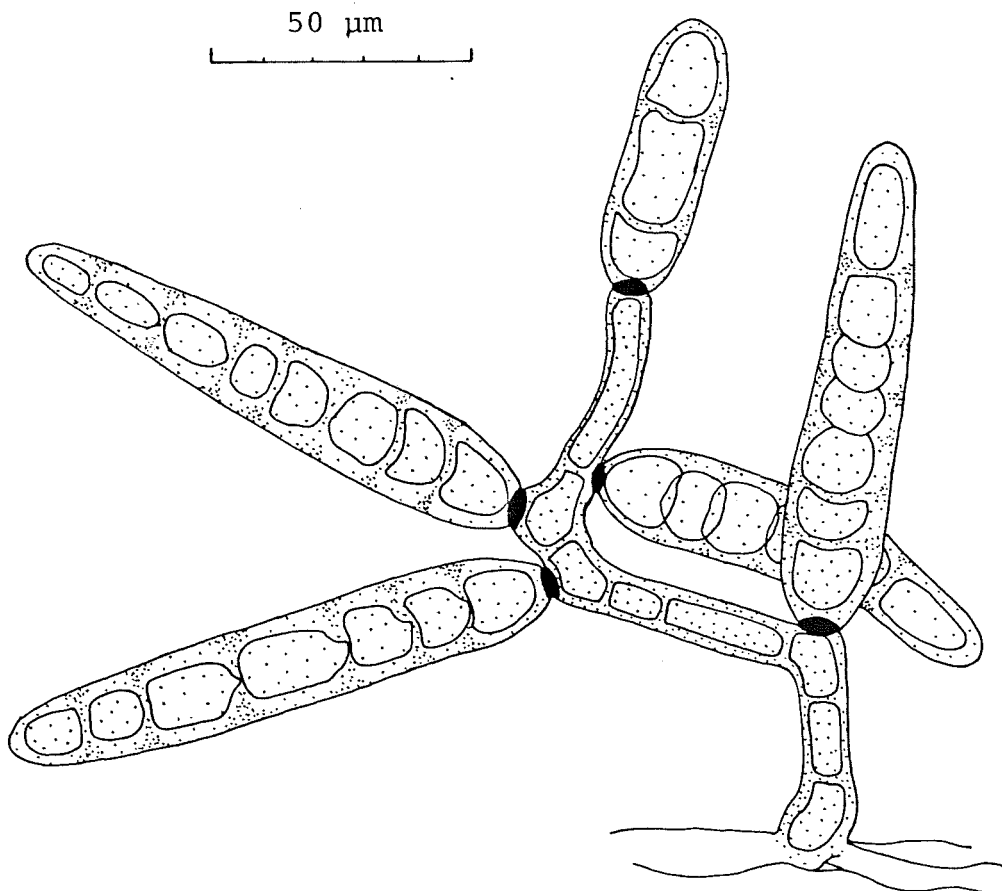
The description given here is within the range given by other workers. This study is the first in New Zealand to report the presence of two forms of *D. dictyoides* as outlined by Scharif (1963) in Britain. The majority of lesions produced form '1' conidia, although the relative occurrence was not assessed. Differences in morphology and pathogenicity between the two forms was also not assessed. *D. dictyoides* is illustrated in figs. 3.1 and 3.2.

A comparison was made between conidia and conidiophores produced in humidity chambers and those produced under natural conditions. The results are presented in table 3.1. Conidiophores produced in nature were shorter and thicker than those produced in humidity chambers. Conidia were of similar dimensions whether produced naturally or in humidity chambers, although those produced in nature had a slightly greater diameter. These results indicate that conditions of spore production should be taken into consideration when making taxonomic assessments.

Drechslera siccans (Drechsler) Shoemaker (based on five lesions).

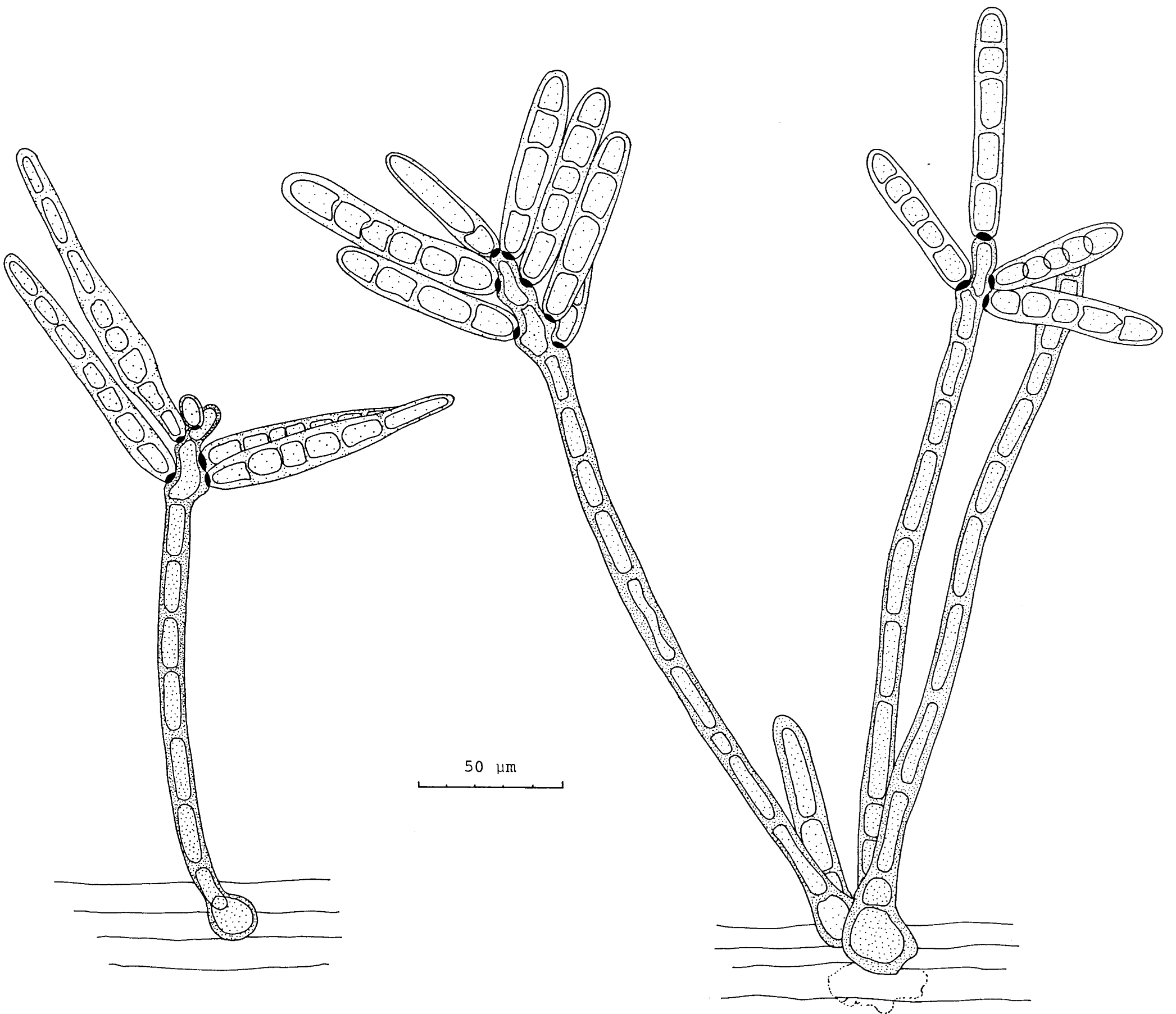
Conidiophores: mainly single but sometimes in groups; brown; lighter at apex; 3 - 10 septate; length to first conidium 110 - 400 μm (usually 150 - 320 μm); width

Fig. 3.1. Species of *Drechslera* pathogenic to ryegrass,
Lolium, species.
Conidiophores and conidia from leaves of *Lolium*
species.



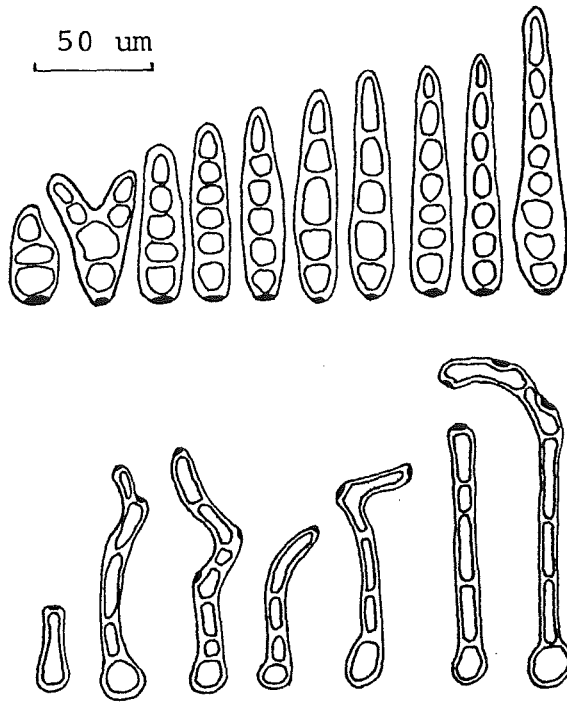
Drechslera dictyoides (Drechsler) Shoemaker f. sp. *perenne*
(Braverman & Graham) Shoemaker.

Fig. 3.2. Morphology of conidiophores and conidia of *D. dictyoides* f. sp. *perenne* (forms 1 and 2), *D. siccans* and *D. nobleae* on ryegrass leaves.

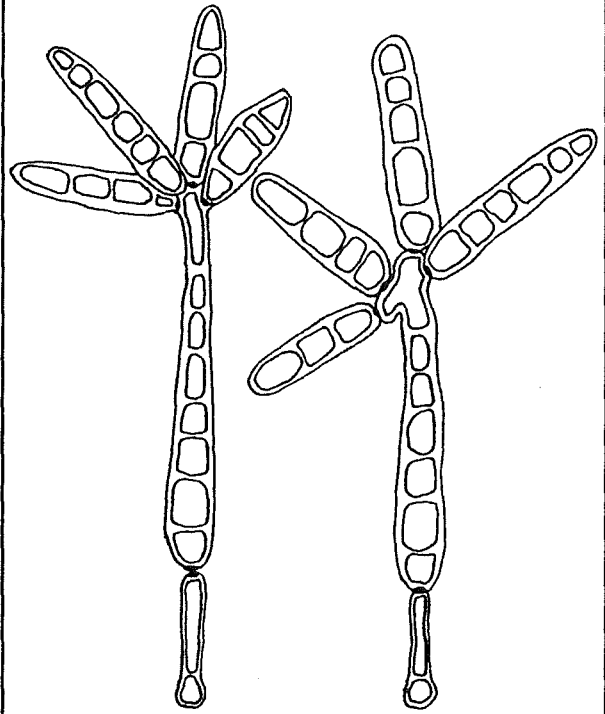


Drechslera nobleae McKenzie & Matthews

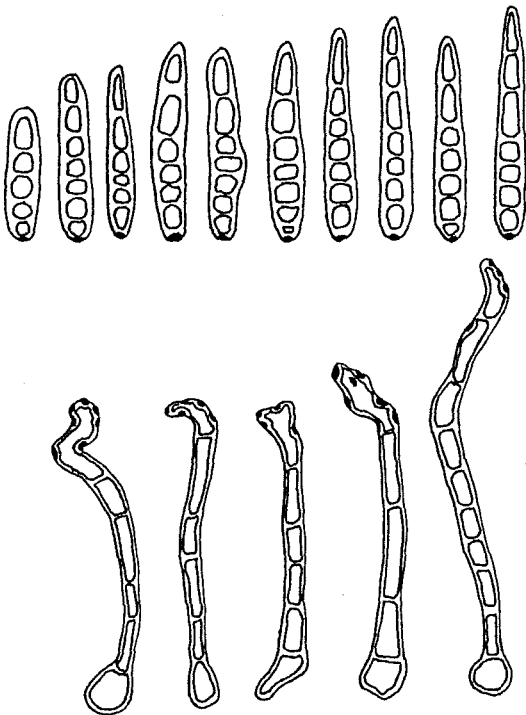
Drechslera siccans (Drechs.) Shoemaker



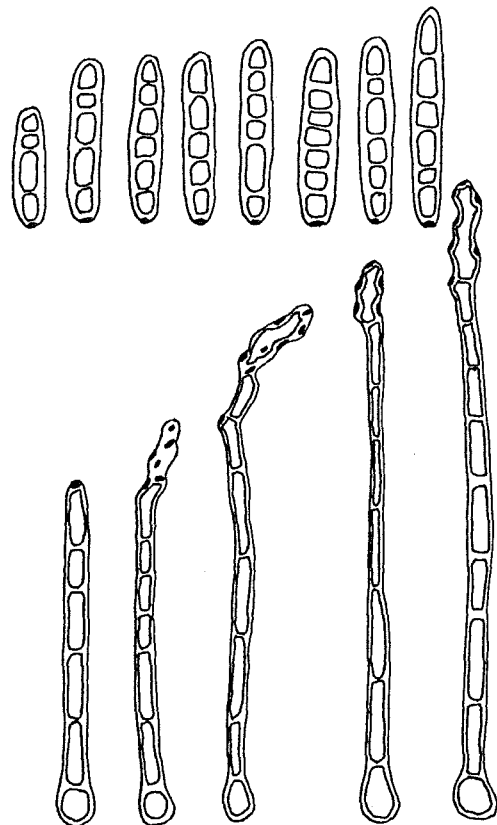
D. dictyoides f. sp. *perenne*
form 1



D. dictyoides f. sp. *perenne*
form 2



D. nobleae



D. siccans

Table 3.1. Comparison of fructifications of *D. dictyoides* produced in a humidity chamber with those produced in nature.

		Produced in humidity chamber (20 lesions)	Produced in nature (7 lesions)
Conidio- phores	length to first conidium (μm)	62 b*	46 a
	width (μm)	8.4 a	9.8 b
	basal width (μm)	11.7 a	12.6 b
Conidia	hilum width (μm)	6.3 a	6.6 a
	max. diam. (μm)	18.4 a	20.1 b
	min. diam. (μm)	12.4 a	13.7 b
	length (μm)	99 a	97 a
	number of septa	5.2 a	4.9 a

* means ^{horizontally} followed by the same letter are not significantly different ($P = 0.05$) T - test.

uniform (7 - 10 μm); often globose at base (10 - 18 μm); often geniculate near apex; conidial scars close together at apex.

Conidia: pale straw coloured; normally straight; cylindrical; 11 - 19 μm in diameter; length 40 - 120 μm (usually 70 - 100 μm); number of septa 3 - 7 (usually 4 - 6); hilum 4.5 - 6.5 μm ; germination by production of one to two germ tubes from each of the basal and apical cells.

Lesion type: "Spot", also isolated from chlorotic/necrotic regions of the leaf.

The present study is consistent with previous work. *D. siccanis* is illustrated in fig. 3.1 and 3.2.

Drechslera nobleae Mckenzie & Matthews (based on two lesions).

Conidiophores: mainly single; very dark brown; erect; straight or slightly curved; 3 - 10 septate; length to first conidium 44 - 370 μm (usually 100 - 220 μm); width uniform (7 - 10 μm), often globose at base (10 - 18 μm); geniculate near apex; conidial scars close together at apex.

Conidia: pale straw coloured; normally straight; maximum diameter (11 - 13 μm) usually at second cell from base, tapering to apical segment (6 - 7 μm); length 40 - 120 μm (usually 70 - 100 μm); number of septa 3 - 8 (usually 4 - 8); hilum 4.5 - 5.5 μm wide; germination from second cell back from base and usually apical cell.

Lesion type: usually spot or 'net-spot'.

The present study is in good agreement with previous results.

D. nobleae is illustrated in figs. 3.1 and 3.2.

3.2.2 Comparison of *D. dictyoides*, *D. siccans* and *D. nobleae*.

Mean figures for the dimensions of the fructifications of *D. dictyoides*, *D. siccans* and *D. nobleae* are given in tables 3.2 and 3.3. The greatest difference in the conidiophores

Table 3.2. Comparison of mean dimensions of conidiophores of *D. dictyoides*, *D. siccans* and *D. nobleae* produced on lesions incubated in moist chambers.

	length to first conidium (μm)	width (μm)	width at base (μm)
<i>D. dictyoides</i> (20 lesions)	62	8.4	11.7
<i>D. nobleae</i> (2 lesions)	152	8.3	12.7
<i>D. siccans</i> (5 lesions)	215	8.4	14.2
L.S.D. (5%)	5	0.4	0.6

Table 3.3 Comparison of mean dimensions of conidia of *D. dictyoides*, *D. siccans* and *D. nobleae* produced on lesions incubated in moist chambers.

	hilum width (μm)	max. diameter (μm)	min. diameter (μm)	length (μm)	number of septa
<i>D. dictyoides</i> (20 lesions)	6.3	18.4	12.4	99	5.2
<i>D. nobleae</i> (2 lesions)	5.5	11.4	6.1	90	5.8
<i>D. siccans</i> (5 lesions)	5.2	14.3		84	5.2
L.S.D. (5%)	0.3	0.5	0.4	6.5	0.3

was the length to the first conidium. *D. dictyoides* had the shortest conidiophore, being only about a third of the length of that of *D. nobleae* and about a quarter of that of *D. siccans*. *D. siccans* had a significantly wider conidiophore base than did *D. dictyoides* or *D. nobleae*.

Conidia of the species were similar in size although not in shape. The mean conidial length of *D. siccans* was significantly less than that of *D. dictyoides* or *D. nobleae*, although as the ranges overlapped (see taxonomic descriptions), this was not particularly useful in identification. Conidia of both *D. dictyoides* and *D. nobleae* were tapered, but there was a clear difference in conidial diameter, conidia of *D. nobleae* being significantly narrower and tapering more strongly than those of *D. dictyoides*. The hilum was wider in *D. dictyoides* than in *D. nobleae* or *D. siccans*.

The mode of spore germination differed between the three species in terms of the conidial cell involved (table 3.4). *D. nobleae* germinated predominantly from the second, or inflated, cell from the base. As this is unique to *D. nobleae* among the three species, it provides a ready

Table 3.4. Germination of *D. dictyoides*, *D. nobleae* and *D. siccans* according to conidial cell.

	% of conidia producing germ tube(s) from these cells			
	basal	cell 2	apical	intermediate
<i>D. dictyoides</i> (11 isolates)	88 b,b*	2 a,a	13 a,a	9 a,a
<i>D. nobleae</i> (3 isolates)	3 a,a	77 b,b	33 a,a	9 a,a
<i>D. siccans</i> (5 isolates)	81 b,b	1 a,a	73 b,b	7 a,a

* means for germ tubes from different cells (across ,a) or different species (down a,) followed by the same letter are not significantly different ($P = 0.05$) Duncan's multiple range test.

distinction from *D. dictyoides* and *D. siccans*. *D. dictyoides* and *D. siccans* usually produced germ tubes from the basal cell, the main difference being the frequency with which they also produced germ tubes from the apical cell and the total number produced per conidium. It was observed that *D. siccans* usually produced more conidia in total than *D. dictyoides*.

For rapid identification of species based on fructifications, only those characters readily distinguishable under the stereo-microscope are of value. The 'typical' diagnostic differences between the species can be summarised as follows:

D. dictyoides: conidia tapered and widely spaced on the conidiophore with only a relatively short distance to the first conidium.

D. nobleae: conidia tapered and produced close together at the apex of long conidiophores.

D. siccans: conidia cylindrical and produced close together at the apex of long conidiophores.

These differences suffice in general, but to ensure correct identification, dimensions of fructifications can be measured using a compound microscope and a few conidia removed and germinated in distilled water on a glass slide. The correct and easy identification of *Drechslera* to species level is vital to ensure past errors are not continued. Morrison (1980) reported that *D. poae* had previously been misidentified as *D. dictyoides*. Therefore, information published on the effects of temperature and light on sporulation of *D. dictyoides* (Vargas & Wilcoxson 1969) and the effects of sugar content of the host on *D. dictyoides* (Gibbs & Wilcoxson 1972) actually referred to *D. poae* and results may not apply to *D. dictyoides*. In fact, observations in the present study on sporulation are at variance with those of Vargas & Wilcoxson (1972). It seems probable, also, that previous reports indicating that *D. catenaria* or *D. siccans* were the predominant species of *Drechslera* on *L. perenne* are due to mis-identification.

3.2.3 Perfect state

Over the seventeen months during which the field trial was carried out (chapter 5) the trial site was regularly searched for ryegrass stubble bearing *Pyrenophora* perithecia. Over the 1981 winter, barley stubble was present on the site and fertile perithecia of *Pyrenophora teres* (perfect state of *D. teres*, the causal fungus of net blotch of barley) were common. No *Pyrenophora* perithecia were found on the ryegrass stubble, although other ascomycetes were present. This study is therefore in agreement with Latch *et al.* (1981) who considered that the asexual cycle was capable of maintaining the disease.

3.3 Species distribution

Leaves of *Lolium* species, containing lesions, were collected from the field throughout the course of this study and incubated under high humidity with a cycle of twelve hours light and twelve hours dark. The lesions were grouped according to 'lesion type' - typical net or net blotch (definite reticulate lesion with or without chlorosis), spot, net-spot (spot with some transverse or longitudinal flecking) or withered tip - and those producing *Drechslera* fructifications were recorded according to species. It should be noted that the time required for production of fructifications varied between species. *D. dictyoides* lesions sporulated after one to four days, depending on the degree of necrosis, whilst *D. siccans* seldom sporulated in less than a week. For this reason it was necessary to assess incubated leaves every few days for at least two weeks to avoid overlooking the presence of *D. siccans*. The time required for *D. nobleae* to sporulate was not recorded due to the infrequency of its occurrence.

Table 3.5 gives the results of this study. *D. dictyoides*

Table 3.5. Species of *Drechslera* according to lesion type on ryegrass.

	Lesion type	Number of <i>Drechslera</i> lesions assessed	Species
<i>L. perenne</i>	net blotch	96	all <i>D. dictyoides</i>
	spot	8	5 <i>D. dictyoides</i> 3 <i>D. siccans</i>
	withered tips	15	all <i>D. dictyoides</i>
<i>L. multiflorum</i>	net blotch	1	<i>D. dictyoides</i>
	net-spot	2	both <i>D. nobleae</i>
	spot	6	5 <i>D. siccans</i> 1 <i>D. nobleae</i>

was always present in net blotch lesions. These were sometimes surrounded by chlorosis but often only the reticulate lesion was present. Only one typical net lesion was found on *L. multiflorum* and this was caused by *D. dictyoides*. Spot lesions were not as distinctive as net lesions and were caused by a variety of pathogens. *D. siccans* was only isolated from spot or spot blotch lesions. *D. nobleae* appeared to have a lesion type intermediate between those of *D. siccans* and *D. dictyoides* but, as it was only recorded three times, little can be concluded.

To give information on the relative occurrence of the species, a small survey of Hagley Park, Christchurch, New Zealand, an area predominantly of recreation grassland, was undertaken. Five sites, where *L. perenne* was the dominant grass species, were chosen - two in the regularly cut areas of sports fields, and three others not regularly cut; two being among trees and one on a river bank. In each site, five randomly selected one square metre quadrats were assessed for disease. Net lesions were assumed to be caused by *D. dictyoides* whilst spot lesions were removed and incubated in the laboratory and their identity confirmed based on fructifications. In two of the sites of longer grass showing considerable disease, five randomly selected 20 cm X 20 cm quadrats were removed at ground level and the disease distribution was assessed more accurately in the laboratory. The survey was carried out during September 1982.

D. dictyoides was the predominant foliage pathogen, representing over ninety percent of the lesions at four of the sites and over sixty percent at the other. The sites on the sports fields had, on average, eight lesions per square metre caused by *D. dictyoides* and 0.5 unidentified

spot lesions. *D. siccans* and *D. nobleae* were not recorded. In two of the sites of longer grass there were, on average, 865 leaves per square metre of ground with lesions caused by *D. dictyoides*, three caused by *D. siccans* and forty-three other unidentified spot lesions.

L. multiflorum is also present in Hagley Park. It was generally healthy during the course of this survey, but any lesions present were incubated and *D. siccans* was identified occasionally.

From this study it is clear that *D. dictyoides* was the predominant foliage pathogen present on *L. perenne* in Hagley Park during the survey period, although crown rust is probably a problem during some seasons.

3.4 Cultural studies

3.4.1 Cultural characteristics

Five isolates of *D. dictyoides*, two isolates of *D. nobleae* and three isolates of *D. siccans*, were grown on Potato Dextrose Agar (PDA), Sucrose Proline Agar (SPA) and Coons Agar (CA) at 21°C in the dark. Morphological characteristics were noted after one week and are listed below. Colours were assessed using a mycological colour chart (Rayner 1970).

D. dictyoides

on PDA: Surface colour was smoke grey to grey-olivaceous

Reverse colour was dark olivaceous (sometimes brown)

Aerial hyphae were felty, with occasionally some tufting. White patches. In some isolates, uneven edge to colony.

Some fine dark hyphae running deep into agar.

on SPA: Surface colour was mostly dirty-white with some smoke-grey patches.

Reverse colour was olivaceous brown, olivaceous grey or light brown.

Aerial hyphae showed luxurious floccose growth, seldom tufting. Often uneven edge to colony.

Sometimes dark branched hyphae running deep into agar.

Pink pigment produced by some isolates.

on CA: Surface colour was grey-olivaceous with white tufts.

Reverse colour was olivaceous.

Aerial hyphae smooth and felty apart from numerous white tufts. Occasionally little or no aerial mycelium, just finely branched rhizoids in agar.

D. nobleae

on PDA: Surface colour was olivaceous grey.

Reverse colour was black.

Aerial hyphae flat, felty, dense.

No dark strands in agar.

on SPA: as on PDA.

No pigment produced

on CA: Surface colour was smoke-grey to grey-olivaceous

Reverse colour was grey-olivaceous.

Aerial hyphae scant, flat.

A few thick brown strands running into agar, mainly fine hyphae.

D. siccons

on PDA: Surface colour was smoke olivaceous-grey.

Reverse colour was dark grey olivaceous, some brown.

Aerial mycelium luxuriant and floccose, no tufting.

on SPA: Surface colour was smoke grey

Reverse colour was grey or grey olivaceous.

Aerial hyphae were luxuriant and floccose, no tufting.

Sometimes a slight pink pigment produced.

on CA: as on SPA but no pigment.

Radial growth was also measured after one week (table 3.6). Greatest growth was shown by *D. siccans* on all media,

Table 3.6. Growth of *D. dictyoides*, *D. siccans* and *D. nobleae* on agar media at 21°C in the dark.

	no. of isolates	Ave. colony diam. after one week (mm)			
		PDA	SPA	CA	MEAN
<i>D. dictyoides</i>	9	50 a,b*	37 a,ab	33 a,a	40 a
<i>D. nobleae</i>	2	77 b,a	76 b,a	73 b,a	75 b
<i>D. siccans</i>	3	80 c,a	78 b,a	77 b,a	78 c
	mean	60 b	51 a	48 a	

* Means for different fungal species (a, down) or for different agar media (,a across) followed by the same letter are not significantly different ($P = 0.05$) Duncan's multiple range test.

although the difference between *D. siccans* and *D. nobleae* was not great. *D. dictyoides* was the slowest growing species on all media. Growth of all species was faster on PDA than on SPA or CA. Sporulation was assessed after four weeks (table 3.7). Where sporulation had not occurred, plates were

Table 3.7 Sporulation of three species of *Drechslera* at 21° C in the dark.

	after weeks 4 weeks			leached plates		
	PDA	SPA	CA	PDA	SPA	CA
<i>D. dictyoides</i> (5 isolates)	+	+	+	-	-	-
<i>D. nobleae</i> (2 isolates)	-	-	-	-	-	-
<i>D. siccans</i> (3 isolates)	-	-	-	+	+	+

'leached' under running water following the technique of Halisky & Funk (1966) then reincubated and sporulation re-assessed a week later. *D. dictyoides* was the only species which sporulated in the first four weeks, but conidia were abnormally short and few were produced. *D. siccans* produced some conidia on leached plates but *D. nobleae* did not sporulate. Increased sporulation could be achieved by incubating plates under a light regime of twelve hours near ultraviolet light plus white light followed by twelve hours dark (see p.9).

Presence of protothecia was assessed after four weeks (table 3.8). *D. siccans* produced protothecia on all three media, *D. nobleae* on SPA and CA while none were produced by *D. dictyoides*.

Table 3.8 Presence of protothecia in 4 week old cultures of three species of *Drechslera*.

	no. isolates	PDA	SPA	CA
<i>D. dictyoides</i>	9	-	-	-
<i>D. nobleae</i>	2	-	+	+
<i>D. siccans</i>	3	+	+	+

3.4.2 Effect of temperature on the growth of *Drechslera* species on agar media.

One isolate of each of *D. dictyoides*, *D. siccans* and *D. nobleae* was grown at 4°C, 10°C, 15°C, 25°C, 30°C, 35°C and 40°C on PDA, SPA and Malt Extract Agar (MEA) in the dark. The diameter of growth was measured after five days and average growth rate in mm day⁻¹ calculated. Differences between species were greater than differences between media so results for the media were combined and growth of the three species assessed according to temperature.

Except near the minimum temperature supporting growth, *D. siccans* grew significantly faster than *D. dictyoides* or *D. nobleae* (fig. 3.3). Growth of *D. dictyoides* was slower than *D. siccans* or *D. nobleae* except near the highest temperature supporting growth. There were no differences in the maximum or minimum temperatures supporting growth between the three species.

Following this experiment, a more detailed study was made of the effect of temperature and agar media on growth of *D. dictyoides*. Three geographically different isolates of *D. dictyoides* were used: isolate 11/3 was from *L. perenne* in the Wairarapa, isolate 14/4 was from *L. perenne* in Wellington and isolate 5/1 was from *L. multiflorum* near Lincoln, Canterbury. The isolates were grown on MEA, SPA and V₈-juice agar (V₈A) in the dark at six different temperatures: 10°C, 15°C, 20°C, 25°C, 30°C and 35°C. Maximum/minimum thermometers were used to measure the temperature range in each incubator. Linear spread of mycelium along two perpendicular marked axes was measured daily over a period of seven days and linear regression equations calculated for the growth of each culture to give a linear growth rate. The data were subjected to analysis of variance in an orthogonal multifactor design.

The temperatures ranged +/- 1.5°C from the set temperatures. Mycelium spread at a linear rate across agar plates at temperatures of 10°C - 25°C. At 30°C the rate was linear to begin with, but slowed down after five to seven days, growth often ceasing altogether after seven days. Growth was minimal at 35°C, which was regarded as the maximum temperature for growth.

All regression coefficients except those at 35°C

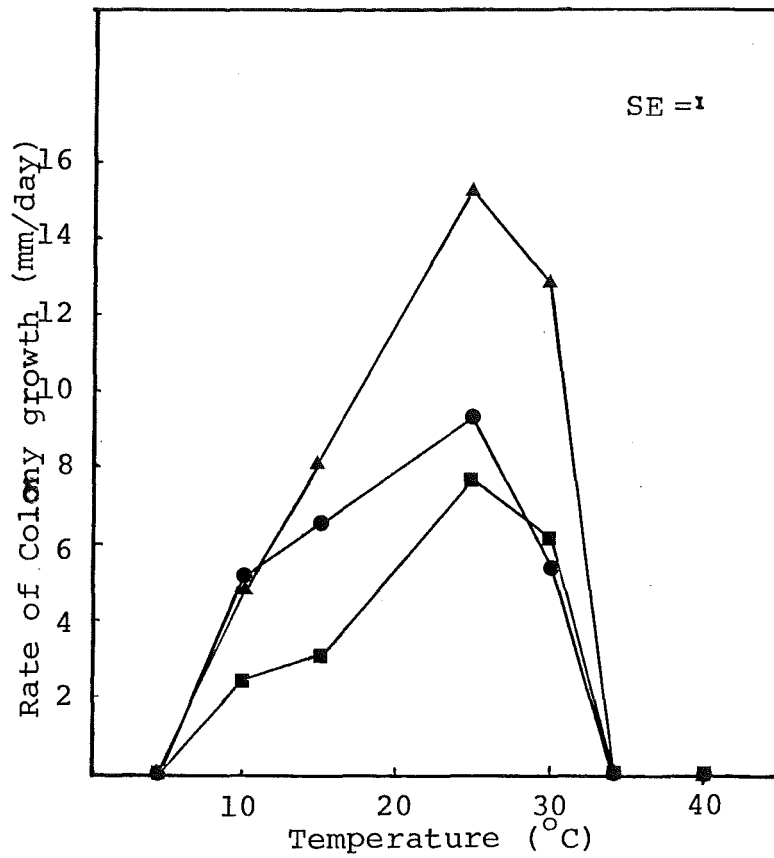


Fig. 3.3. Growth of *D. dictyoides* (■), *D. siccans* (▲) and *D. nobleae* (●) on agar media according to temperature.

were submitted to statistical analysis. Three way interaction between the factors was highly significant and for this reason lower order interactions were not separated. Fig. 3.4 illustrates the isolate/media/temperature interaction. The temperature supporting optimum growth was 25°C for all three isolates on MEA and for isolate 5/1 on all media. Isolate 14/4 had optimum growth on V₈A at between 20°C and 25°C. All other isolate and media combinations showed optimum growth at 20°C.

Of the three isolates, isolate 5/1 showed the highest growth maximum overall and the highest on V₈A and SPA in particular. On MEA, isolates 5/1 and 14/4 had significantly higher growth maxima than isolate 11/3. Of the three media, all isolates showed their highest growth maxima on MEA. Isolates 11/3 and 14/4 had significantly higher growth maxima on V₈A than on SPA. Isolate 5/1 showed similar growth on V₈A and SPA.

Plate 3.1 illustrates some of the differences in the morphological characteristics of the cultures. On SPA, isolate 5/1 had fine, long, sparingly branched, dark brown strands running through the agar, released a red pigment into the agar and had sparse aerial mycelium. All other isolates produced abundant grey-olivaceous aerial mycelium with white tufts, on the three media.

3.4.3 Discussion of the three species in culture

D. dictyoides showed morphological variation between isolates and between media. Shoemaker (1962) found that cultures on SPA had very fine, sparingly branched, brown strands that ran longitudinally deep into the agar. A pink pigment formed in the agar on SPA (also noted by Braverman & Graham 1960) but no aerial mycelium developed. Two of the isolates in the present study conformed partly to this

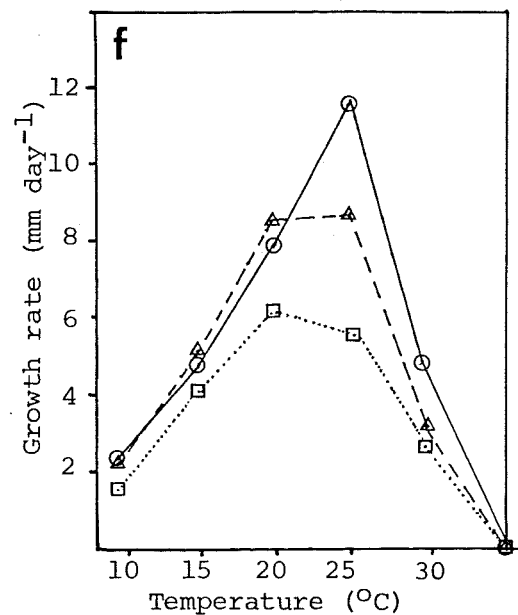
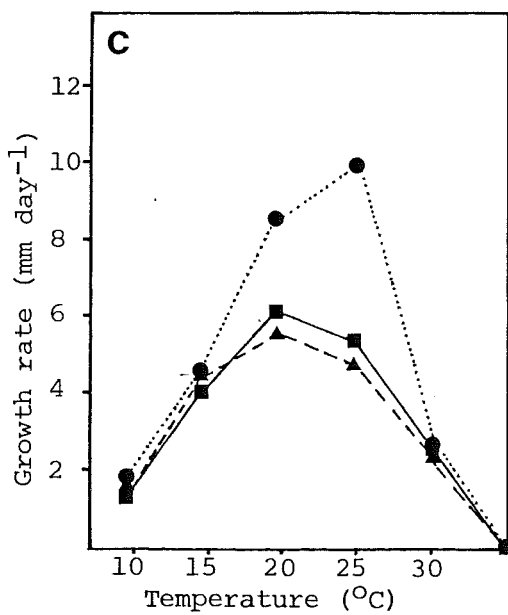
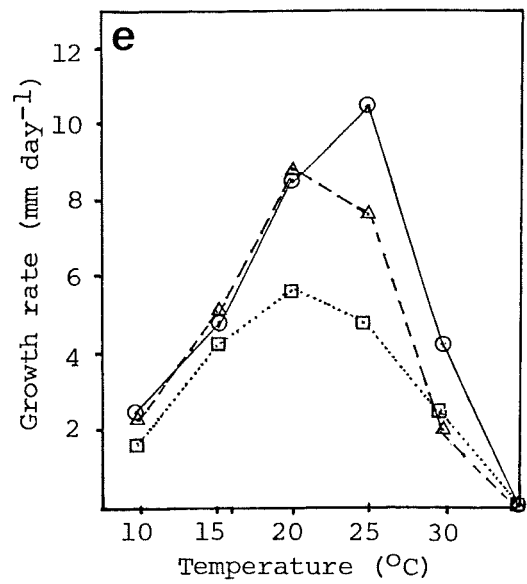
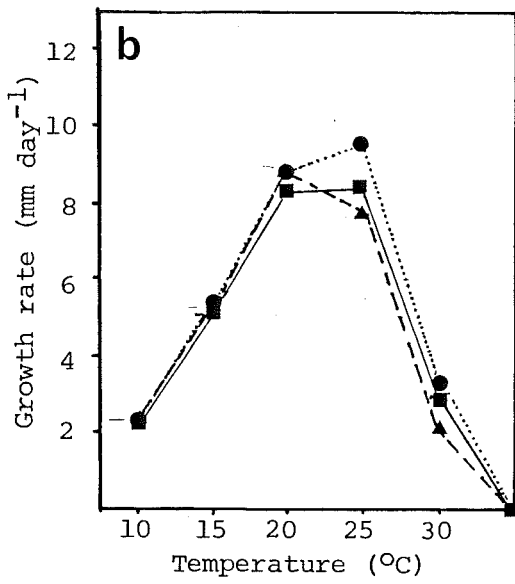
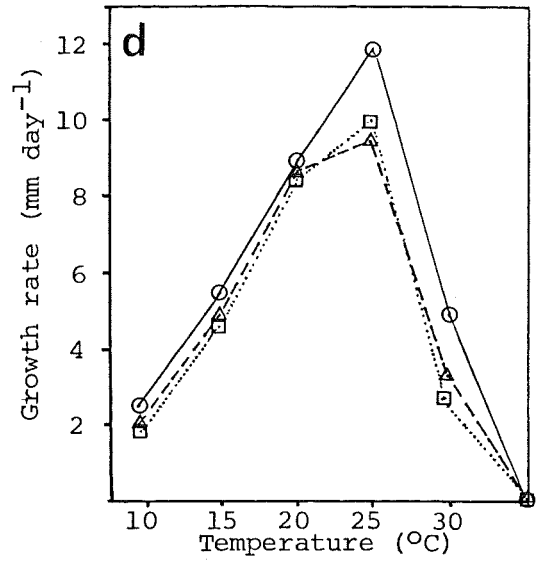
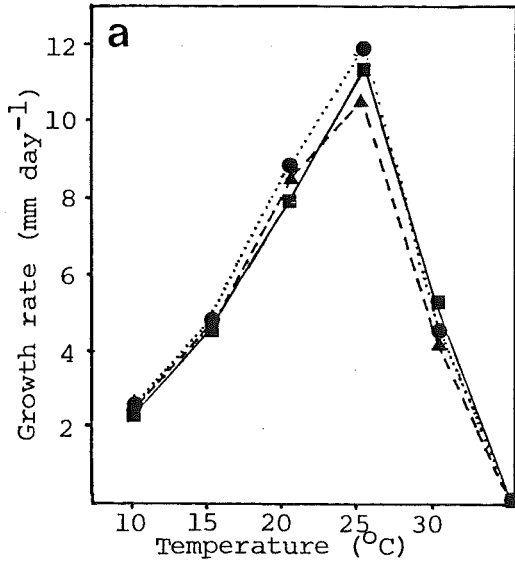
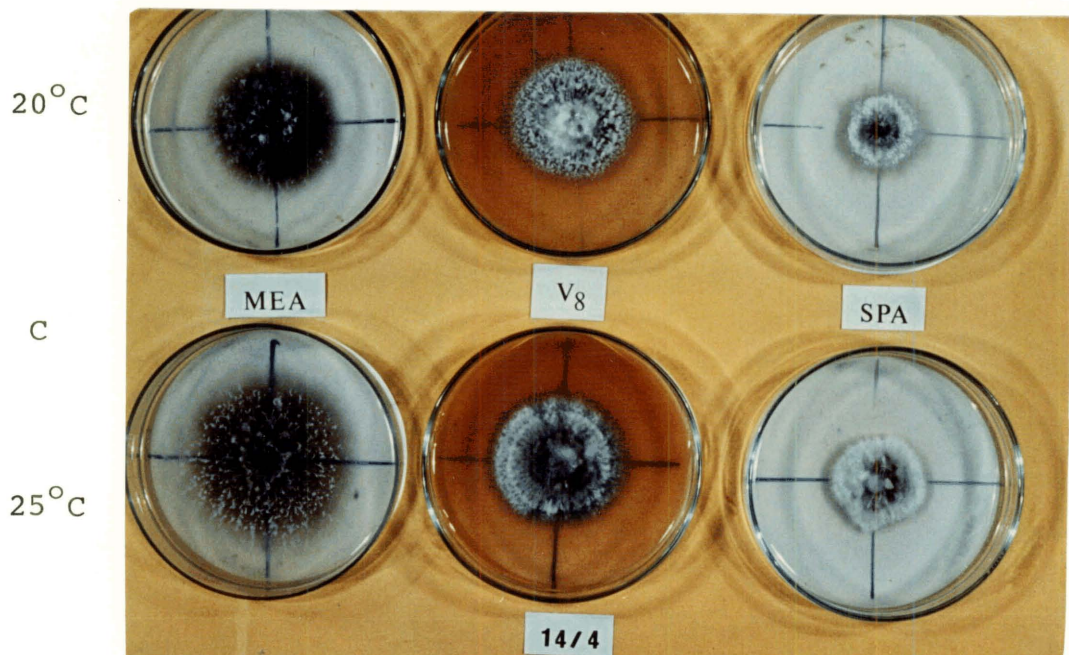
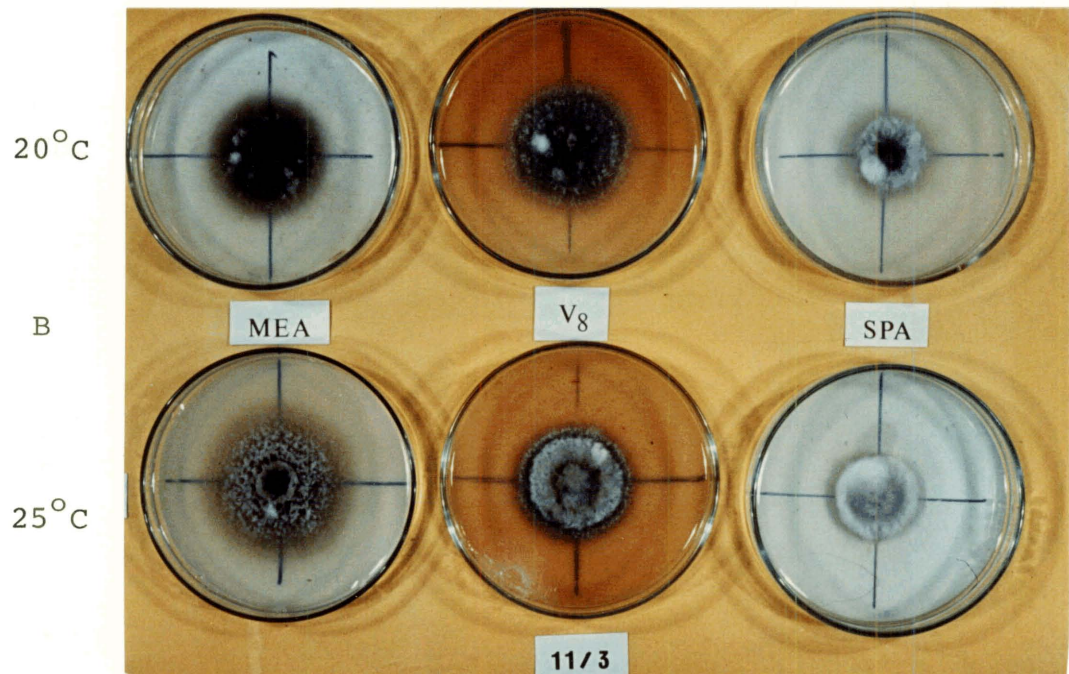
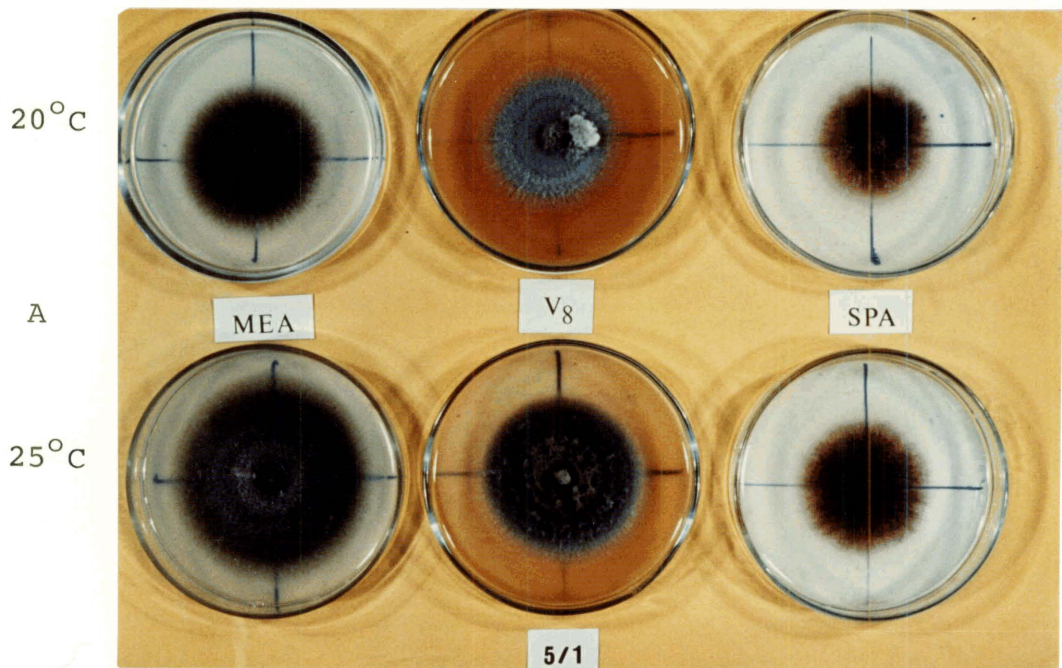


Plate 3.1. Cultural characters of three isolates of
D. dictyoides on MEA, V₈A and SPA.

A: Isolate 5/1

B: Isolate 11/3

C: Isolate 14/4



description, although aerial mycelium was present. The majority of isolates had luxurious, floccose growth on SPA. Tufting was common on some agar media, such as MFA and CA. These results are in agreement with Latch (1966), using New Zealand isolates, and Lam (1981) although she did not record finding a pink pigmentation.

In the present study, the temperature range supporting growth was from about 4°C to 35°C with the optimum temperature being between 20°C and 25°C, depending on isolate and medium. This is comparable with the result of Latch (1966) who recorded a temperature range of 3°C to 36°C and an optimum of 25°C on SPA.

The cultural characteristics of *D. nobleae* have been described by Lam (1981) and by Morrison (1982). The results of this study are in agreement with these, although growth rates were a little slower in Morrison's work than in the current study.

Shoemaker (1962) reported that *D. siccans* produced a deep fuchsin pigment on SPA, while Lam (1981) found a pinkish pigmentation to be formed. In the current study, one of the three isolates of *D. siccans* produced a pigment but this was only a slight pink pigmentation. The grey or grey-olivaceous colour of the aerial mycelium in the present study is in accordance with previous work (Latch 1966, Shoemaker 1962, Scharif 1963, Lam 1981).

In culture, *D. nobleae* and *D. siccans* are very similar but can be distinguished from *D. dictyoides* on surface texture. On many media, *D. dictyoides* produces characteristic white tufts which are not produced by *D. nobleae* or *D. siccans*. *D. nobleae* and *D. siccans* produce a 'felty' or 'floccose' aerial mycelium and that of *D. siccans* is more luxuriant.

Cultural characteristics are of some value in separating the taxa, but must be used in conjunction with other characteristics.

3.5 Host ranges

In this study, the following grasses and cereals were inoculated (detached leaves) with the three species of *Drechslera*:

perennial ryegrass (*Lolium perenne*), Italian ryegrass (*L. multiflorum*), oats (*Avena sativa*), wheat (*Triticum aestivum*), Yorkshire fog (*Holcus lanatus*), prairie grass (*Bromus cartharticus*), cocksfoot (*Dactylis glomerata*), timothy (*Phleum pratense*), crested dogstail (*Cynosurus cristatus*), Kentucky bluegrass (*Poa pratensis*).

Assessments were made of number of spores penetrating after twenty-four hours, lesions developed after five days and sporulation after seven days.

Results:

Table 3.9 lists numbers of penetrations of the inoculated leaves. *D. dictyoides* penetrated the three ryegrass cultivars to an equal extent while *D. nobleae* and *D. siccans* showed a preference for the Italian ryegrass cultivars ('Tama' and 'Paroa'). Penetrations were rare on the non-host grasses with the following exceptions:

D. siccans on oats (significant) and on crested dogstail and Kentucky bluegrass (not significant) and *D. dictyoides* and *D. nobleae* on Kentucky bluegrass (not significant).

Table 3.9 Penetrations by *D. dictyoides*, *D. nobleae*

D. siccans twenty-four hours after inoculation onto various grasses and cereals.

	mean penetrations per germinated conidium		
	<i>D. dictyoides</i>	<i>D. nobleae</i>	<i>D. siccans</i>
'Ruanui'	0.42 b/b*	0.05 a/a	0.23 a/b
'Tama'	0.38 b/b	0.09 a/a	0.42 b/b
'Paroa'	0.40 b/a	0.26 ab/a	0.53 b/a
Oats	0.05 a/a	0.07 a/a	0.47 b/b
Wheat	0.03 a/a	0.00 a/a	0.00 a/a
Yorkshire fog	0.00 a/a	0.02 a/a	0.00 a/a
Prairie grass	0.18 a/a	0.07 a/a	0.09 a/a
Cocksfoot	0.13 a/a	0.01 a/a	0.00 a/a
Timothy	0.01 a/a	0.01 a/a	0.00 a/a
Crested dogstail	0.07 a/a	0.00 a/a	0.27 a/a
Kentucky Bluegrass	0.24 ab/a	0.24 a/a	0.21 a/a

* means for different grasses (a/ down) or for different fungal species (/a across) followed by the same letter are not significantly different ($P = 0.05$). Duncans multiple range test.

Lesion development is outlined in table 3.10. Pin-point or no lesions were produced on all non-host grasses with the exception of *D. siccans* on oats where full lesions developed and on crested dogstail where fleck lesions were formed.

Table 3.10. Development of lesions by *D. dictyoides*,
D. nobleae and *D. siccans* on various grasses
and cereals.

	lesion type*		
	<i>D. dictyoides</i>	<i>D. nobleae</i>	<i>D. siccans</i>
'Ruanui'	4	1	3
'Tama'	3	2	3
'Paroa'	2	3	4
Oats	1	1	3
Wheat	1	0	1
Yorkshire fog	1	0	0
Prairie grass	1	1	1
Cocksfoot	1	0	1
Timothy	0	0	0
Crested dogstail	1	0	2
Kentucky bluegrass	1	1	1

* rated on a visual scale of 0 - 4 (see p. 13)

Levels of sporulation on hosts and non-hosts are given in table 3.11. *D. dictyoides* produced a few spores on senescent leaves of some non-host plants. So did *D. siccans*, although not on oats, where lesions had developed. *D. nobleae* produced a few spores on wheat but the only ryegrass it sporulated on was the cultivar 'Tama'.

Discussion

The infection of oats by *D. siccans* is noteworthy and is in accordance with the results of Ibrahim & Threlfall (1966) and Lam (1981). The fungus did not sporulate on oats and so the claim of Ibrahim & Threlfall (1966) that *D. siccans* and *D. avenae* are the same species is not verified. Sporulation of all three fungal species was limited to a trace on all

Table 3.11 Sporulation of *D. dictyoides*, *D. nobleae* and *D. siccans* on various grasses and cereals seven days after inoculation.

	Degree of sporulation*		
	<i>D. dictyoides</i>	<i>D. nobleae</i>	<i>D. siccans</i>
'Ruanui'	3	0	1
'Tama'	4	3	2
'Paroa'	2	0	0
Oats	1	0	0
Wheat	1	1	0
Yorkshire fog	0	0	0
Prairie grass	0	0	0
Cocksfoot	1	0	1
Timothy	0	0	0
Crested dogstail	0	0	1
Kentucky bluegrass	1	0	1

* rated on a visual scale of 0 - 4 (see p.13)

non-host grasses and it is unlikely that infection of grasses other than ryegrass occurs in nature.

3.6 Summary

In this study, three species of *Drechslera* were found to cause lesions on the foliage of ryegrasses. They were; *D. dictyoides*, *D. siccans* and *D. nobleae*. The species have been compared in relation to lesion types, fructifications on the leaf and cultural characteristics as well as their occurrence in nature. They are taxonomically distinct and can most easily be distinguished on the morphology of their fructifications. In the absence of perfect states, taxonomic work was based purely on the conidial states.

D. dictyoides was the most common species on perennial ryegrass, accounting for over ninety percent of lesions caused by *Drechslera* species in a disease survey in Hagley Park. Throughout the course of the research for this chapter, all net lesions on perennial ryegrass produced *D. dictyoides* fructifications. The remainder of this thesis is concerned with *D. dictyoides* which is clearly the most important *Drechslera* disease of perennial ryegrass at the present time.

CHAPTER FOURTHE LIFE HISTORY OF *D. DICTYOIDES* ON *L. PERENNE*4.1 Introduction4.1.1 The life history

'Disease develops in individual plants by a series of sequential steps beginning with the arrival of inoculum at the plant surface and ending with the terminal stages of pathogenesis. The innumerable stages in between are unique to each particular pathogen, host and variation of the environment.'

Cowling and Horsfall (1978)

Only with an understanding of the complete disease cycle and therefore of the fungal life history is it possible to recognise the complexities of a disease syndrome. Many studies have concentrated on a particular aspect of disease incitement, such as spore germination (Hawker & Hendy 1963, Murray & Maxwell 1974, Fletcher 1971), penetration (Murray & Maxwell 1975, Wheeler 1977, Politis 1976) or sporulation (Cole & Sampson 1979, Carroll & Carroll 1974, Brotzman *et al.* 1975). While these studies are of value, they can lead to an overestimation of the importance of a single stage in the disease cycle. In this study, each phase of the fungal life history was investigated separately and linked to the ongoing process by time-course studies. The stages investigated separately were spore germination, infection, lesion development and conidium ontogeny.

Time-course studies of infection using living organisms are most useful in enabling the separation of cause from effect. For instance, where a hypersensitive response is found, does it occur before or just after penetration? Such studies tend to be hampered by inherent difficulties in

the observation by means of light microscopy of many host pathogen interactions. It is not possible to view events in many intact green leaves because of the presence of chlorophyll and most studies have avoided the problem by using host tissues with little or no chlorophyll, such as roots (Aist & Israel 1977a), coleoptiles (Aist & Israel 1977b) or 'just unfolded young leaflets' (Tomiyama 1963). Time-course studies enabled Tomiyama and co-workers (reported in Tomiyama 1963) to show that there is no difference between the rate of penetration of the cell wall of potato plants resistant or susceptible to *Phytophthora infestans* in the case of major gene resistance. Their results indicated that the hypersensitive response followed penetration rather than *vice versa*.

Two systems allowing sufficient definition of the infection process with optical microscopy were used in the present study. Epidermal peels of the abaxial epidermis of ryegrass were used to follow the progress of individual germinated conidia and to elucidate the general pattern of infection. The system involved considerable disruption of the host after about twelve hours and so leaf sheaths of ryegrass were used for a detailed study of the interaction of the host cytoplasm with the pathogen (chapter six). Little chlorophyll was present in the leaf sheath and the host cytoplasm continued to behave normally for several days after excision.

4.1.2 The spore and its germination

There are two broad groupings of fungal spores; those that remain at the place of origin to survive over unfavourable periods and those which are dispersed to new geographic locations (Marchant 1979). The first group are

relatively thick-walled spores and may require a specific set of conditions to germinate whilst the second group are usually thin-walled and germinate more easily. Conidia of the genus *Drechslera* belong to the second group, whose primary function is in the dissemination of the pathogen to new, uninfected host tissues. Changes on germination are mainly metabolic and biosynthetic (Bracker 1967, Gottlieb 1974) *vis*, a change from a relatively inactive state to an active one.

'Fungal morphology is primarily a reflection of cell wall fabrication, wall growth and wall modifications. The characteristic and stable forms of a variety of reproductive structures and propagules are a direct consequence of developmental events involving some kind of cell wall elaboration.'

Aronsen (1981)

Modifications of the fungal wall are responsible for the distinct morphology of the various structures formed during the life history of the fungus and so are responsible for the success or failure of the pathogen in the completion of the disease cycle. The fungal wall should not be considered an inert envelope surrounding the fungal protoplast as further deposition can occur at the inner or outer surface of an existing wall, or even within it (Marchant 1979). The synthesis of wall material ceases between spore maturity and germination and this is a suitable place to begin an investigation of wall structure.

Many conidia have a two layered cell wall - an outer electron dense layer and an inner electron transparent layer (Hawker & Hendy 1963, Murray & Maxwell 1974). When a spore germinates the outer wall is usually ruptured by protrusion of the germ tube. Bartnicki-Garcia (1968)

listed three types of spore germination in terms of the wall layers involved. In type I, the germ tube is directly derived as an extension of the spore wall or one of its innermost layers (as reported by Hawker & Hendy 1963, Murray & Maxwell 1974). Type II involves *de novo* formation of a cell wall on a naked protoplast (as in aquatic Phycomycetes). Type III involves *de novo* formation of a vegetative wall under the spore wall (as reported by Fletcher 1971, Gull & Trinci 1971).

The distinction between types I and III may not always be clear. Hawker & Hendy (1963) found that *Botrytis cinerea* conformed to type I, while Gull & Trinci (1971), using the same species, found that three new walls were laid down in the conidium during germination. The outer two were present around the spore while the innermost layer was continuous with the germ tube and only arose in the region of the germ tube. They, therefore, suggested that *B. cinerea* conforms to type III and that, with more suitable fixatives, new walls may be visualised in many other examples. Bartnicki-Garcia (1968) warned, however, that inner walls which appear at the time of germination may not always be morphologically entirely new walls.

A fibrillar layer, or mucilage layer, is often found at the tip of the developing germ tube and it has been suggested that this may attach the germ tube or appressorium to a leaf or other firm surface (Hawker & Hendy 1963, Murray & Maxwell 1974). Wheeler & Gantz (1979) reported the occurrence of extracellular sheaths surrounding hyphae of *D. maydis* and *D. victoriae* and these may have a similar function. Pringle (1981) found germinating conidia of *D. sorokiniana* bound tightly to glass surfaces by means

of an adhesive substance.

Shortly after germ tube emergence, a septum may form at the point of egress from the conidium and the developing germ tube is septate (Gull & Trinci 1971). Hunsley & Gooday (1974) considered the function of septa in younger areas was probably to add rigidity to the hyphae. In older mycelial areas, where the pores are plugged, septa may serve as compartmentation devices. Growth of fungal septa is rapid, 3.5 - 4 minutes in *Neurospora crassa* (Hunsley & Gooday 1974). During formation of septa by *Botrytis cinerea* electron transparent material (angular wedges in cross section) is deposited on the inner surface of the germ tube wall and the plasmalemma is continuous over it (Gull & Trinci 1971).

The typical septum of ascomycetes or their conidial states consists of a simple plate with a central pore about 0.05 - 0.5 μm in diameter (Bracker 1967). Unless plugged, the septum does not seem to prohibit cytoplasmic streaming or nuclear migration (Bracker 1967). Smith *et al.* (1981) suggested that the function of the septum between the conidium and the germ tube may be to separate the developing structure from the degenerating and autolytic contents of the aging parental cells.

Once the spore has germinated and its contents are utilised, its useful life is over. The same applies to the germ tube after penetration of the host. Senescence follows. When hyphae age, organelles begin to disintegrate (Gunasekaran *et al.* 1973) and much of the cell may become filled with one or more vacuoles, the thin lining of cytoplasm containing little apart from a few mitochondria and membraneous remains of organelles (Campbell 1970).

Carroll & Carroll (1973) examined senescence and death of the conidiogenous cell in *Stemphylium botryosum*. The vacuolar system expanded and coalesced and there were signs of mitochondrial degeneration. Total wall thickness increased from around 0.3 μm in the young stages to about 1.5 μm after 46 hours. It was suggested that the vacuolar system carried out the lysosomal functions.

4.1.3 The appressorium

'The initiation, formation and action of the appressorium are integral parts of the infection process in many parasitic fungi.'

Emmett & Parbery (1975)

Emmett & Parbery (1975) gave a general review of appressoria - their morphology, development and role. The main role of an appressorium, they stated, was the direct penetration of a host. To achieve this appressoria must attach firmly to the host surface and produce infection pegs which pierce or dissolve the cuticle and usually the epidermal cell wall. Appressoria are frequently formed over the longitudinal junction of epidermal cells (Preece *et al.* 1967). Attachment often appears to be by means of binding substances (Edwards & Allen, 1970, Mckeen 1974).

Appressorium formation marks the conclusion of spore germination (Gaumann 1950). Emmett & Parbery (1975) proposed that:

'Appressorium formation is primarily controlled by genotype whose expression may require a specific conducive environment'.

They went on to outline the factors external to fungi which may play a role in appressorium formation. These were:

- a) thigmotropism - or specifically apical contact at a given point in germ tube development.
- b) factors inherent in plants - such as surface phenomena, cuticular waxes, endogenous chemicals, nutrient status and phyllosphere micro-organisms.
- c) factors external to plants - such as exogenous chemicals, water, temperature and light.

It was suggested that no one factor is of over-riding importance.

4.1.4 Penetration

In order to obtain the nutrients required for growth and reproduction, it is necessary for parasitic fungi to enter their hosts, thus establishing contact with them (Aist 1976). Pathogens may invade plants through wounds, natural openings or by direct penetration, the latter being the method most frequently encountered (Tarr 1972). In direct penetration of the leaf, a penetration peg arises from the underside of the appressorium and must pass through the cuticle and epidermal wall. Once the host is penetrated, the peg increases in diameter and develops as a hypha either inside or between the epidermal cells.

The question of whether penetration is by mechanical or enzymatic means is still not satisfactorily answered. Aist (1976) listed five arguments in favour of each of the mechanical and enzymatic methods and suggested that in most cases penetration was likely to be the result of a combination of the two.

The ultrastructural changes in the fungus and the host accompanying penetration have been investigated in a number of host-pathogen combinations. *D. maydis* on

corn always penetrates the cuticle between epidermal cells (Wheeler 1977). Subcuticular hyphae radiate out from the initial point of penetration of the cuticle, with haustoria-like branches functioning in secondary infection.

D. carbonus on corn remains mostly subcuticular up to forty-eight hours after inoculation of the leaf (Murray & Maxwell 1975). After the cuticle is penetrated a hypha containing nuclei, mitochondria, endoplasmic reticulum small vacuoles and lipid bodies, enlarges under the cuticle in the junction of epidermal cells.

Penetration of corn by *D. turcica* (Knox-Davies 1974) is usually directly through the cuticle and outer epidermal cell wall although initial subcuticular growth is occasionally seen. Germ tubes of *D. teres* grow above the cuticle and penetration is directly into epidermal cells (Van Caeseele & Grumbles 1979). The situation is similar in *Colletotrichum graminicola* on corn (Politis & Wheeler 1973) where the outer electron dense wall of the appressorium disappears during penetration in the region of the developing infection peg. At all stages, the infection peg and hypha that develops from it are enveloped by a distinctive, electron-transparent cell wall which is continuous with the inner wall of the appressorium. In the area of attachment of the appressorium to the epidermis 'lomasome-like' bodies arise between the dense outer wall and the plasmalemma of the appressorium. It was suggested they may have played a role in the dissolution of this outer wall of the appressorium and in penetration of the host cell wall.

4.1.5 Lesion development

After penetration the pathogen must obtain its nutrient requirements from the host in order to complete its life cycle with the production of spores. This is usually achieved by extensive ramification through host tissues. Pathogens may be grouped into three broad types according to the manner in which they derive their nutrients. Necrotrophic, hemibiotrophic and biotrophic pathogens derive their nutrients from dead cells, initially from living cells and then from dead cells, or only from living cells, respectively (Hancock & Huisman 1981).

Many species of *Drechslera* fall within the necrotrophic group because of the presence of host-specific phytotoxins. These have been reviewed extensively (Pringle & Scheffer 1964, Shotwell & Ellis 1976, Yoder 1980). It is postulated that toxins kill host cells in advance of the hyphae which then derive their nutrients from the dead cells. Some examples are given below.

Luke *et al.* (1966) found that the toxin victorin, produced by *D. victoriae*, caused general disruption of internal membrane systems of susceptible oats. *Drechslera maydis*-race T also has an early effect on the structural integrity of cell membranes (White *et al.* 1973) and caused rapid collapse of mesophyll (Toth & Smith 1982) presumably due to toxin production and possibly also enzyme production. *Drechslera sorokiniana* on *Agrostis palustris* caused disruption of chlorenchyma cells in advance of the hyphae (Healy & Brittan 1968) and the same phenomenon in sugarcane infected with *D. sacchari* was attributed to the toxin helminthosporoside (Strobel *et al.* 1972). Jennings & Ullstrup (1957) examined three

species of *Drechslera* on corn - *D. turcica*, *D. maydis* and *D. carbonus*. In all three cases hyphae proliferated only within necrotic tissues.

Fungal proliferation is not always confined to necrotic tissues, even amongst species of *Drechslera*. Van Caesele & Grumbles (1979) found the most striking feature of the infection of barley by *D. teres* was the lack of cell disruption in advance of intercellular hyphal growth. Chloroplasts, mitochondria and microbodies in host cells appeared intact despite the close proximity of intercellular hyphae. Penetration of mesophyll cells below the first layer was rare and it was suggested that the isolate used did not produce toxins, or at least not in the early stages of infection. A similar situation was found in cucumber infected with *Cladosporium cucumerinum* Laborda & Maxwell 1976. At the margin of the rotted area, intercellular hyphae were observed in association with normal or modified host cells while intracellular hyphae were found only in disorganised host cells.

Parasites growing intercellularly without specialised absorptive structures presumably must rely on intercepting solutes diffusing in hydrated host cell walls. The concept of the apoplast-symplast in higher plants has been used by Hancock & Huisman (1981) to explain nutrient movement in host-parasite relations. The apoplast represents the plant's cell walls and is continuous throughout large portions of the plant. Water and solute movement in the apoplast is limited to diffusion along concentration gradients and perhaps bulk flow. The symplast is represented by the living cytoplasm and is continuous from cell to cell through plasmodesmata.

Hancock & Huisman (1981) calculated that the growth of fungal pathogens in infected tissue could be supported by the supply of nutrients via the apoplast. Diffusion of nutrients from host cell walls into parasite cell walls would be expected to occur at points of contact on the assumption of a suitable concentration gradient. Fibrillar material was found to be present between host and fungus with *Cladosporium cucumerinum* on cucumber (Laborda & Maxwell 1976) and this could greatly increase the absorptive area.

4.1.6 Conidium ontogeny

Once development of the lesion on the leaf is complete, the final phase in the life history of the disease may begin. This is sporulation.

'The principal function of the process of sporulation is to produce a discrete structure - a spore.'

Marchant (1979)

The process of sporulation can begin as soon as the fungus and host are in the appropriate physiological state and the required environmental conditions have been met. The fungal spore and its ontogeny also forms the basis for taxonomic position and this is, for mycologists, another important aspect of the sporulation process.

'The last quarter century has seen the basis for classifying Deuteromycetes change from one which emphasises the morphology of the mature conidium to one which emphasises the processes by which conidia are generated.'

Madelin (1979)

The impetus for this research was provided by Hughes who wrote that

'The need for further studies on the precise

method of conidium development is stressed because this promises to provide the most stable character for the classification of Fungi Imperfecti as a whole.'

Hughes (1953)

The electron microscope has allowed a critical examination of the ontogeny of the conidium.

Two basic modes of conidiogenesis exist in the Deuteromycetes and these are referred to as 'blastic' and 'thallic' development. In the blastic process, characteristic of *Drechslera* species, there is a marked enlargement of a recognisable conidium before the initial is delimited by a septum. On the other hand, in the thallic process, any enlargement of the recognisable initial of the conidium occurs only after the initial has been delimited by a septum (Cole & Sampson 1979).

The remainder of this section will be confined to a discussion of the blastic process. There are two major forms of blastic ontogeny. In holoblastic ontogeny, all layers of the wall of the conidiogenous cell are involved in formation of the conidium wall, while only the inner layers are involved in enteroblastic ontogeny. Madelin (1979) proposed a hypothesis to explain the existence of the two forms. It was suggested that:

'... the mode of blastic ontogeny is related to the juvenility or maturity of the wall at the conidiogenous locus. If the wall is juvenile, it retains its capacity to be plasticised and have new materials intersussepted, so that all of it can be involved in generation of the conidium initial. If, on the other hand, it has been omitted from the developmental processes for some time, it may undergo maturation changes and become no longer plasticizable, in which case new development at that locus is possible only

when the mature parts of the wall are split or lysed (or both) to allow the egress of a juvenile hypha from below.'

In most classifications of the Deuteromycetes a group of fungi that produce thick-walled heavily pigmented conidia at minute pores in the wall of the conidiophore have been recognised. These are usually termed 'porospores', a name intended only to denote the presence of a conspicuous pore between the conidiophore and mature conidium (Carroll & Carroll 1974) and could be produced either holoblastically or enteroblastically).

There has been a considerable controversy concerning the two forms of blastic ontogeny. Cole (1973) suggested that, in *D. sorokiniana*, the innermost wall layer of the conidiophore was continuous with the developing wall of the conidial initial, while the outer wall layers broke down. Carroll & Carroll (1974) in *Ulocladium atrum* found no discontinuity in the wall between the conidiophore and the initial of the conidium, which suggested a holoblastic mode of conidiogenesis. They reinterpreted Cole's micrographs as non-median sections which did not give adequate evidence of an enteroblastic origin. From work on *D. maydis*, a species closely related to *D. sorokiniana*, Brotzman *et al.* (1975) suggested that conidiogenesis was holoblastic. A critical re-examination of Cole's micrographs led them to believe that the conidia of *D. sorokiniana* also had a holoblastic origin. Cole & Samson (1979) re-examined available ultrastructural data and chose to consider the poroconidium as a special kind of holoblastic propagule. It was suggested that the very early stages in formation of the initial of the conidium are holoblastic, although the outermost wall

layer soon breaks down, whilst the inner wall layers continue to thicken (Cole 1981). It was concluded that there were limitations in the use of features of cell wall differentiation alone for classification (Cole 1981).

The interpretation of the above controversy by Madelin (1979) was that distinct enteroblastic ontogenies might represent the extremities of a continuum involving the occurrence of different degrees and rates of stratification within the wall of the fungus.

After conidial secession a tiny occluded pore is revealed at the apex of the conidiogenous cell, typically surrounded by a thickened ring of wall material (Cole & Samson 1979). A conidiophore may be determinate or proliferous. In the latter case a new proliferating apex would elongate and give rise to a new conidium.

4.2 Results

4.2.1 Seed-borne disease

The three species of *Drechslera* pathogenic to ryegrass are regularly found in seed samples. Matthews (1971) and Mckenzie (1978) assessed the presence of the species on ryegrass seed in New Zealand and Lam (1982) did the same in the United Kingdom. Whilst they are commonly present, the occurrence of *Drechslera* on seed does not appear to affect seedling emergence, incidence of disease in the field or yield (Tribe & Herriot 1968, Labruyere 1977, Lam 1982). Wells & Allison (1952) suggested that seed-borne *Drechslera* on tall fescue may account for epiphytotic build-up on plants from new seedlings.

In many crops, seed-borne inoculum has a marked effect on disease levels in the ensuing crop. One such case is that of net blotch of barley caused by *D. teres*, in New Zealand. Hampton (1980) found that seed-borne inoculum was the major source of primary infection in the absence of an effective seed treatment. Ryegrass is ubiquitous in New Zealand, ensuring an ever present source of inoculum and this obviates the need for infection from seed to carry on the disease cycle.

In the present study seed-borne infestation was found in some seed samples (plate 4.1). Infection from seed probably follows the pattern outlined by Turner & Millard (1931) for *D. avenae* on oats and Teviotdale & Hall (1976), for *D. graminea* on barley. In my study the rate of germination was often slowed by a heavy infestation of *Drechslera* but the infested seed usually germinated (plate 4.1:A). Conidia were observed borne on the coleoptile after becoming detached from conidiophores on the seed

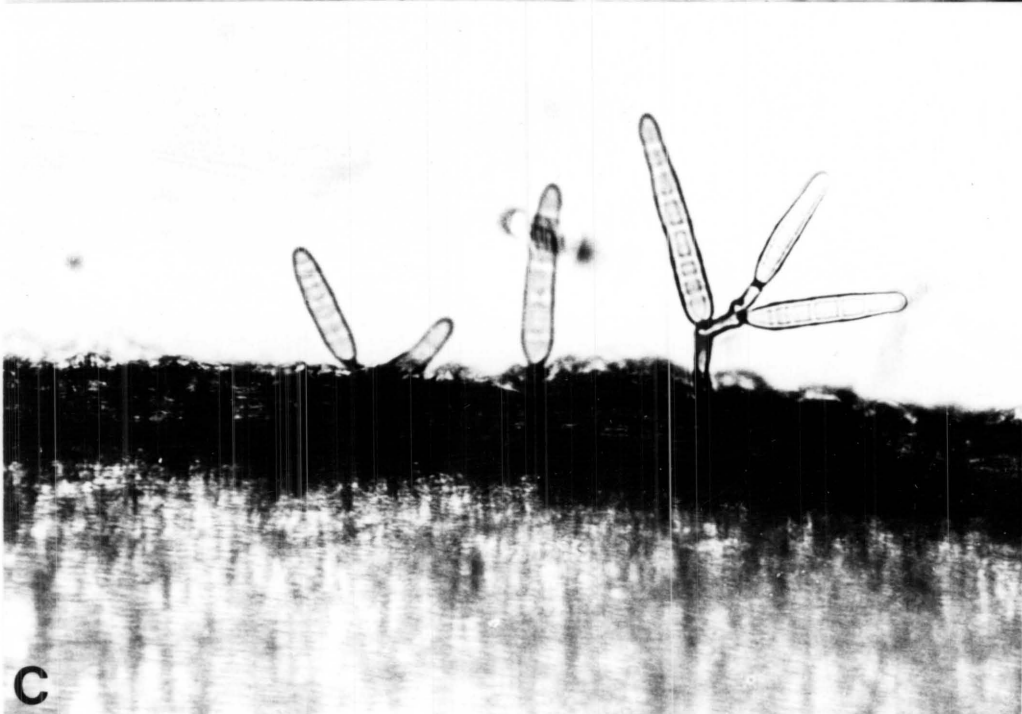
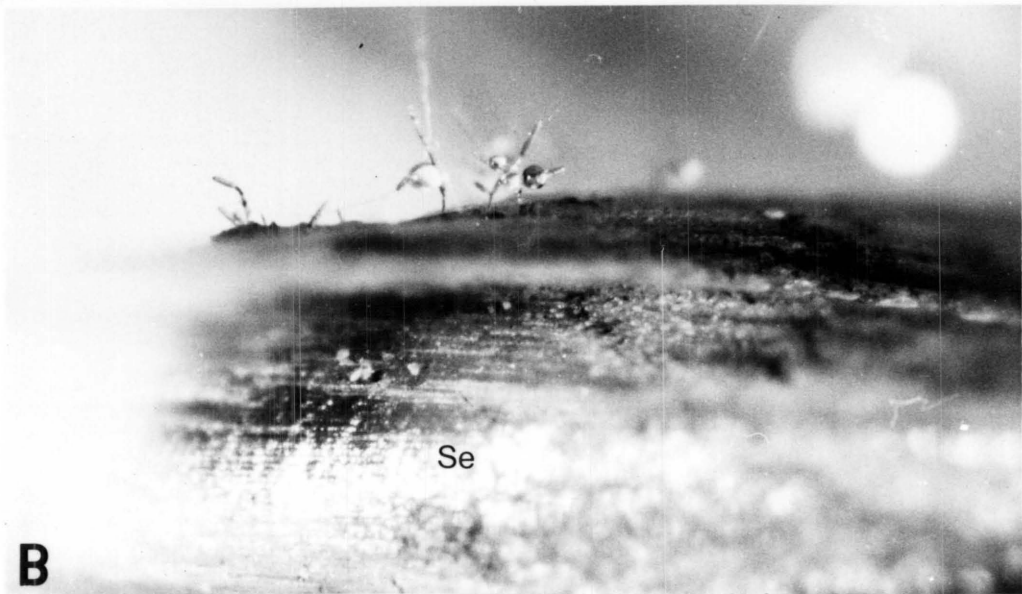
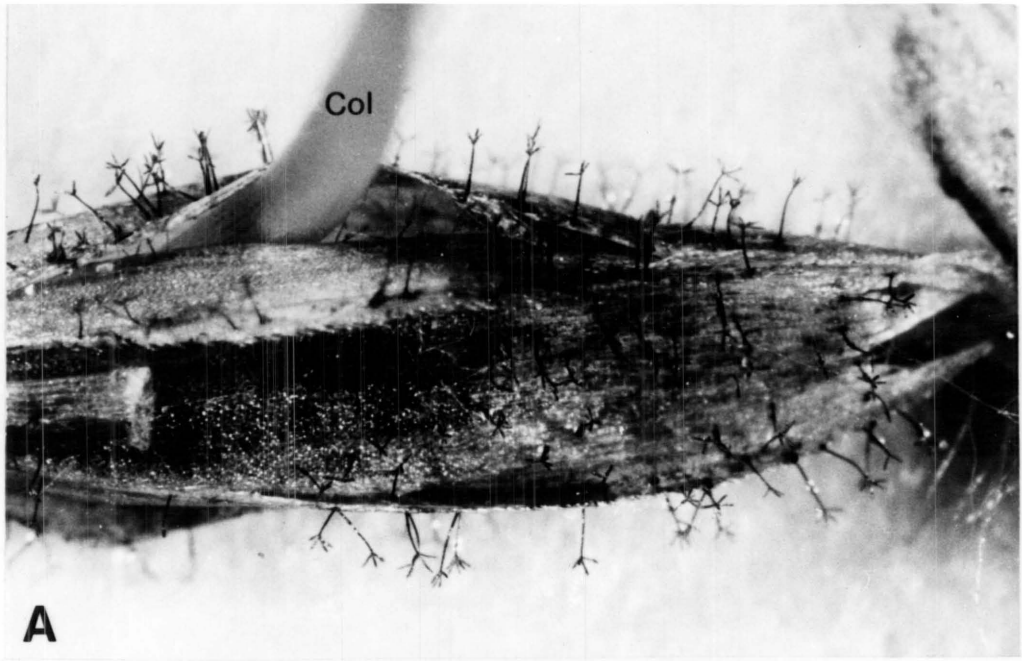
Plate 4.1 Infestation of ryegrass seed with *Drechslera* species.

A: Germinated seed heavily infested with *D. siccans* (stereo-micrograph). Note the cluster of conidia at apex of long conidiophores.

B: Ryegrass seed infested with *D. dictyoides* (stereo-micrograph).

C: Seed infestation with *D. dictyoides* (compound-photomicrograph). Note the production of conidia at intervals on the conidiophore.

Abbreviations: Col - coleoptile, Se - seed



surface. With suitable environmental conditions, infection of the leaf may occur. Seed-borne infection is unlikely to play a part in the disease cycle and this aspect was therefore not pursued.

4.2.2 Time-course study of *D. dictyoides* on *L. perenne*

For a time-course study of the infection process, abaxial epidermal peels were inoculated with a spore suspension of *D. dictyoides* and individual infections were followed microscopically at regular time intervals. Plate 4.2 shows a 'typical' course of infection. Germination was usually from the basal or apical cell or from both, the germ tube growing for a variable distance before producing an appressorium. Germ tubes travelled over stomata from time to time but penetration was rarely achieved through them. Appressoria were almost invariably formed over anticlinal epidermal cell walls (in 94% \pm 1% of cases out of a total of 1,000 appressoria assessed). Penetration occurred below the appressorium but usually only one host epidermal cell was penetrated. Septa formed at regular intervals along the germ tube and septation occurred before penetration of the leaf.

As the conidium aged it was possible to observe the contents of the conidial cells gradually 'emptying', initially from the germinated cells and then from those adjacent. After penetration the germ tube became vacuolate. From the initial infection vesicle, the intracellular hypha ramified within the penetrated epidermal cell before spreading into the host mesophyll. After about twenty-eight hours, the conidium and germ tube showed signs of advanced senescence. In a comparison with infections which

Plate 4.2 Time-lapse photomicrographs showing penetration of the abaxial leaf surface of *L. perenne* by *D. dictyoides*.

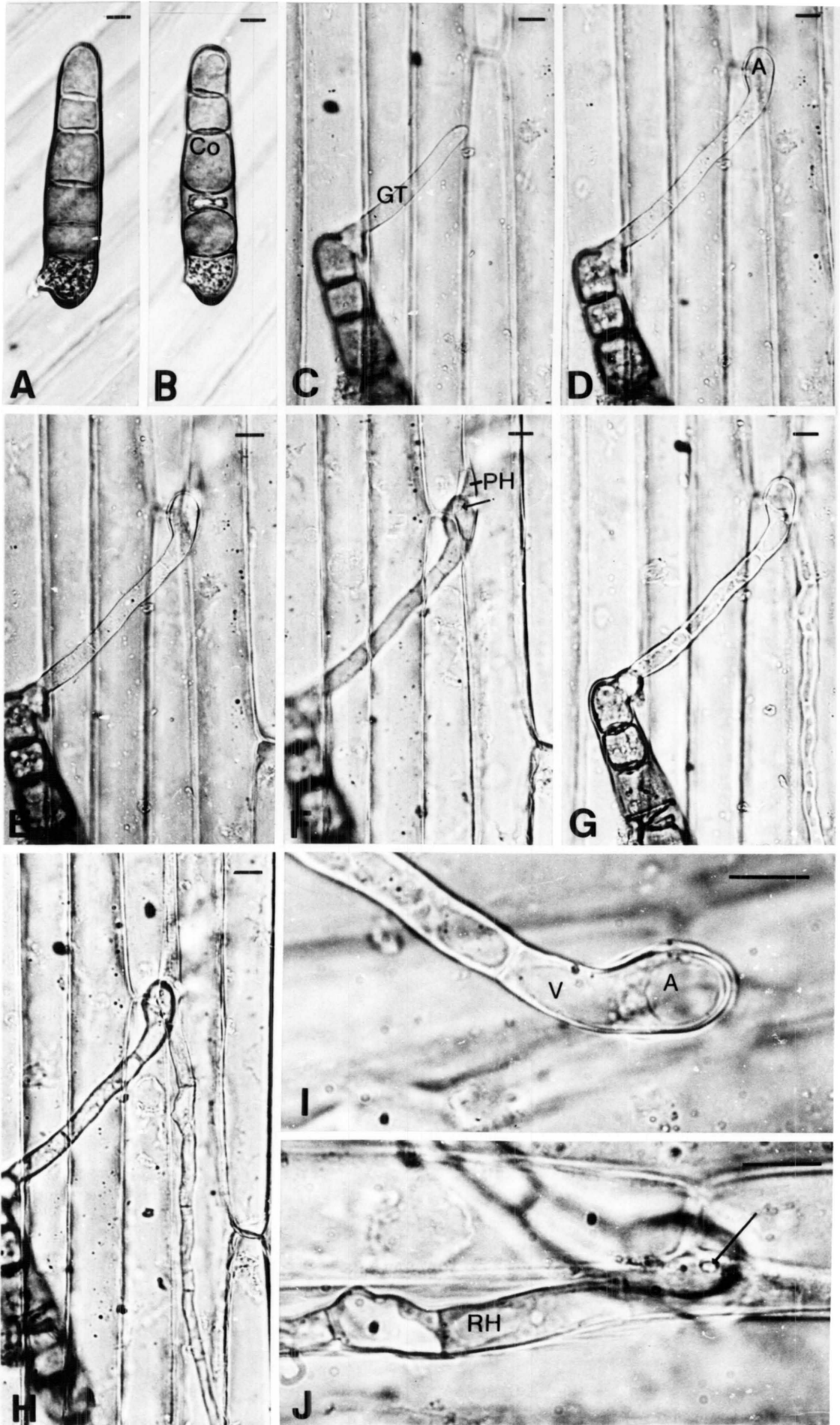
(times given are hours after inoculation of the leaf).

- A: Apical cell of conidium ungerminated (3h)
- B: Germination of apical cell (4½h)
- C: Elongation of non-septate germ tube (6h)
- D: Germ tube septate. Appressorium forming over anticlinal host wall (9½h)
- E - F: Penetration of host epidermal wall (10½h)
- G - H: Development of intracellular hypha in host epidermal cell (10½h)
- I: Detail of appressorium (22½h)
- J: Penetration peg and intracellular hypha (22½h)

(scale bar = 10 µm)

Abbreviations: A - appressorium, Co - conidium, GT - germ tube, PH - penetration hypha, RH - intracellular hypha, V - vacuole.

Arrow denotes point of penetration of leaf.



occurred naturally outdoors, penetrations on leaves inoculated in the laboratory had longer germ tubes. In nature, germ tubes seldom extended more than two epidermal cell widths across the leaf before producing an appressorium.

4.2.3 Spore germination

Plates 4.3, 4.4 and 4.5 show stages in the germination and subsequent degeneration of conidia of *D. dictyoides*. There were two prominent wall layers in the ungerminated conidium; a usually narrow outer electron dense wall and a wider electron-transparent wall. Conidia were septate and the septa were continuous with the inner conidial wall layer.

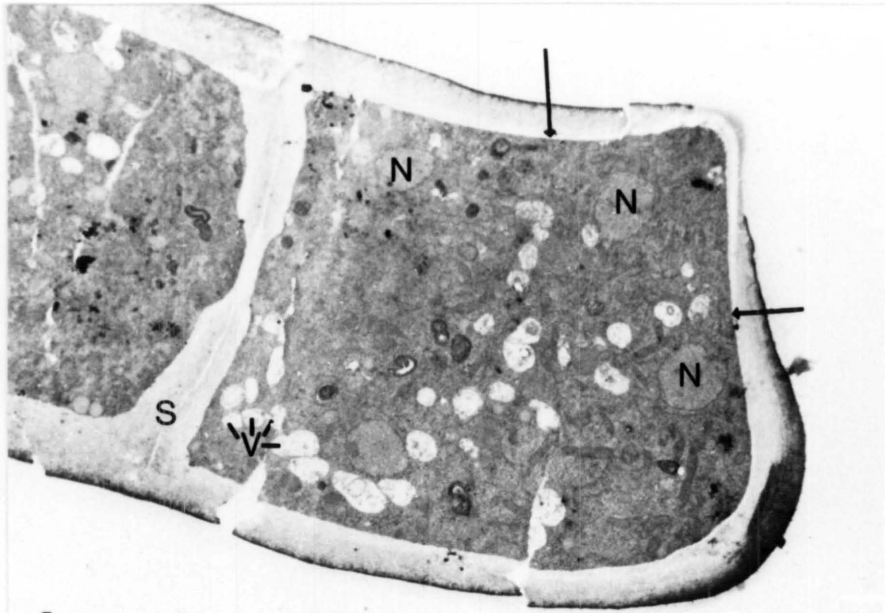
No evidence was found for a pre-formed germination pore but germination was almost solely from the basal or apical cell; those from the basal cell usually germinated close to the hilum. Germination began with the appearance of a new inner wall layer in the region of germination (plate 4.3:A). The developing germ tube forced its way past the existing wall layers of the conidium (Plate 4.3:B). Interferencecontrast microscopy of germinating conidia indicated a 'tear' in the outer conidial wall, the germ tube clearly emerging from within, rather than as an extension to the outer layer of the wall.

The emerging germ tube initially showed a single electron-transparent wall layer, but this was surrounded by a fibrillar layer (plate 4.3:C) which was also found near the apex in developing hyphae. Cells of the conidium were multinucleate (plate 4.4:B) and nuclei were usually found close to the developing germ tube (plate 4.3:B) indicating that the nuclei of the new hyphae could originate

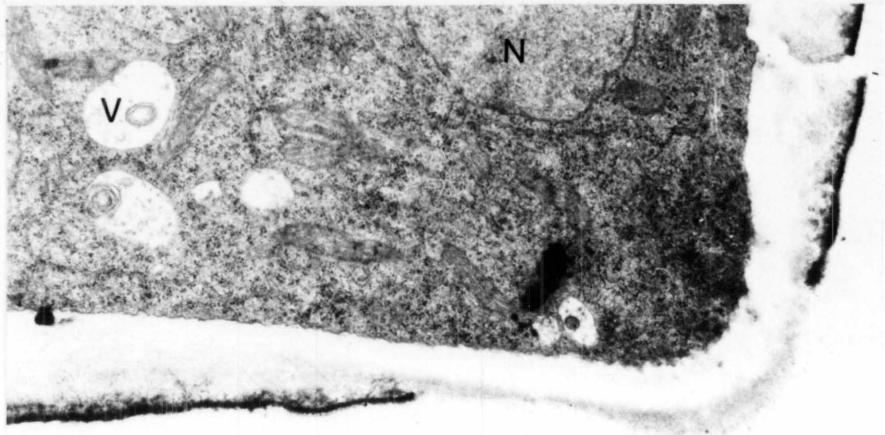
Plate 4.3 Germination of a conidium of *D. dictyoides* at the time of germ tube emergence.

- A: Germ tube emerging from basal cell of conidium. Arrows indicate origin of germ tube wall. Bar = 5 μ m
- B: Break in wall layers of conidium allowing the emergence of a germ tube. Note nucleus in proximity of germ tube. Bar = 1 μ m
- C: Emerging germ tube wall surrounded by a fibrillar layer. Bar = 1 μ m

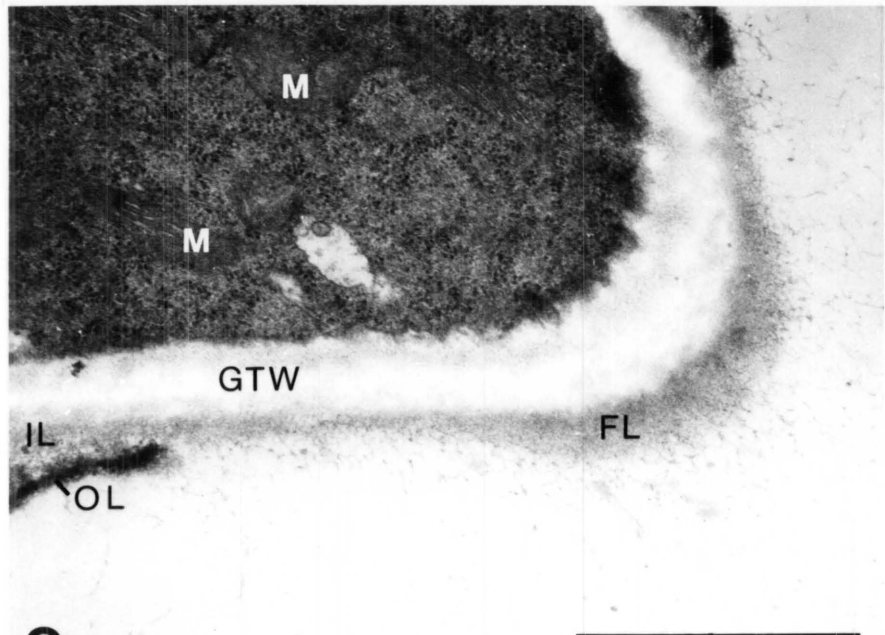
Abbreviations: FL - fibrillar layer, GTW - germ tube wall, IL - inner wall layer, M - mitochondrion, N - nucleus, OL - outer wall layer, S - septum, V - vacuole



A



B



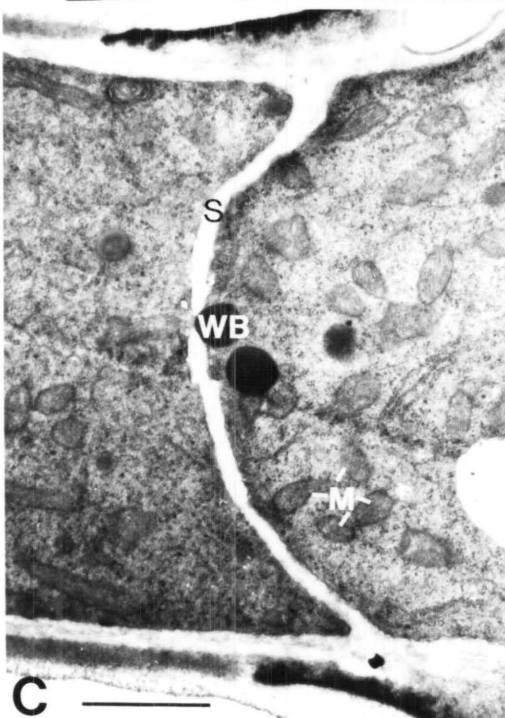
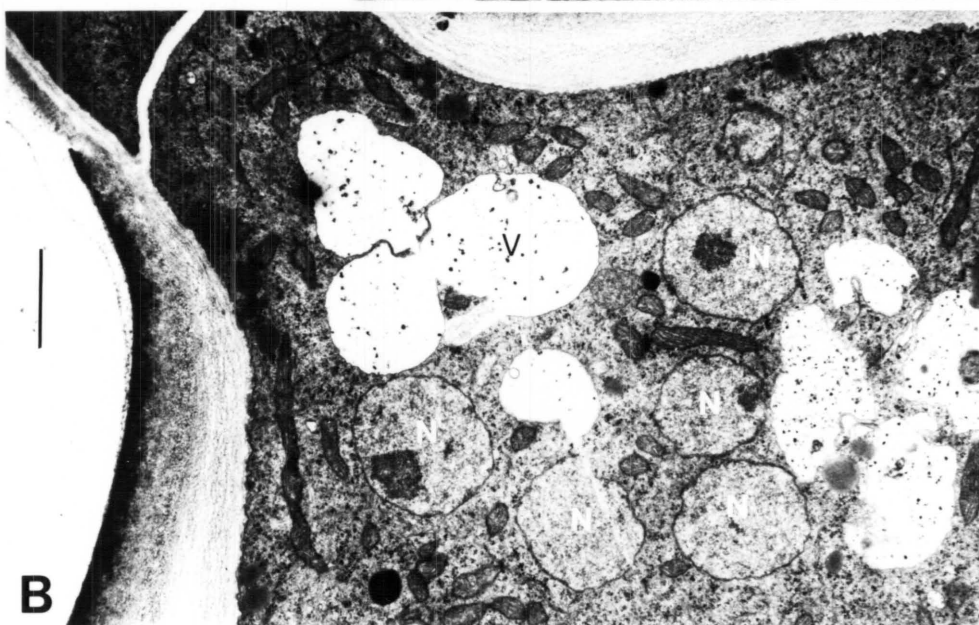
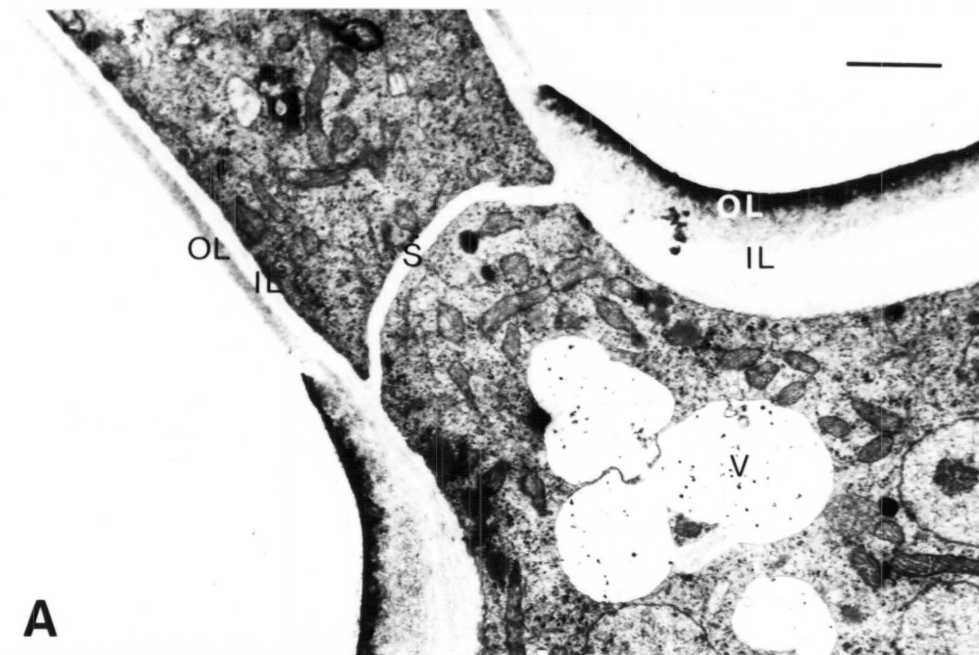
C

Plate 4.4 Region of emergence of germ tube from conidium.

Bar = 1 μ m

- A: An outer wall layer has formed on the germ tube and a septum delimits the germ tube from the conidium.
- B: Multinucleate nature of conidial cell from which germ tube has arisen.
- C: Septum delimiting germ tube from conidium showing septal pore plugged by a Woronin body.
- D: Wall layers of the conidium in proximity to a germ tube. A new innermost wall layer is continuous with the germ tube wall.

Abbreviations: IL - inner wall layer, M - mitochondrion, N - nucleus, NW - new wall layer, OL - outer wall layer, S - septum, V - vacuole, WB - Woronin body



from several conidial nuclei. Mitochondria were abundant in all cells of the conidium at the time of germination and showed no sign of differential accumulation.

A septum usually formed at, or close to the origin of the germ tube (plate 4.4). Plate 4.4:C shows a septal pore at this point, which has been plugged by a Woronin body from within the conidium. Small vacuoles were common in germinating conidia (plate 4.3:A) but these enlarged as germination proceeded. An outer electron dense wall layer developed on the germ tube, giving the two distinct wall layers of fungal hyphae (plate 4.4:A).

Forty-eight hours after inoculation of the leaf, the conidium and germ tube had become senescent (plate 4.5). Cytoplasm had degenerated and few recognisable organelles remained (plate 4.5:C). A layer of lipid bodies surrounded the conidial wall (plate 4.5:A) and the septa within the conidium remained unplugged, although surrounded by Woronin bodies. The inner wall layer of the conidium often became convoluted and frequently increased in diameter with the addition of new wall material (plate 4.5:D). After penetration of the host senescence followed in the germ tube and the same cytoplasmic degeneration occurred (plate 4.5:E).

4.2.4 Appressoria

The next major phase in the infection process is the production of appressoria. Appressoria of *D. dictyoides* were usually club-shaped and frequently were separated from the germ tube by a septum. They developed on glass as well as on the leaf and so did not require a natural substrate for their production.

Plate 4.5 Senescence of a germinated conidium (forty-eight hours after inoculation of the leaf).

Bar = 1 μ m unless otherwise stated

- A: Germinated conidium alongside the host epidermis. Note presence of lipid bodies lining the walls.
- B: Region of germination showing ruptured outer wall layers.
- C: Germinated conidium with an unplugged septal pore, surrounded by Woronin bodies. Note advanced stage in degeneration of cytoplasm.
- D: Wall region of senescent conidium, showing concentration of lipid bodies and thickened wall.
- E: Germ tube, showing same degree of cytoplasmic degeneration. Bar = 0.5 μ m

Abbreviations: HE - host epidermis, LB - lipid body, WB - Woronin body.

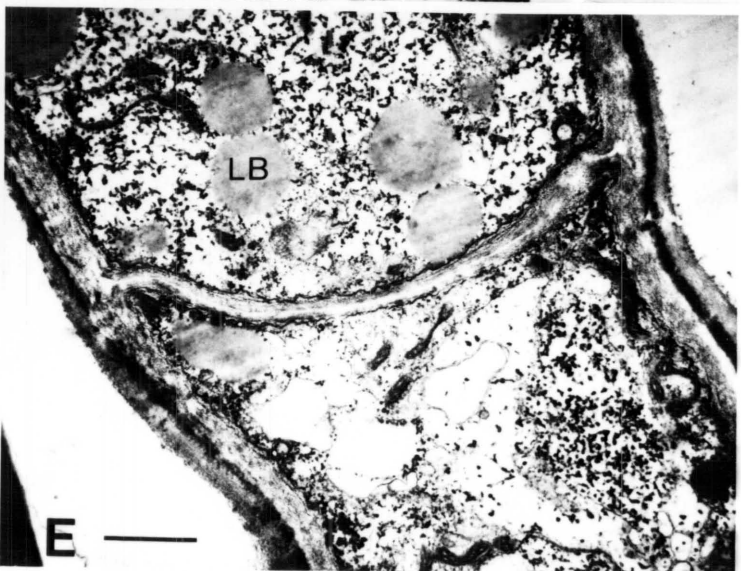
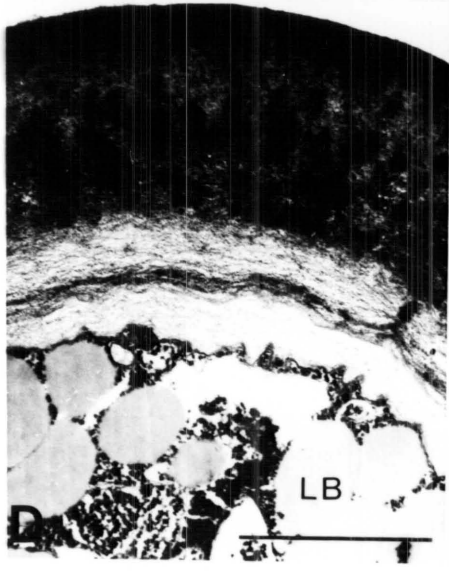
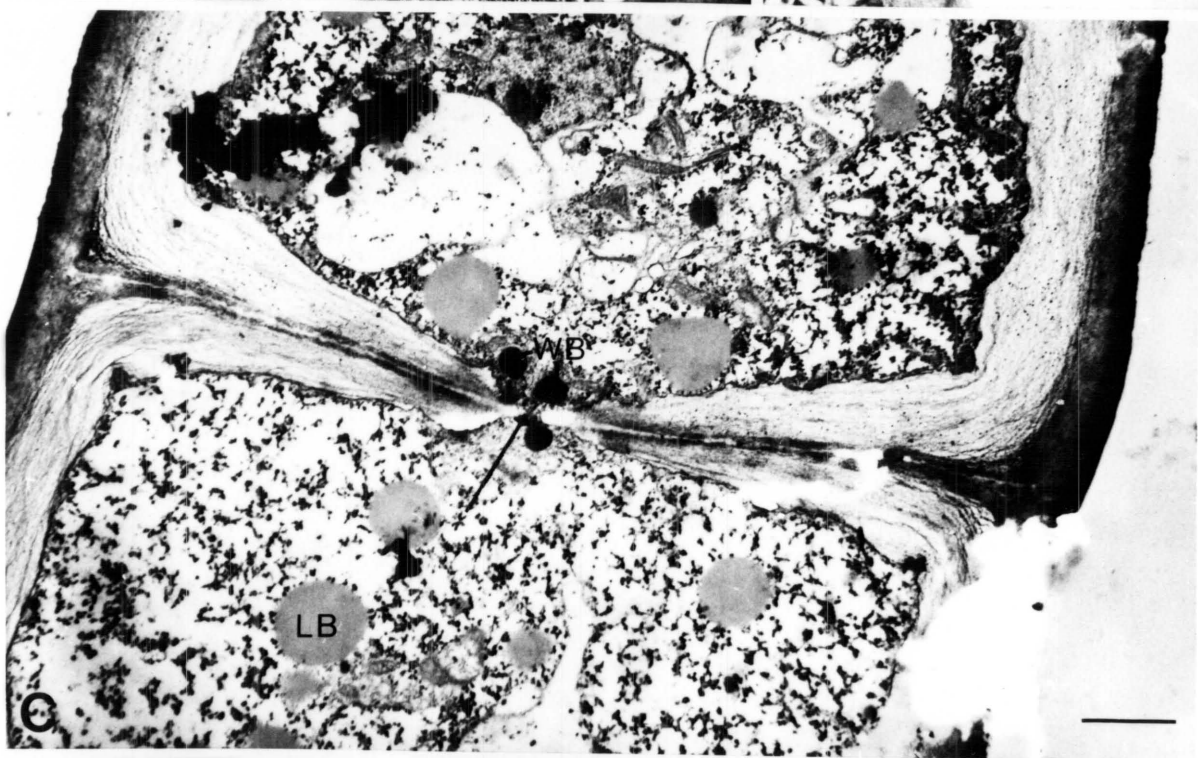
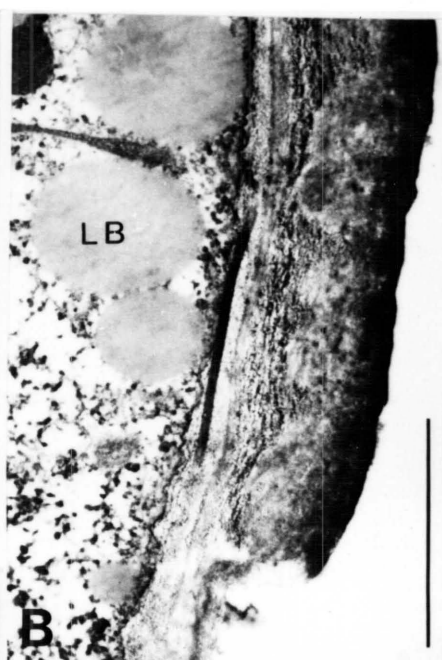
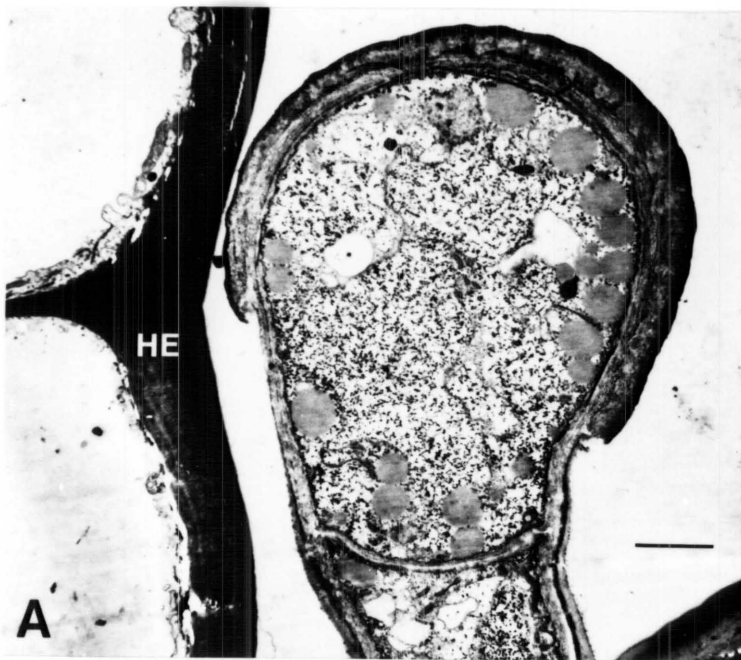


Plate 4.6 shows TEM micrographs of an appressorium prior to penetration of the leaf. It formed over the longitudinal junction of two epidermal cells of the host and was closely adpressed to the host epidermis although some separation caused by fixation is visible. Unlike the appressorium, the germ tube had only a loose association with the host epidermis. The section illustrated is a median one through the appressorium. The appressorium wall consisted of the two distinct layers normally present in hyphae. The outer wall layer was absent from the region of contact between the appressorium and host epidermis and finished abruptly with a slight expansion where it abutted the epidermis (plate 4.6:B). The inner wall layer was present along most of the region of contact between the appressorium and host, but was absent in a region of extracellular vesicular material (plate 4.6:C). In the example illustrated there was little sign of dissolution of the host epidermis, although the narrow cuticle proper had been dissolved in the region directly beneath the vesicular material. Mitochondria were abundant in the appressorium.

4.2.5 The infection process as seen by Scanning Electron Microscopy.

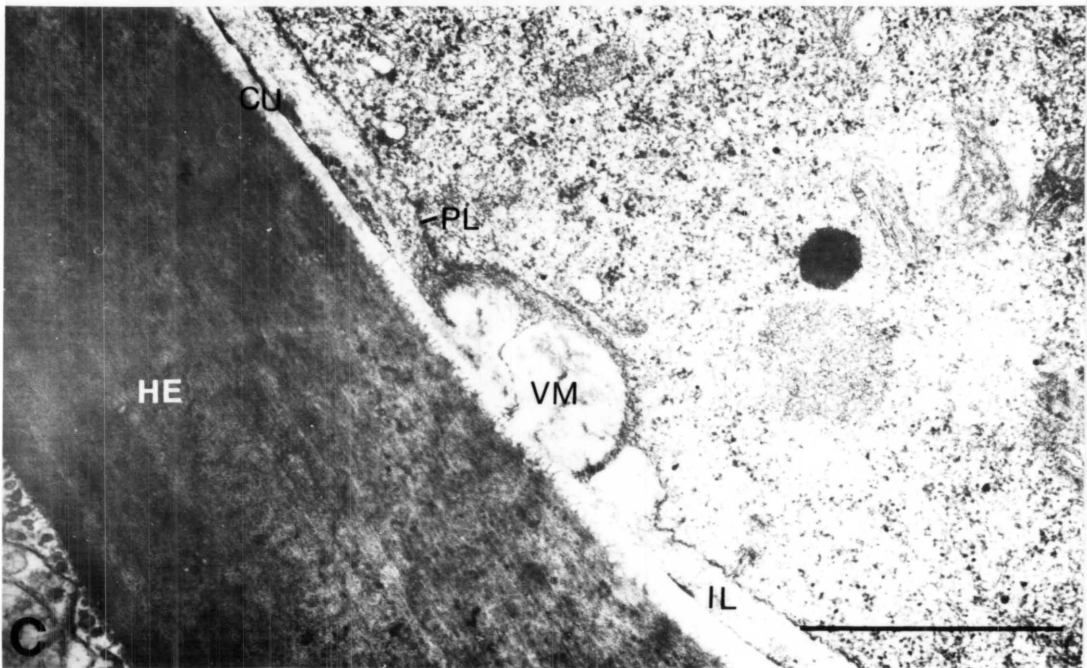
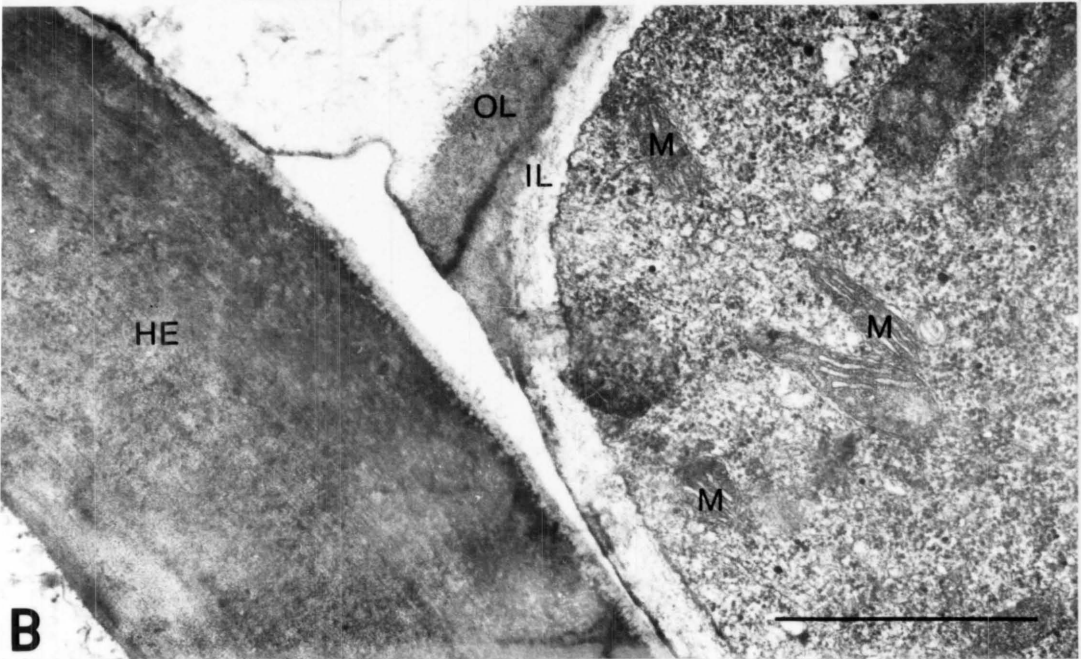
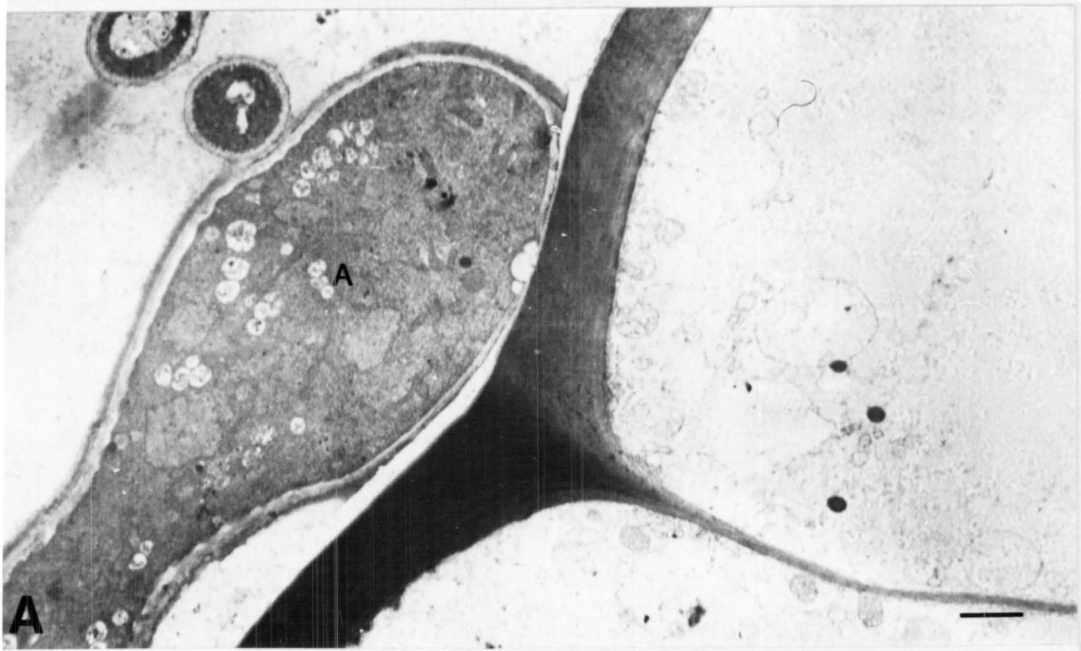
The surface morphology of the infection process was followed using the SEM (plate 4.7). The three-dimensional shape of the ryegrass epidermis could be clearly seen as could the depression marking the anticlinal wall over which the appressorium usually formed. In plate 4.7:A a conidium had produced a germ tube from its basal cell and an appressorium over an anticlinal wall. Bacteria

Plate 4.6 Appressorium of *D. dictyoides* over an anticlinal epidermal wall of *L. perenne* prior to penetration.

Bar = 1 μ m)

- A: Position of appressorium in depression over host anticlinal wall.
- B: Attachment of appressorium to host epidermis with some separation probably due to fixation.
- C: Region of penetration attempt of host cell wall. Note extracellular vesicular material.

Abbreviations: A- appressorium, CU - host cuticle, HE - host epidermis, IL - inner wall layer, M - mitochondrion, OL - outer wall layer, PL - plasmalemma, VM - extracellular vesicular material.



surrounded the appressoria on many occasions and were also found on its surface (plate 4.7:A-C). Appressoria were not necessarily produced at the first anticlinal wall met, and the germ tube often grew for some distance over the leaf surface. Germ tubes frequently produced swellings, often over anticlinal walls, which did not develop fully into appressoria (plate 4.7:D). In this case the germ tube usually recommenced growing and, often, later produced a normal appressorium. Regermination could take place from fully formed appressoria which had not penetrated the leaf surface. Secondary appressoria were capable of producing infection pegs. In plate 4.7:E a collapsed appressorium shows the penetration hole in the leaf.

4.2.6 Penetration

Once an appressorium had formed, penetration could begin. A penetration peg arose under the appressorium (plate 4.8:E), sometimes with co-incident production of wall apposition material on the host epidermal wall below. Where penetration was successful, a round hole approximately 2 μm in diameter could be seen in the epidermis (plate 4.8C & D). A penetration hypha, or peg, was formed within the penetrated epidermal cell (plate 4.8:F) and from there the hyphae spread intracellularly for a time. The epidermal wall surrounding the penetration peg reacted visibly by becoming darker (plate 4.8:D) and an increase in density can be inferred by the greater difficulty in sectioning epidermal walls around infection sites as compared with healthy epidermal walls.

PA-TCH-AgPr staining was used to detect the polysaccharide component of the fungal and host walls

Plate 4.7 Surface view of the infection process of
D. dictyoides on the abaxial leaf surface of
L. perenne.

(SEM micrograph of critically point dried
material unless otherwise stated)

A: Germinated conidium where the appressorium
is surrounded by bacteria. Note concavity
along line of anticlinal wall. Bar = 10 μ m

B: Appressorium surrounded by bacteria. Bar = 10 μ m

C: Bacteria on surface of appressorium. Bar = 1 μ m

D: Conidium with swelling near origin of germ
tube, probably indicating early abortion
of appressorium. Bar = 10 μ m

E: Collapsed appressorium showing point of
penetration of leaf. (Fresh mounted material)
Bar = 10 μ m

Abbreviations: A - appressorium, AW - anticlinal
wall of epidermis, Co - conidium, GT - germ
tube, PP - point of penetration of host

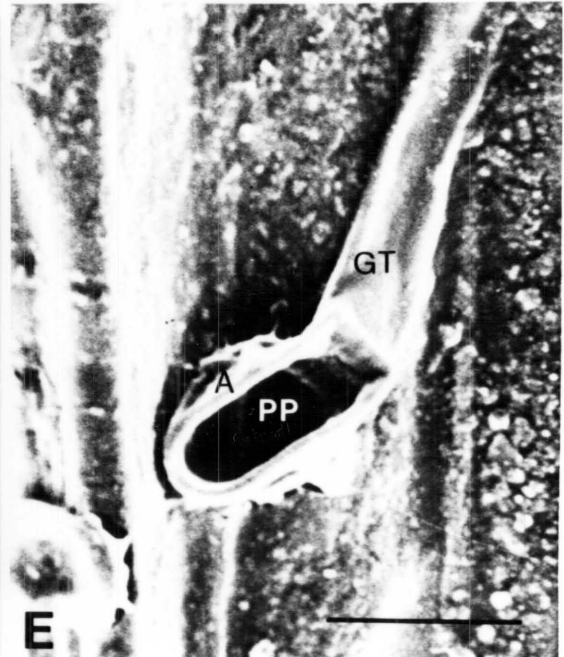
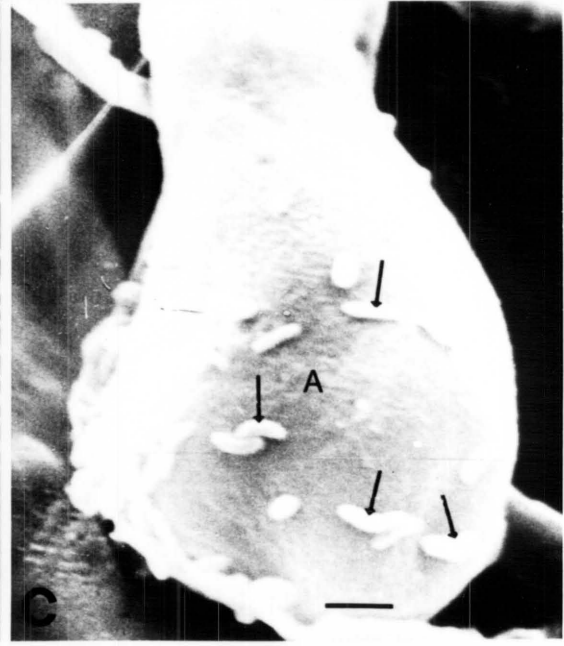
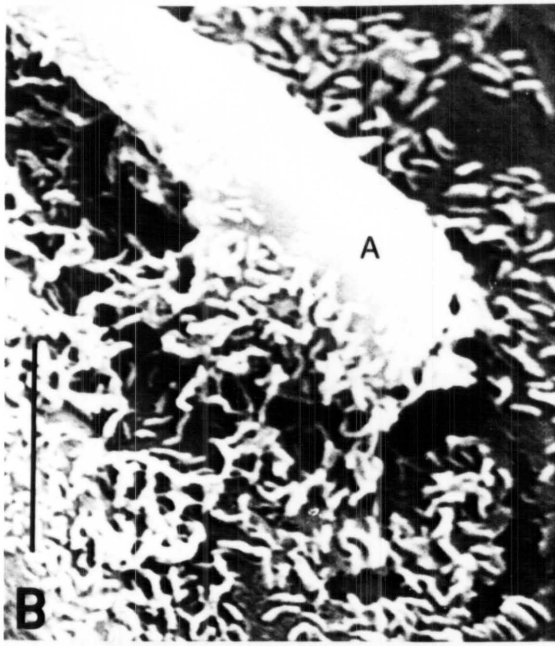
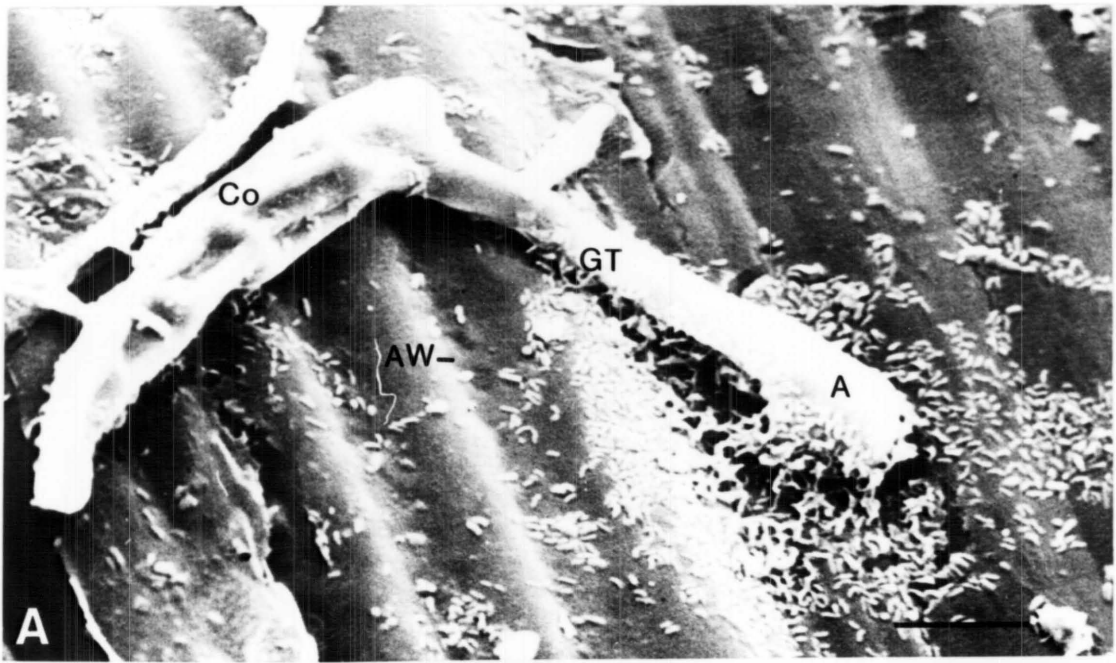
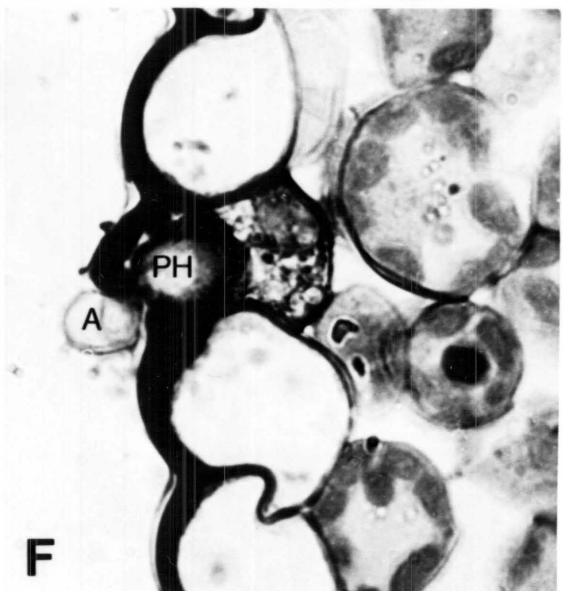
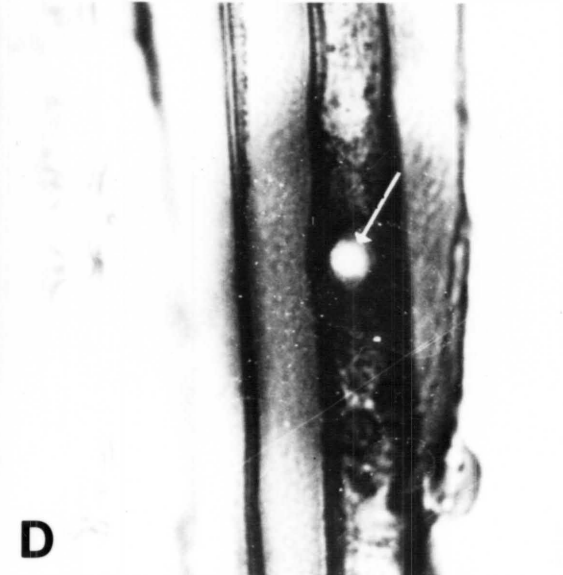
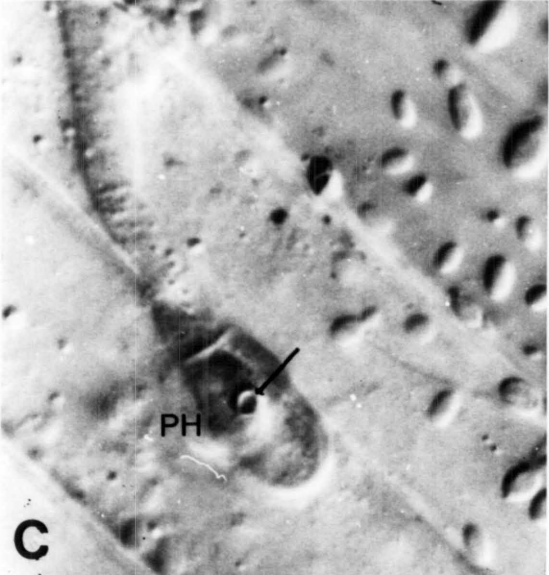
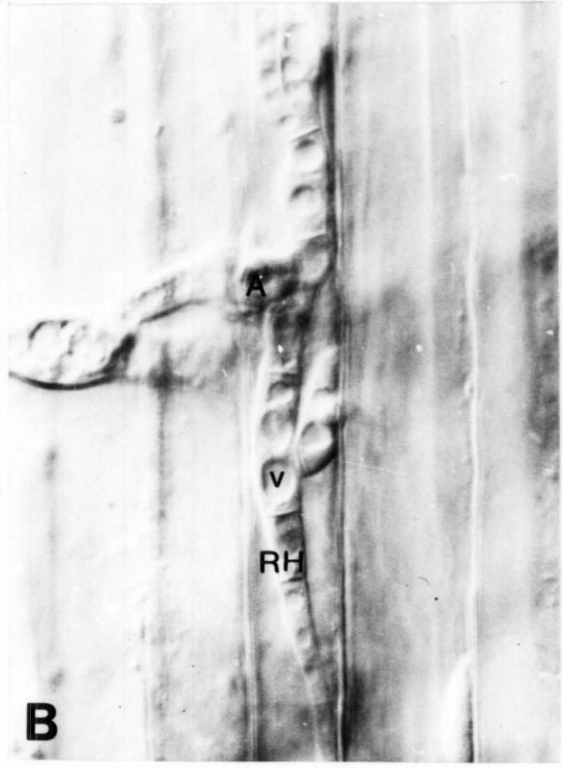


Plate 4.8 Penetration of the abaxial ryegrass epidermis
by *D. dictyoides*.

(A - C Nomarski Interference Contrast, D,F Bright
field microscopy, E TEM)

- A: Germinated conidium which has produced an
appressorium and penetrated the epidermis.
- B: Intracellular hyphae within epidermal cell
following penetration.
- C: Appressorium showing point of penetration of
leaf and primary infection hypha.
- D: Section in plane of leaf showing the hole
where penetration has occurred and darkening
of the epidermis around it.
- E: Partially completed penetration of epidermis.
Note wall apposition material below appressorium.
- F: Successful penetration into epidermal cell.

Abbreviations: A - appressorium, PH - penetration
hypha, RH - intracellular hyphae, V - vacuole,
arrow indicates point of penetration of host
wall.



during penetration (plate 4.9). The inner electron transparent wall of the penetration peg separated it from the surrounding host wall. In the region of active penetration of the host wall, however, the fungal wall was absent although vesicles which appeared to be extracellular were present in close proximity to the host wall (plate 4.9:C). A septum, with a simple pore, formed between the appressorium and the infection peg. Hyphal walls on the leaf surface showed a darker staining reaction than did the wall of the penetration peg. The two layered wall structure of the fungal hyphae was still evident in the hyphae external to the host (plate 4.9:D), but there was less distinction between the layers than when sections were stained with uranyl acetate and lead citrate.

Plate 4.10 shows the successful penetration of the ryegrass epidermis. The appressorium has become mis-shapen during tissue preparation. As shown in plate 4.6, the appressorium had two wall layers, the outer one of which X dissapeared before the penetration peg was reached. The inner layer became continuous with the wall of the primary infection hypha within the host epidermal cell. The outer epidermis in the region of the penetration became buckled but did not appear to be any thicker. At the point of penetration a septum formed (plate 4.10:B) and the septal pore was plugged by a Woronin body in the primary infection hypha (plate 4.10:C). The primary infection hypha contained large vacuoles, but mitochondria were still prominent in the cytoplasm. Coagulated cytoplasmic material was all that remained of the host cytoplasm (plate 4.10:A).

Until now only direct penetration of the epidermis has been considered. Whilst this was the most usual

Plate 4.9 Penetration of *L. perenne* by *D. dictyoides*.

(Transmission Electron Microscopy: PA - TCH -
AgPr staining)

Bar = 1 μ m

A: Appressorium with penetration peg.

B: Separation of appressorium and penetration
peg.

C: Vesicular material at point of active
penetration.

D: Hypha on abaxial surface of leaf.

Abbreviations: Cy - host cytoplasm, D -
dictyosome, ER - endoplasmic reticulum, FW -
fungal wall, HE - host epidermis, HW - host
wall, M - mitochondrion, PL - plasmalemma, PP -
penetration peg, VM - vesicular material

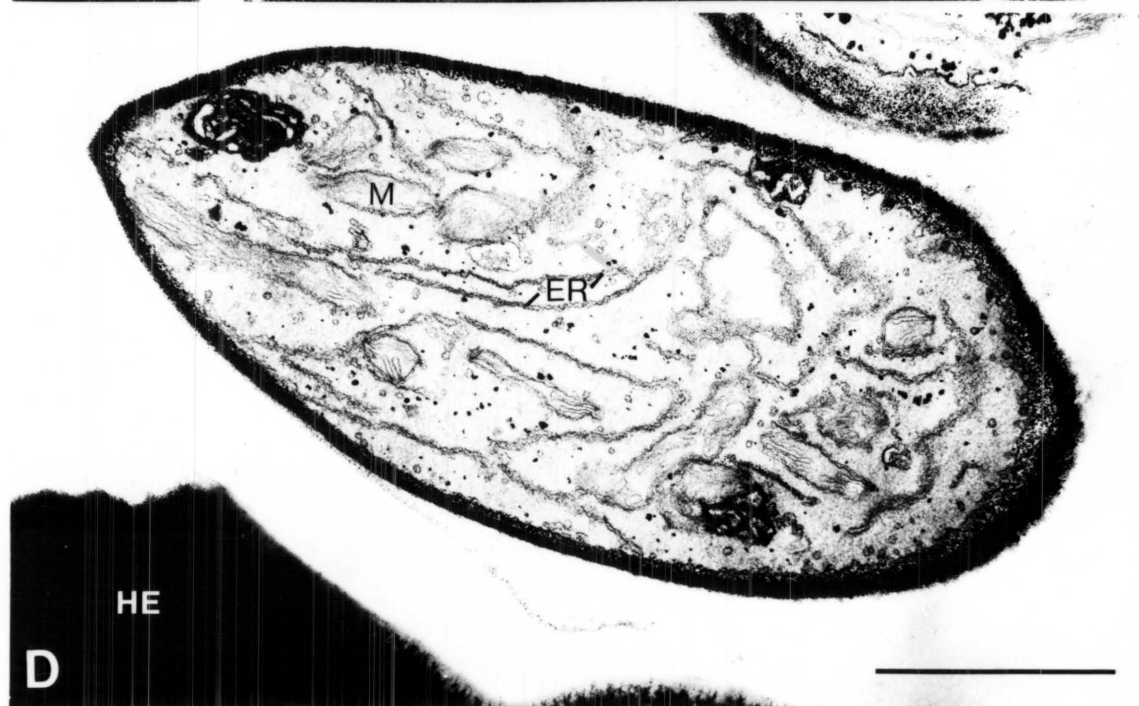
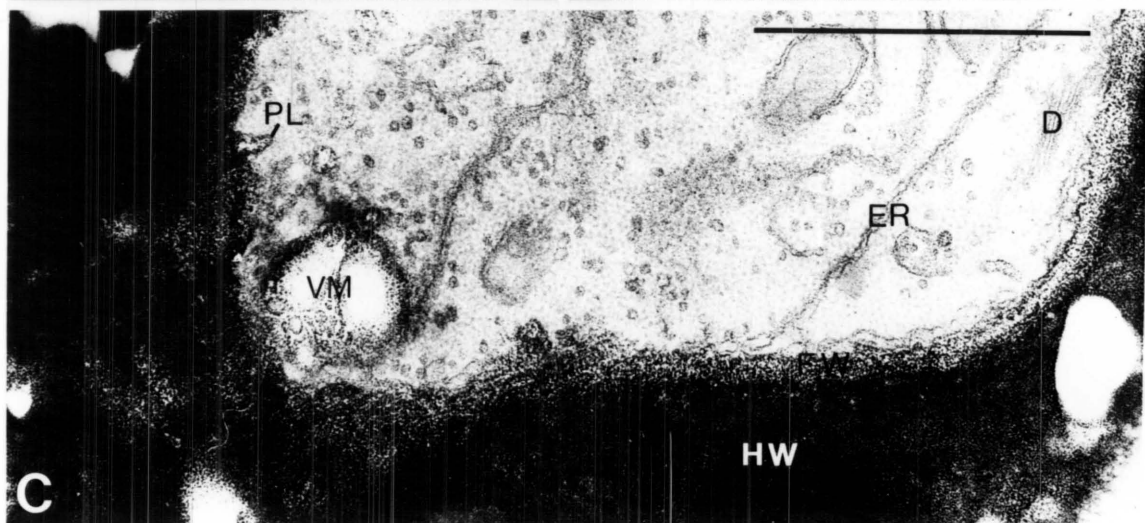
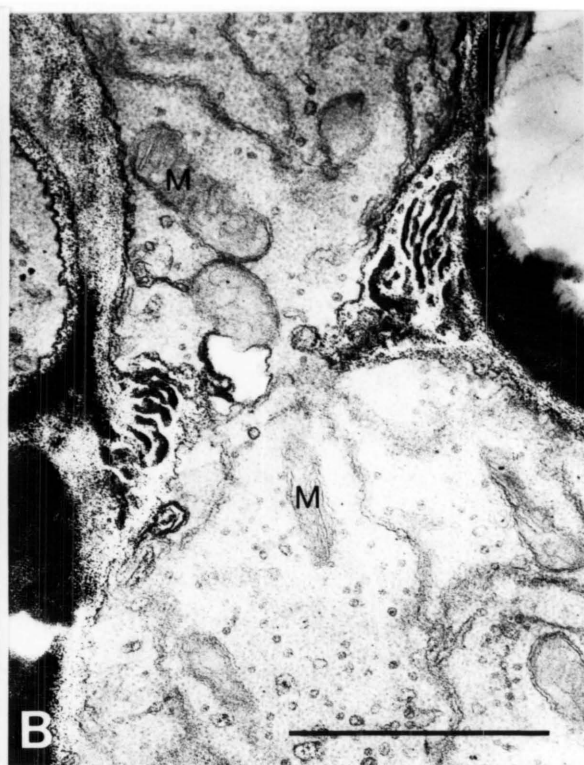


Plate 4.10 Successful penetration of the ryegrass epidermis by *D. dictyoides*. (Transmission electron microscopy)

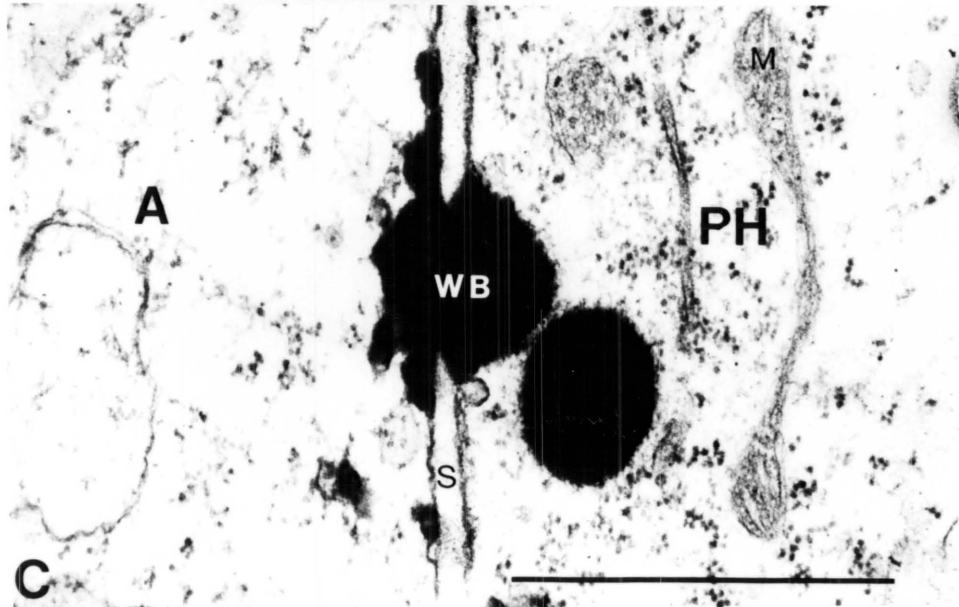
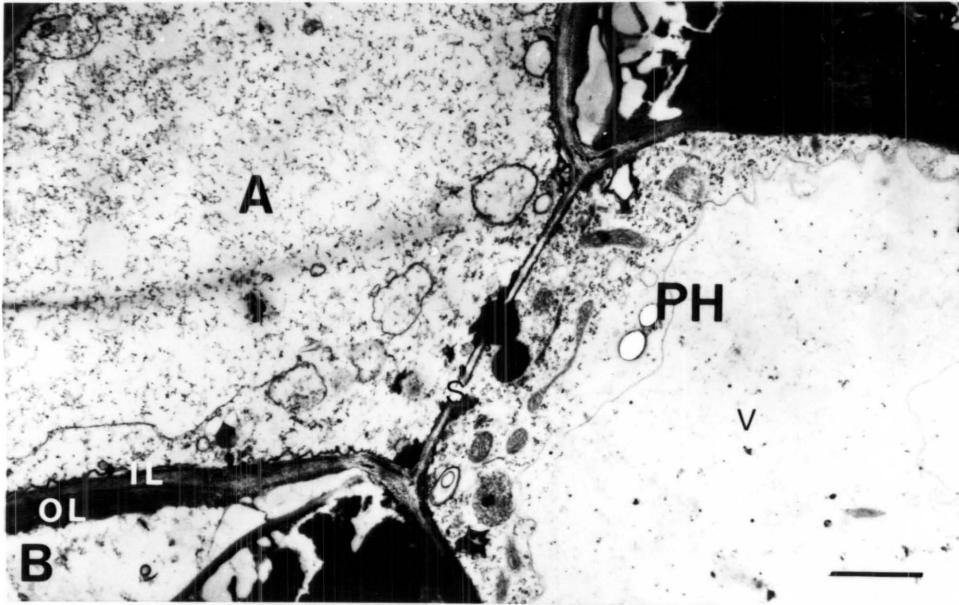
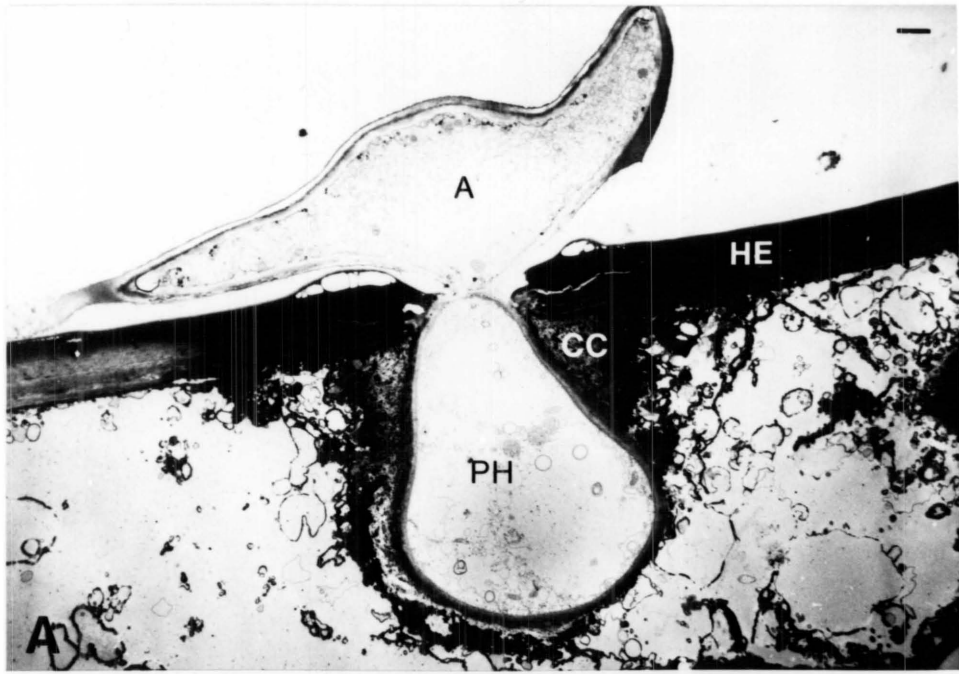
Bar = 1 μ m

A: Appressorium and penetration hypha

B: Median section of penetration showing septum at point of penetration of the leaf.

C: Septal pore plugged by a Woronin body in the penetration hypha. Another Woronin body lies nearby.

Abbreviations: A - appressorium, CC - coagulated host cytoplasm, HE - host epidermis, IL - inner hyphal wall layer, M - mitochondrion, OL - outer hyphal wall layer, PH - penetration hypha, S - septum, WB - Woronin body



situation, subcuticular infection in the early stages of disease was also a frequent phenomenon, both in the case of natural infection and when the leaves were inoculated in the laboratory. The pathogen frequently remained subcuticular for as long as forty-eight hours after leaf inoculation and occasionally this was as far as the infection proceeded. The structure of the monocotyledon leaf cuticle is not well understood and will be discussed in chapter six (p. 150). Subcuticular hyphae are defined in the present study as those growing between the outer layer of the cuticle and the host plasmalemma, the exact position varying individually.

In surface view using cleared leaves stained with lactophenol cotton blue, subcuticular penetrations were typified by largely unstained hyphae spreading from beneath the blue-stained appressorium across several rows of epidermal cells (plate 4.11:A). In addition, hyphae frequently grew along the region above the anticlinal wall of the ryegrass epidermis (plate 4.11:E). If disease was to develop further it was necessary for the fungus to penetrate an epidermal cell. This usually occurred in the vicinity of the appressorium although not necessarily directly under it (plate 4.11:B). A hyphal-filled cavity was formed inside the epidermal wall and there was frequently evidence of degeneration of the host wall (plate 4.11:F).

The extreme case of subcuticular infection is illustrated in plate 4.12. Only the outermost layer of the host epidermal wall was penetrated and the subcuticular hyphae were surrounded by host wall material or their coagulated remains. The host wall had the appearance of being 'folded back' (plate 4.12:B), leaving a cavity for

Plate 4.11 Subcuticular infection of the abaxial epidermis
of *Lolium perenne* by *D. dictyoides*.

(A,B,D Optical microscopy, C,E,F TEM)

- A: Cleared leaf showing appressorium and
subcuticular hyphae. Bar = 1 μ m
- B: Section of epidermis showing subcuticular
hyphae penetrating an epidermal cell.
Bar = 10 μ m
- C: Penetration of the outer layers of the host
epidermal wall. Bar = 1 μ m
- D: Optical section of C.
- E: Subcuticular hyphae within the anticlinal
wall region of the abaxial epidermis. Bar = 1 μ m
- F: Subcuticular hyphae with intracellular
hyphae in the epidermal cell below. Bar = 1 μ m

Abbreviations: A - appressorium, CO - conidium,
GT - germ tube, PH - penetration hypha, RH -
intracellular hyphae, SC subcuticular hyphae,
V - vacuole

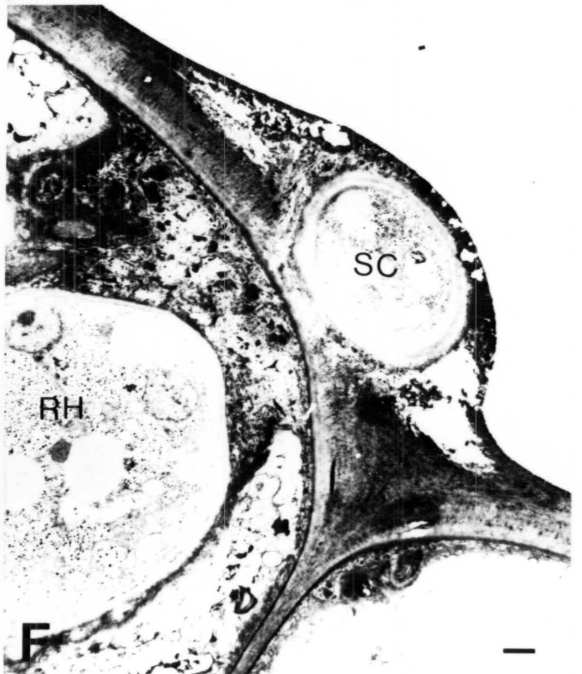
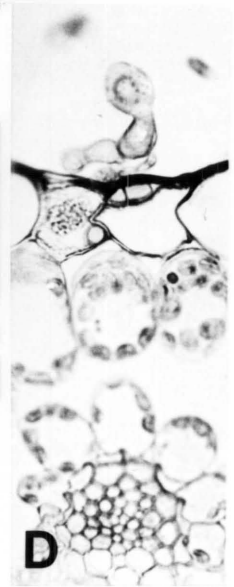
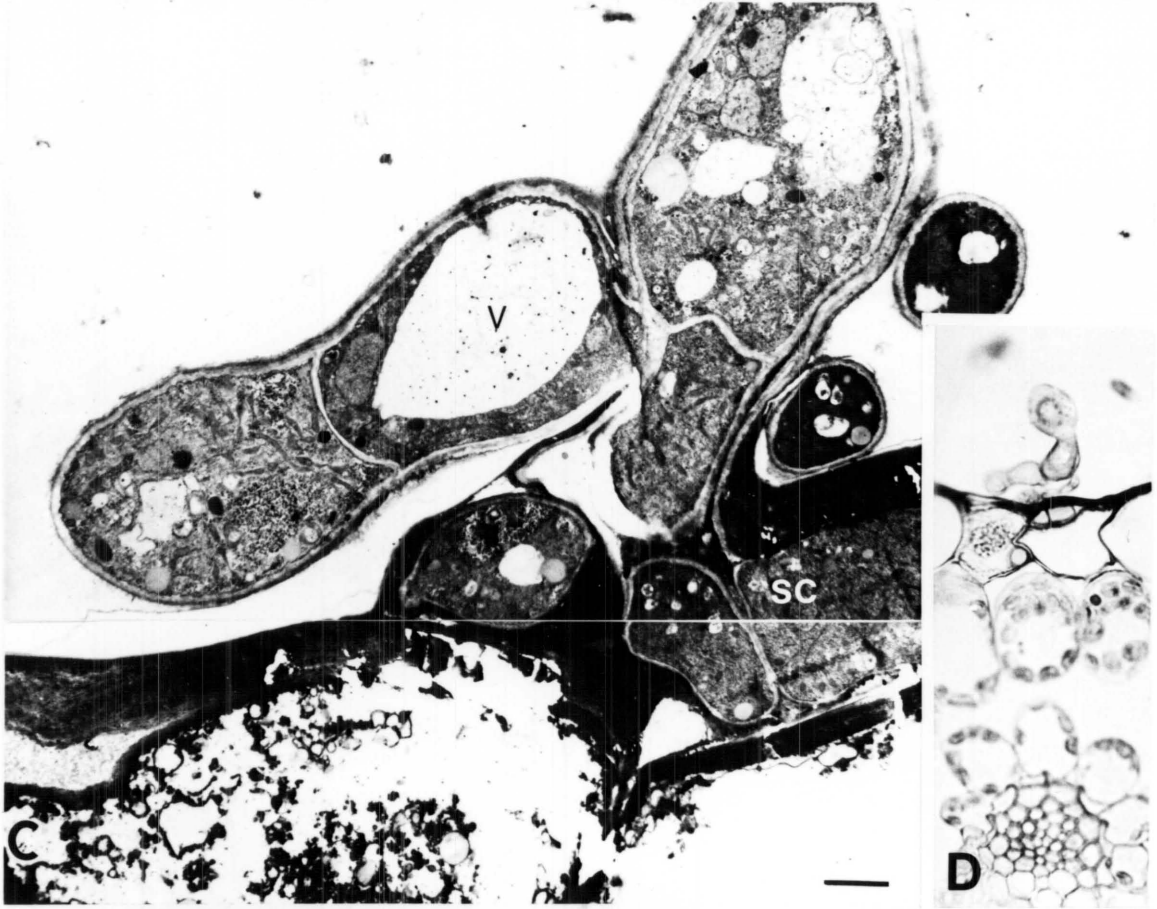
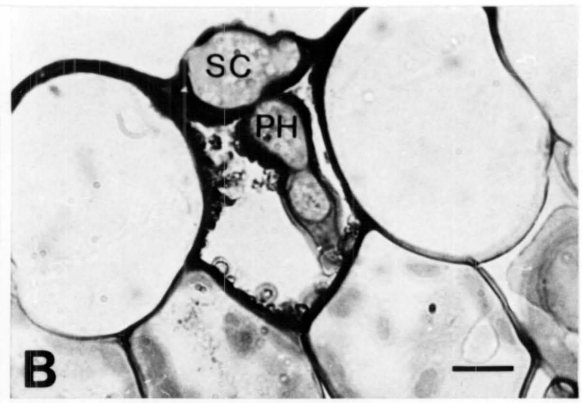
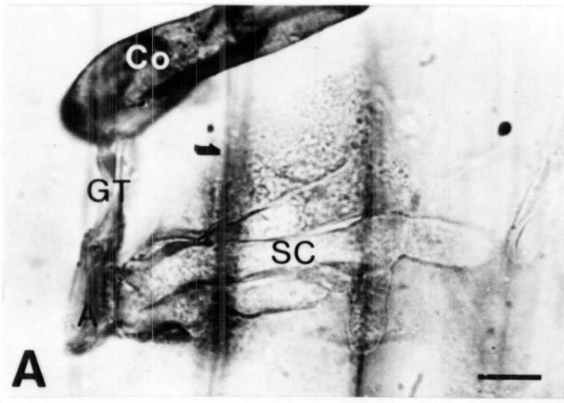
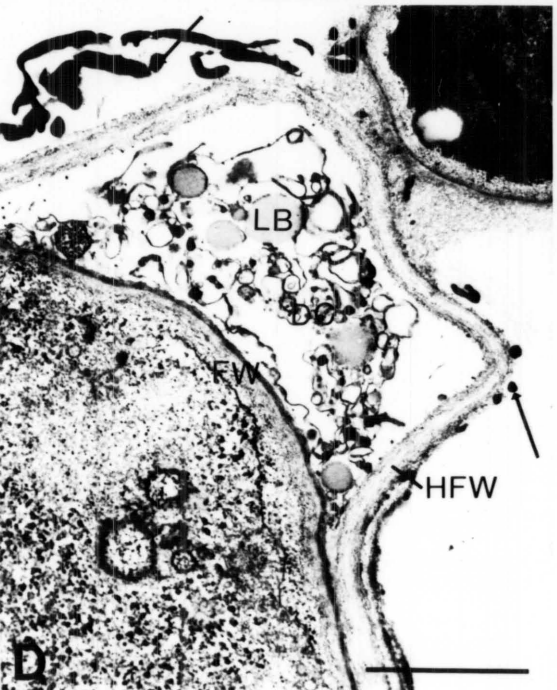
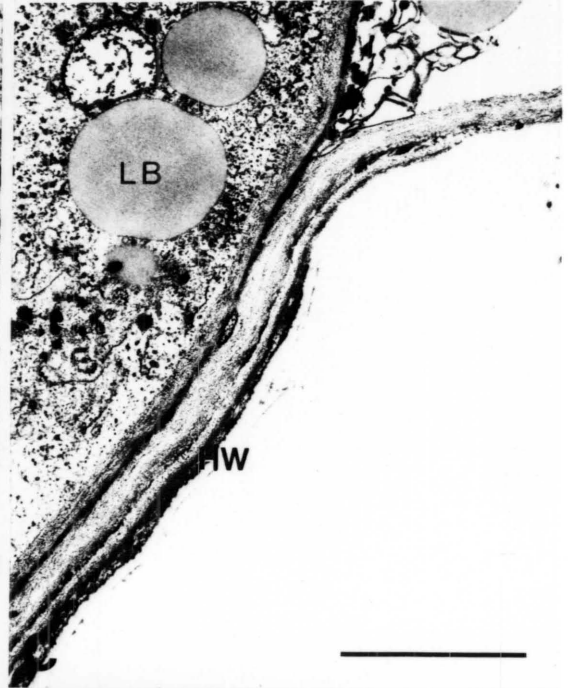
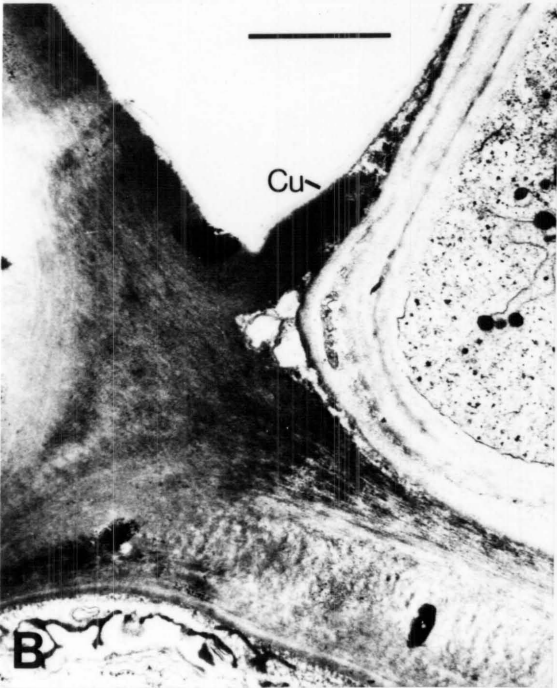
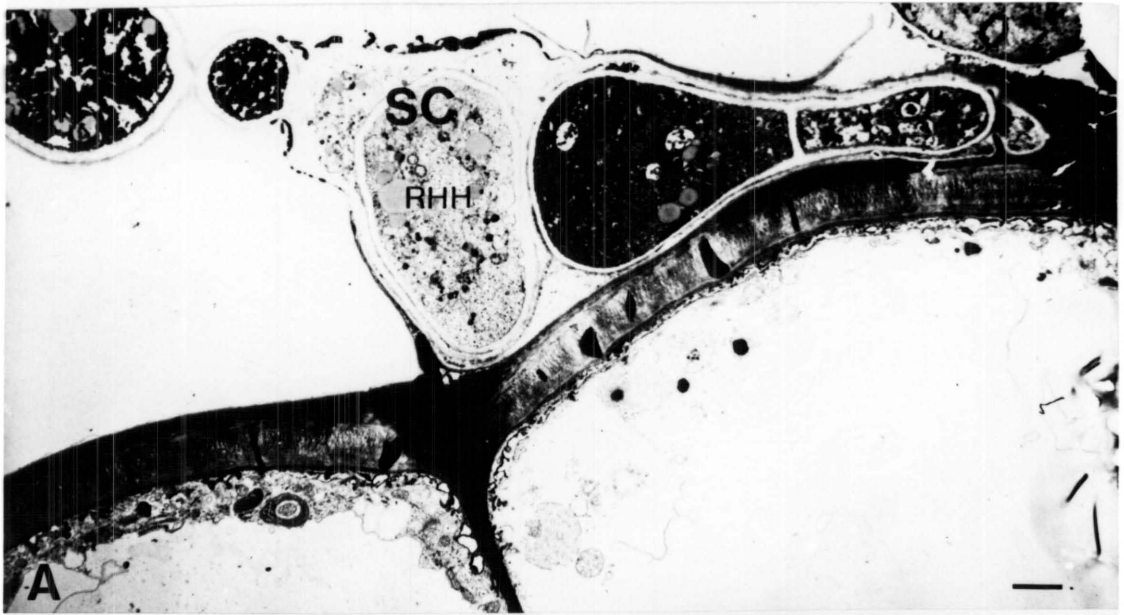


Plate 4.12 Subcuticular hyphae in the outermost wall layers of the host.

Bar = 1 μ m

- A: Subcuticular hyphae surrounded by a thin layer of host cell wall material. Note presence of intrahyphal hyphae.
- B: Outer layers of epidermal wall 'folded back' around subcuticular hyphae.
- C: Point at which host epidermal wall loses its integrity.
- D: Coagulated remains of host cuticle surrounding intrahyphal hyphae. Note close association of wall layers of the two hyphae, and degenerating cytoplasm of host hypha.
- E: Reaction of host cytoplasm below subcuticular hyphae.

Abbreviations: Cu - cuticle, DC: degenerating cytoplasm, FW - fungal wall, HFW - host fungal wall, HW - host wall, LB - lipid body, RHH - intrahyphal hyphae, RM - host reaction material, SC - subcuticular hyphae. Arrows indicate coagulated remains of host cuticle.



the fungus to grow in, but below the hyphae wall appeared relatively normal. The host wall was only able to be stretched by the invading hyphae to a limited extent and so the integrity of the wall was not maintained throughout. The epidermis became thinner and finally was marked only by coagulated wall remains (plate 4.12:D). Intrahyphal hyphae were present in this subcuticular zone and the membrane-bound remains of organelles in the outer hyphal layer were still evident, with lipid bodies common (plate 4.12:D). Host reaction material was produced in the cytoplasm of the epidermal cell below the subcuticular hyphae (plate 4.12:E).

4.2.7 Lesion development

After the fungus had penetrated an epidermal cell of the host, it ramified within it (plate 4.13). Death of the epidermal cell was rapid and the host nucleus was often found in close proximity to the invading hyphae (plate 4.13:D). The nuclear envelope became filled with coagulated material (possible lipid) followed by degeneration of the nuclear contents (plate 4.13:E). This nuclear association will be discussed more fully in chapter six (p. 176). The host cytoplasm became coagulated, leaving few recognisable organelles (plate 4.13:C). Intracellular hyphae often grew through much of the length of the invaded epidermal cell before advancing into the host mesophyll.

During lesion development, hyphae were largely intercellular in the host mesophyll, only becoming intracellular again just prior to sporulation. Reaction of the host mesophyll cells to the advancing hyphae was limited at first and the fungal hyphae grew in advance of tissue necrosis.

Plate 4.13 Intracellular hyphae of *D. dictyoides* within epidermal cells of *L. perenne*.

Bar = 1 μ m

- A: Hypha within an epidermal cell showing general disorganisation of the host cytoplasm.
- B: Detail of hyphae. Note rough cell wall and abundant mitochondria.
- C: Fungal hypha and nearby coagulated host cytoplasm.
- D: Fungal hypha in vicinity of disintegrating host nucleus.
- E: Coagulation of unknown material (possibly lipid) in the nuclear envelope some distance from the fungal hypha.
- F: Normal nuclear envelope in nearby mesophyll cell.

Abbreviations: CC - coagulated host cytoplasm, M - mitochondria Mb - microbody, N - nucleus, NM - nuclear envelope, RH - intracellular hyphae, W - fungal wall

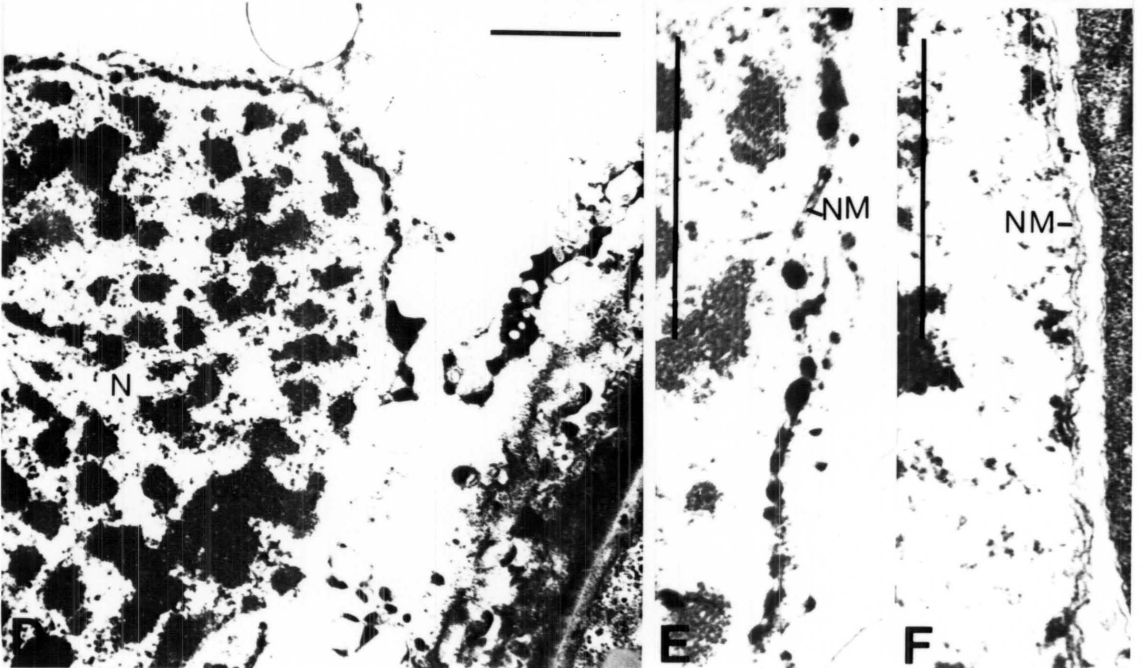
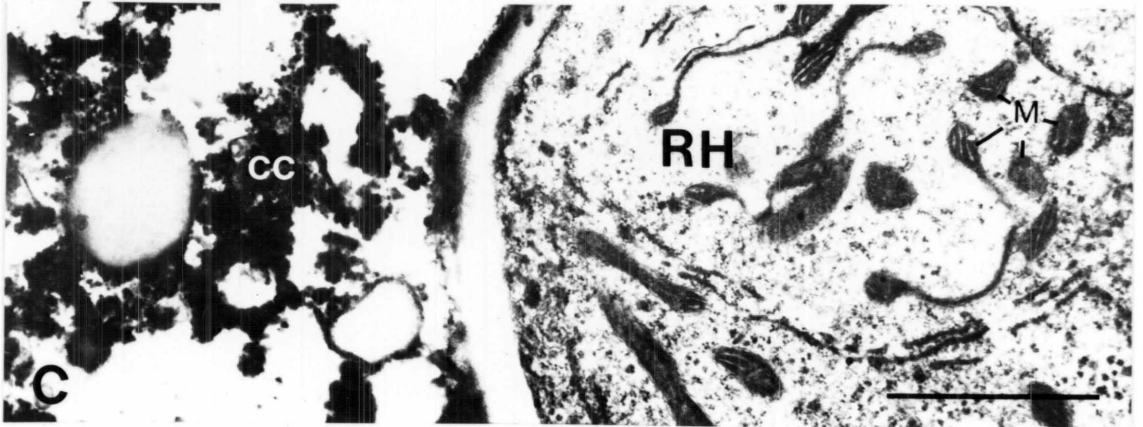
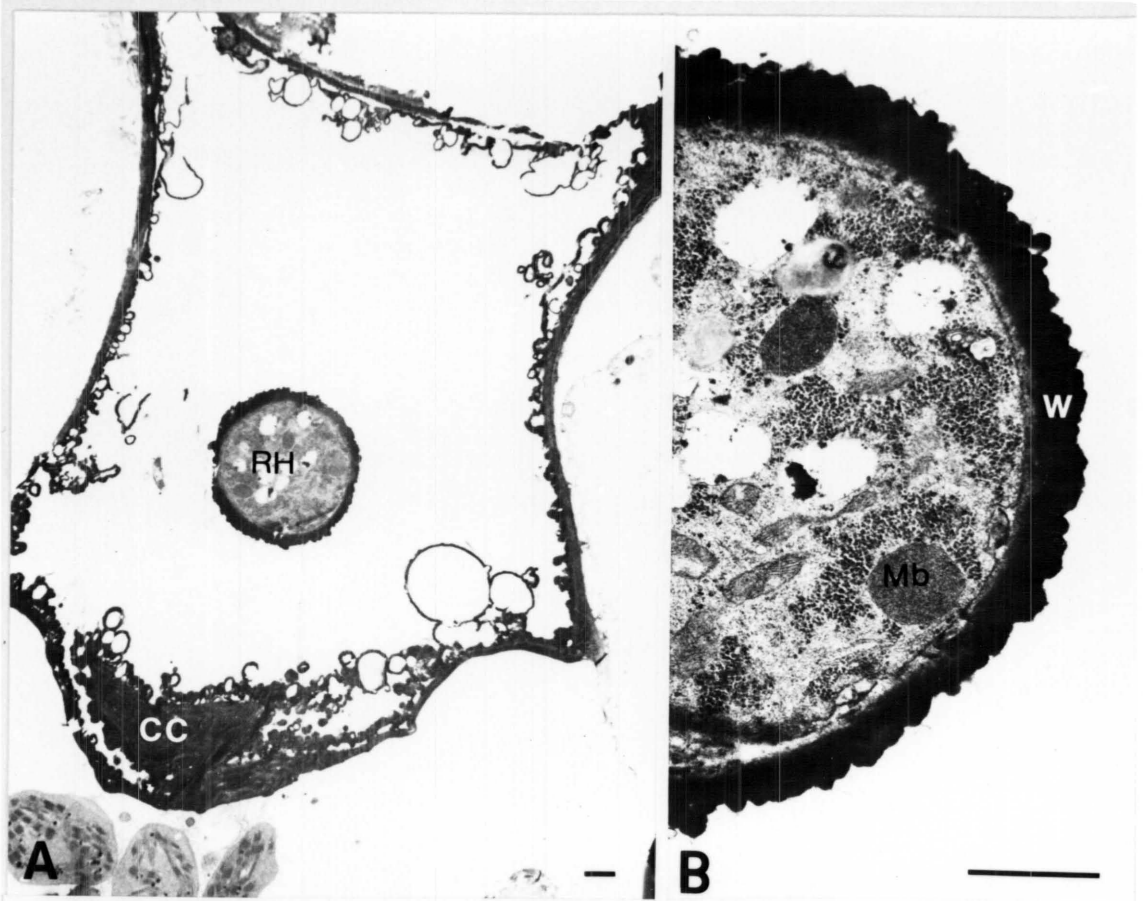


Plate 4.14 shows an early stage in the ramification of intercellular hyphae through the host mesophyll. The hyphae grew in close proximity to the mesophyll cells, with host and fungal walls closely appressed (plate 4.14:C). Integrity of the host chloroplast was maintained at this stage and there was little sign of a host reaction (plate 4.14:B & C). Intercellular hyphae were septate and at this early stage possessed only a single wall layer which was similar in dimensions and nature to the inner, electron transparent, layer of the germ tube (plate 4.14:A). Sometimes a narrow darker region was present, probably marking an early stage in the formation of an outer layer.

At a later stage (plate 4.15), the fungal wall had developed into a well-defined two-layered structure similar to that of the germ tube. Fibrillar material often extended the region of contact between the intercellular hyphae and the host mesophyll cells (plate 4.15:A & D). At this stage host reaction material was produced which was usually in the form of an electron-dense lattice-work extending into the cell lumen (plate 4.15:B & C), although it sometimes consisted of electron-dense granular material (plate 4.15:D). Immediately adjacent to the reaction material the host organelles such as mitochondria, nucleus, dictyosomes, chloroplasts and polyribosomes all retained their structural integrity.

Figure 4.1 illustrates the development of intercellular hyphae through the host mesophyll in a young visible lesion using a cleared leaf preparation of a natural lesion. Mesophyll cells near the hyphae showed no necrosis and necrosis of host cells adjoining the hyphae was usually limited to the region in contact with the fungus. Cells at the margin of the lesion, adjacent to the spreading hyphae

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Plate 4.14 Ramification of fungal hyphae through the host mesophyll.

A: Intercellular hypha between two host mesophyll cells. Bar = 1 μ m

B: Normal chloroplast near fungal hypha. Bar = 1 μ m.

C: Region of contact of host wall and fungal wall showing minimal host reaction. Bar = 0.5 μ m

Abbreviations: C - chloroplast, FW - fungal wall, HW - host wall, IH - intercellular hyphae, PL - plasmalemma, V - vacuole

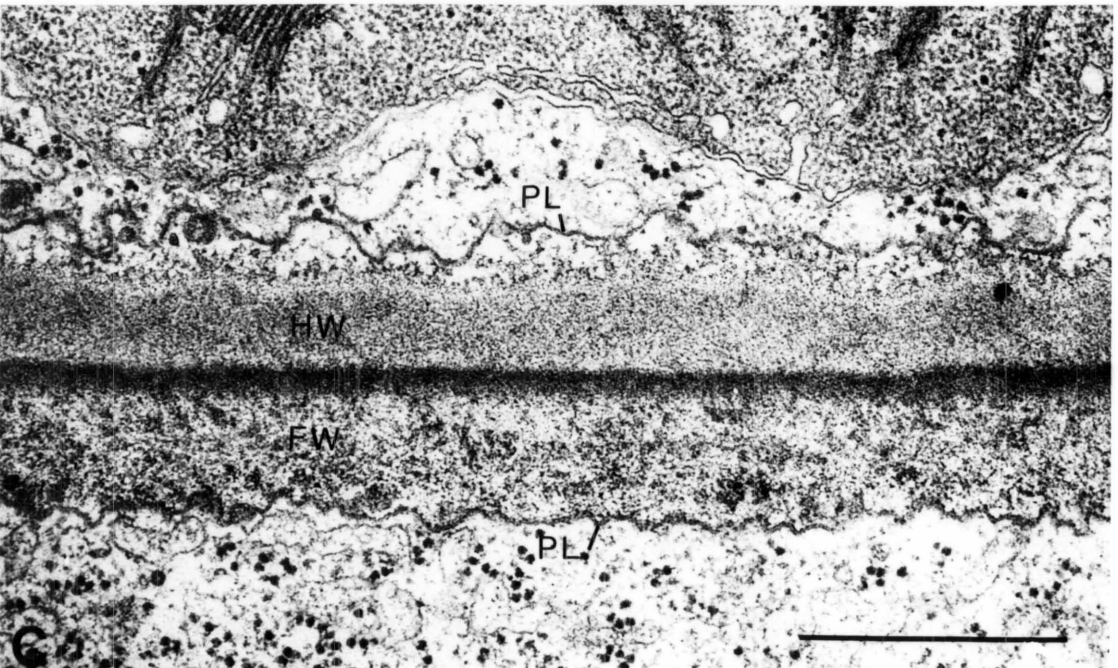
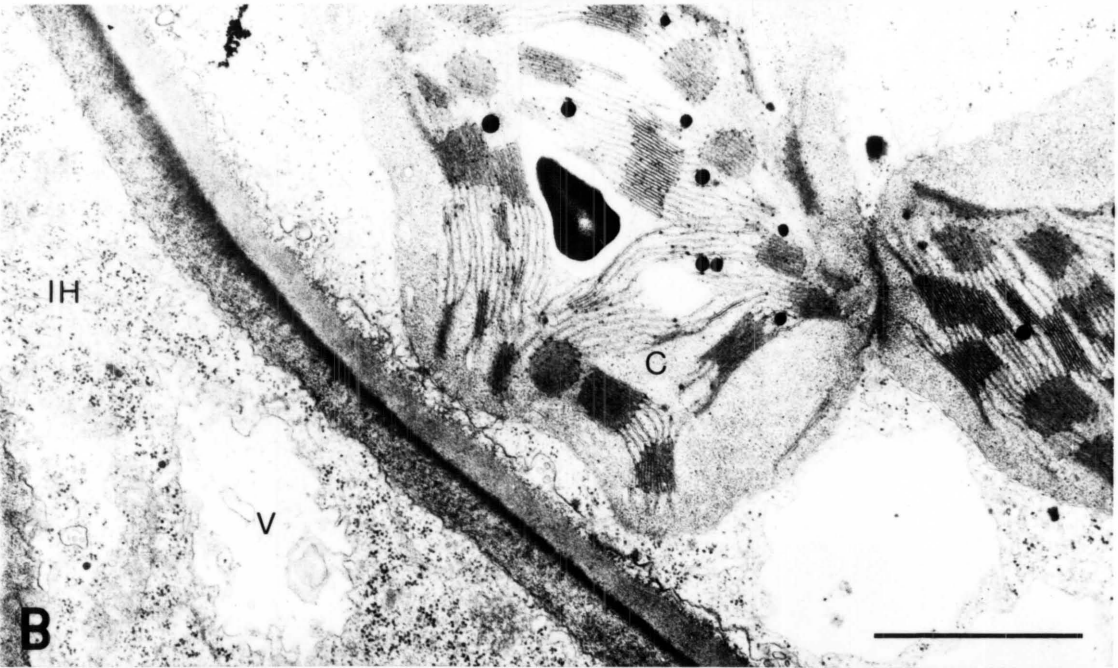
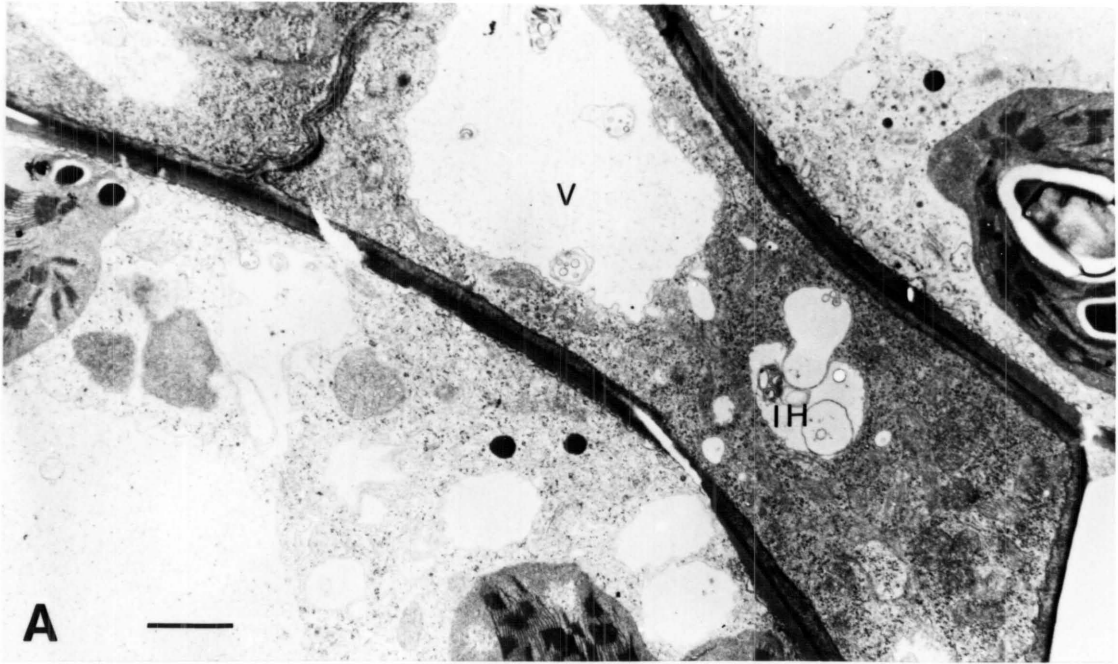


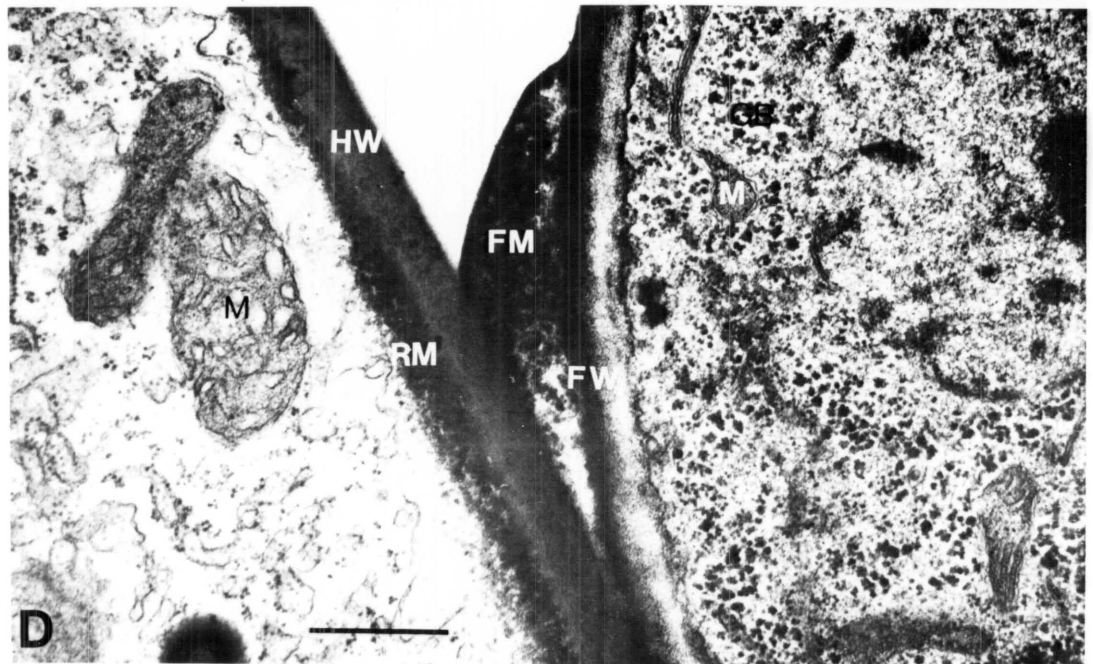
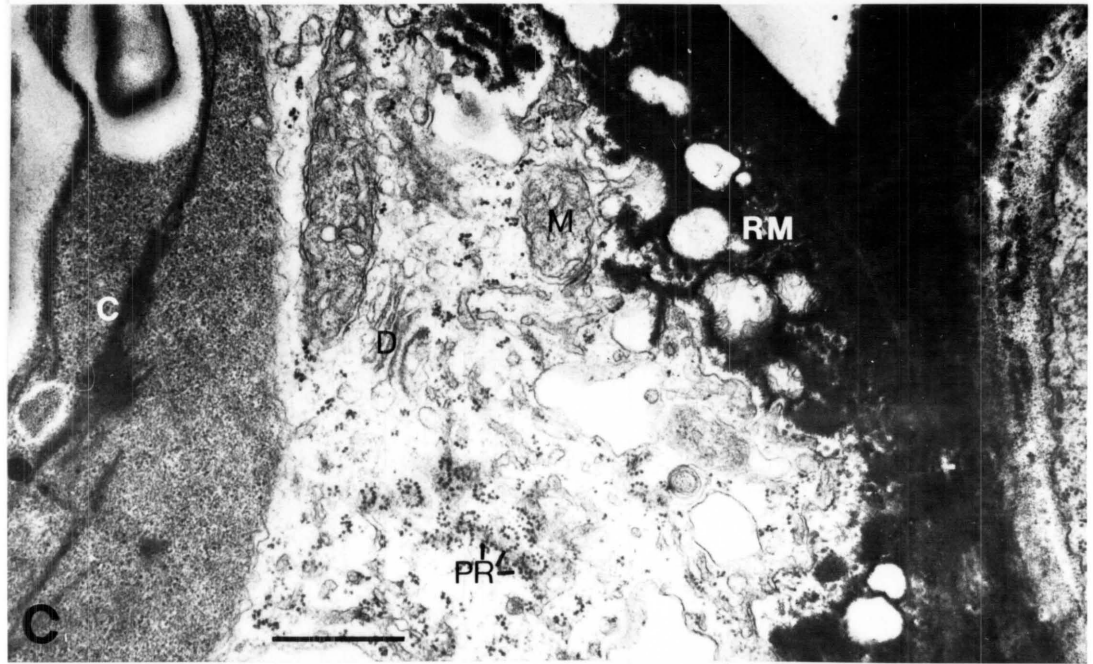
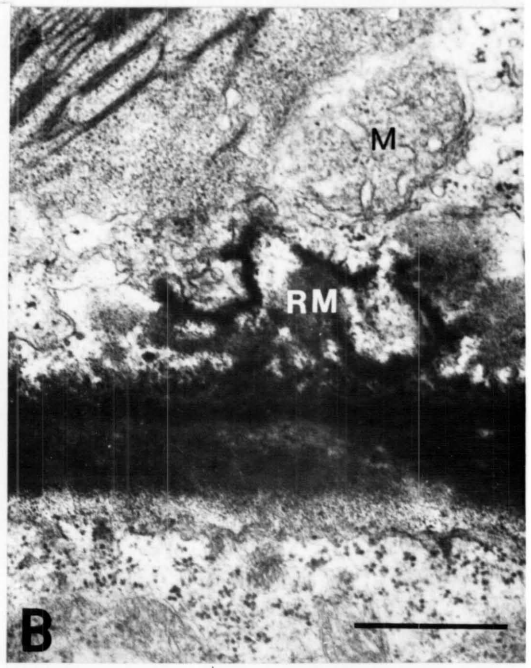
Plate 4.15 Reaction of host mesophyll cells to the presence of intercellular hyphae.

A: Intercellular hypha appressed to host mesophyll cell. Note fibrillar material increasing area of contact between host and pathogen. Bar = 1 μm

B,C: Reaction material produced by the host in response to nearby fungal hyphae. Note structural integrity of nearby host organelles. Bar = 0.5 μm

D. Fibrillar material between fungus and host. Bar = 0.5 μm

Abbreviations: C - chloroplast, D - dictyosome, FM - fibrillar material, FW - fungal wall, GB - glycogen body, HW - host wall, IH - intercellular hypha, M - mitochondria, N - nucleus, PR - polyribosomes, RM - host reaction material, S - septum



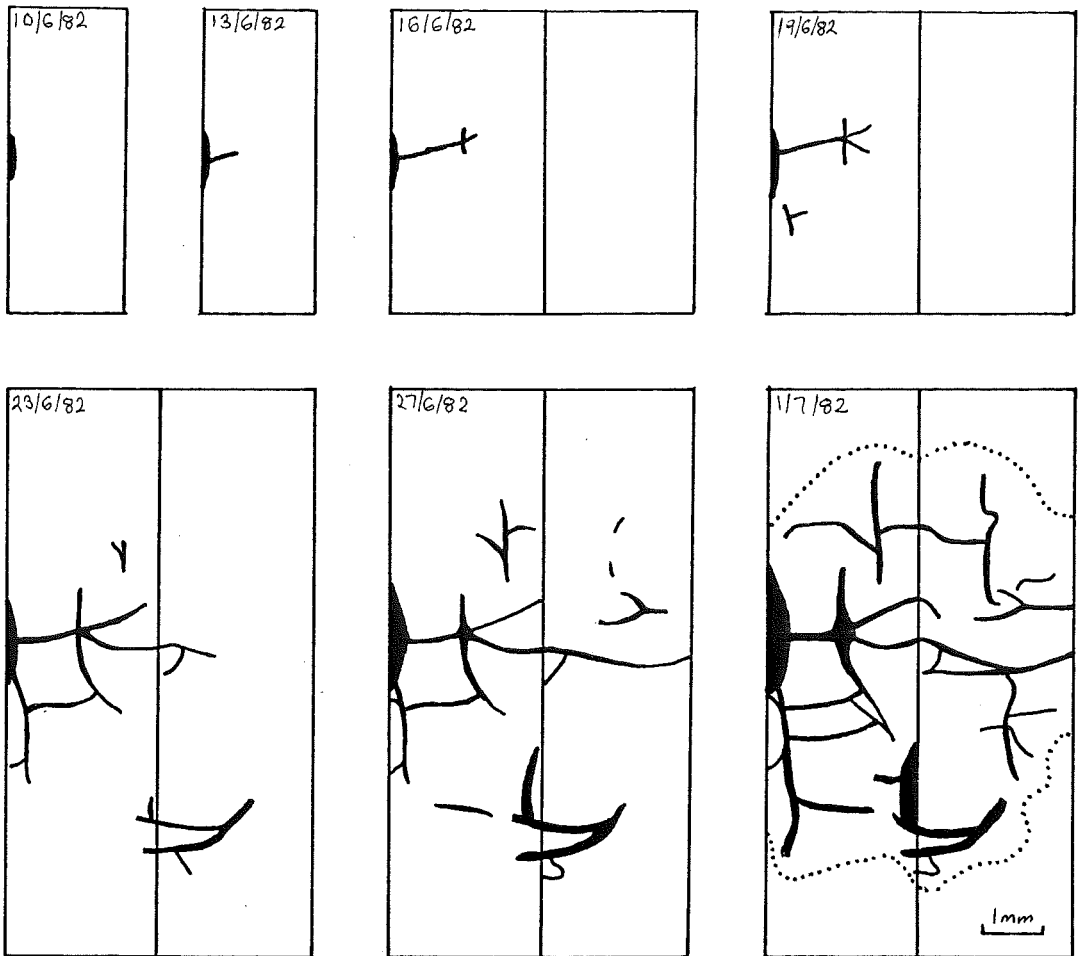
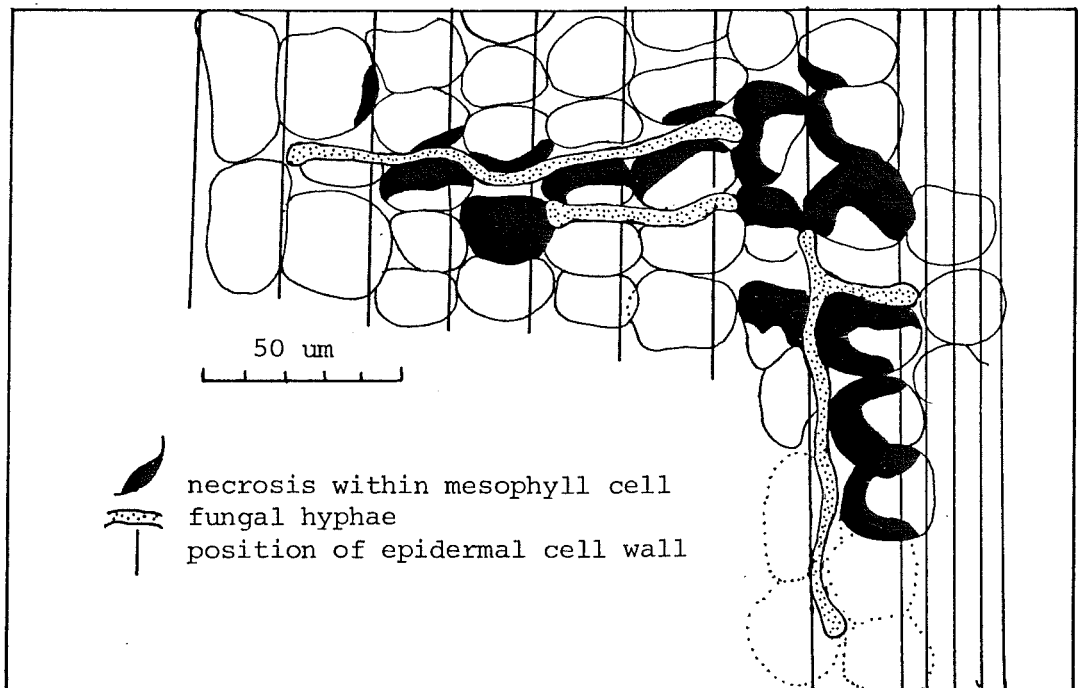


Fig. 4.2 Development of a net blotch lesion on the abaxial surface of a leaf of *L. perenne* during June 1982.

— necrotic regions
 extent of chlorosis






 necrosis within mesophyll cell
 fungal hyphae
 position of epidermal cell wall

Fig. 4.1 Position of intercellular hyphae within a net blotch lesion.

were free of necrosis, indicating that hyphae were still growing in advance of tissue necrosis.

In June 1982, the development of lesions on tagged leaves was assessed and a typical example is illustrated in figure 4.2. The reticulate nature of the lesion was most apparent on the abaxial surface of the leaf where observations were made. From an initial small fleck lesion, the necrosis spread, predominantly transversely and longitudinally, to form a full net lesion three weeks later. Chlorosis was evident in the late stages of lesion development, but varied according to host-pathogen combination. Over the period of lesion development, a youngest visible leaf (leaf two) bearing a small lesion would have changed status to become leaf three or four with the production of new leaves. Leaves were again tagged in October 1982 and at that time lesions developed fully from initial fleck lesions after one to two weeks.

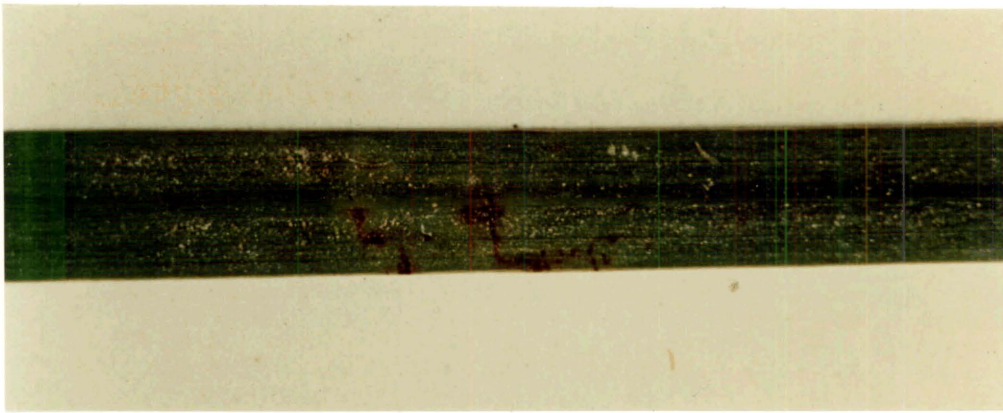
Plate 4.16 illustrates some examples of natural lesions collected in the field. The reticulate nature of the lesions was most obvious on leaves with only a few discrete lesions. Where conditions are particularly favourable for sporulation and infection, multiple penetrations of the leaf can produce a single large necrotic lesion and the 'withered tip' symptom becomes common where the tip of the leaf becomes completely necrotic.

In the final stages of lesion development, immediately prior to sporulation, the host cells were senescent and fungal invasion was more severe (plate 4.17). Hyphae did not remain strictly intercellular and were able to penetrate mesophyll cells. There was invariably a septum at the point of penetration of a host cell and this was perforated by a

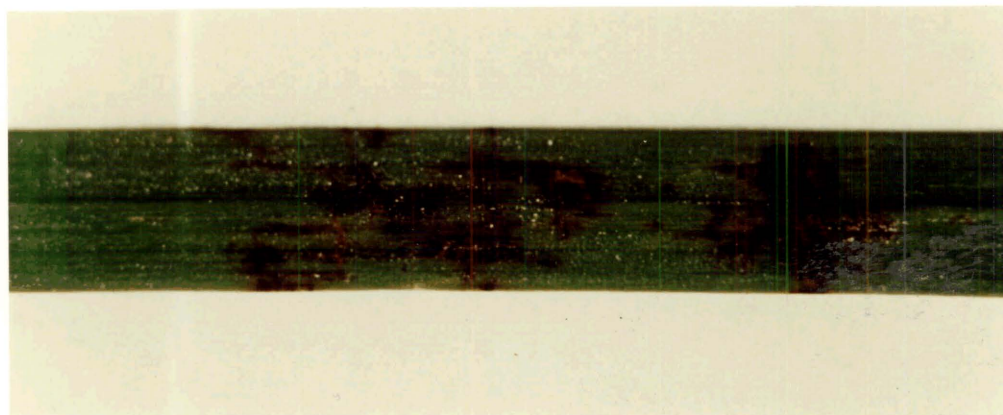
Plate 4.16. Lesions caused by *D. dictyoides* on *L. perenne*.

- A: 'Young' lesions, showing reticulate nature of lesion.
- B: Lesions formed by multiple penetrations.
- C: Numerous small lesions grading into a 'withered-tip' symptom.
- D: 'Withered-tip' symptom.

A



B



C



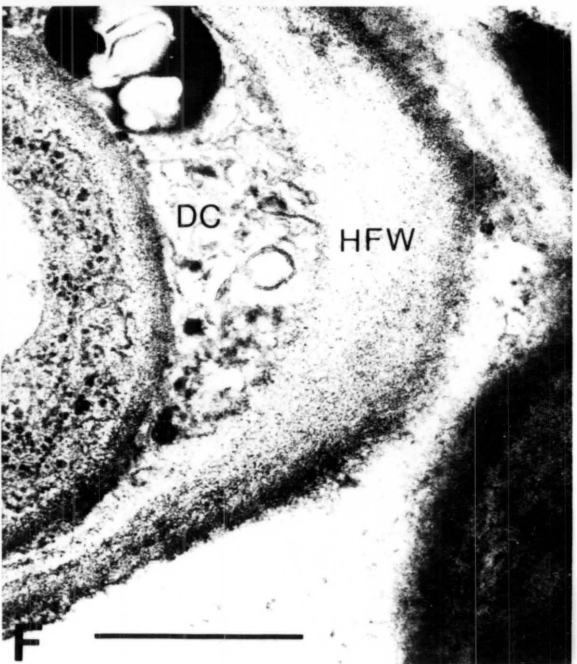
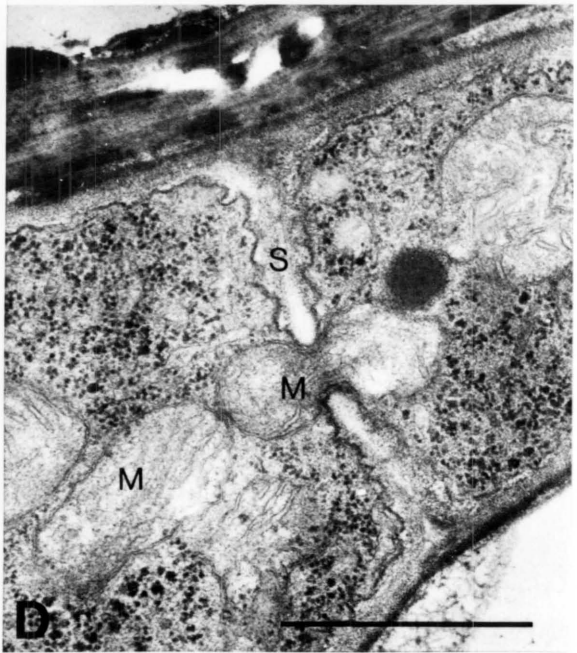
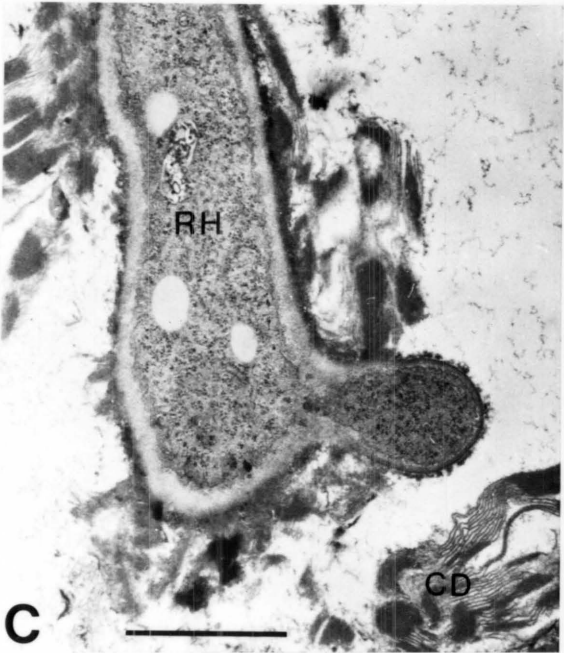
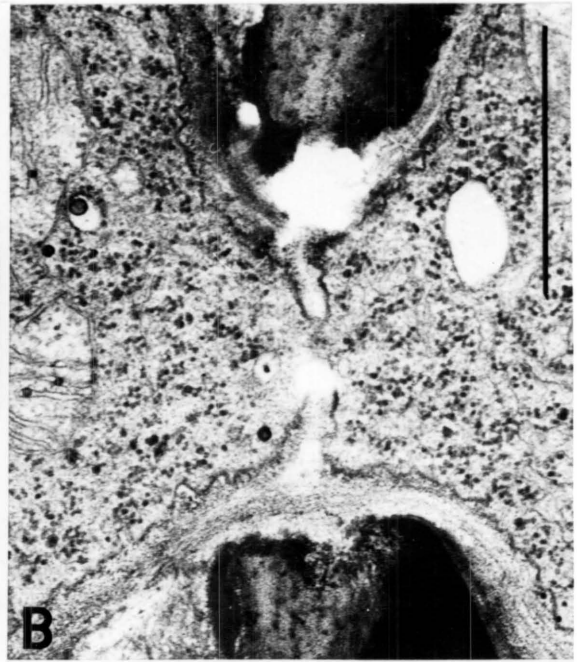
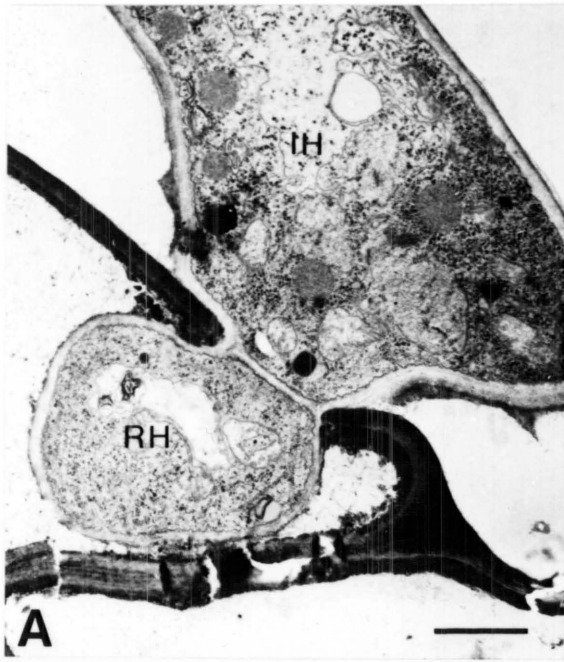
D



Plate 4.17 Host/pathogen interaction at the commencement of sporulation within naturally produced lesions.

- A: Intercellular hypha penetrating a subepidermal host cell. Bar = 1 μ m
- B: Median section through penetration showing septal pore. Bar = 0.5 μ m
- C: Intracellular hypha surrounded by remains of chloroplasts. Bar = 1 μ m
- D: Septate intercellular hypha with mitochondrion probably traversing a septal pore. Bar = 0.5 μ m
- E: Intrahyphal intercellular hypha. Bar = 1 μ m
- F: Detail of association of intrahyphal hypha and host hypha. Bar = 0.5 μ m

Abbreviations: CD - degenerating chloroplast, DC - degenerating cytoplasm, HFW - host fungal wall, IH - intercellular hypha, M - mitochondrion, RH - intracellular hypha, RHH - intrahyphal hypha, S - septum, V - vacuole



simple pore (plate 4.17:A & B). Inside the penetrated mesophyll cell, disorganised remains of organelles such as chloroplasts, could often be seen (plate 4.17:C). Intercellular hyphae remained most common and these were septate with simple pores. The pores were not usually plugged by Woronin bodies and organelles could sometimes be seen midway through them suggesting their passage from one hyphal cell to another (plate 4.17:D).

Intrahyphal hyphae were present in the mesophyll although they were not particularly common (plate 4.17:E & F). They were never found within host cells and were confined to the intercellular spaces (plate 4.17:E). The inner hypha was morphologically similar to nearby intercellular hyphae although the vacuole was prominent. The cytoplasm of the host hypha, while largely degenerated, showed membrane-bound remains of organelles (plate 4.17:F).

4.2.8 Conidium ontogeny

It was observed that sporulation of *D. dictyoides* occurred only on senescent host tissue. In this study leaves were selected where lesions had not commenced to sporulate (usually due to low relative humidity) but which were physiologically ready to commence sporulation within a few hours of achieving suitable humidity. Lesions were cut into 4 mm² pieces and placed in humidity chambers. They were observed at two hourly intervals, using a stereo-microscope, to determine the stage in sporulation. Leaf pieces were sampled at conidiophore emergence and maturity and conidium emergence, growth and maturity. The processes were not completely synchronous and each leaf piece represented a range in stages of development. Sampled

leaf pieces were fixed in glutaraldehyde and then half were prepared for the SEM by critical point drying while the other half were prepared for the TEM.

Scanning electron microscopy is invaluable to elucidate the morphology of the sporulation process. The results are shown the plates 4.18 and 4.19. Conidiophores emerged from epidermal cells, from between epidermal cells or through stomata, without any detected preference. There were few stomata on the abaxial leaf surface but stomatal emergence was common on the adaxial surface. Sporulation only occurred on senescent tissue and frequently occurred first on 'withered tips'.

As the conidiophore grew, the apical segment often had a rough surface and this was evident in the expanded conidiogenous region of the conidiophore (plate 4.19 A - E). Mature conidiophores, where the conidia have become dislodged during tissue preparation for SEM, showed a prominent pore surrounded by an annulus at the point of conidium attachment. Conidia emerged from the apex of the conidiophore and were spherical at first (plate 4.18:G & H), reaching their maximum diameter before elongating to any appreciable extent. Elongation then occurred and several transverse septa formed apparently adding rigidity to the conidium. The conidium/conidiophore attachment is shown clearly in plate 4.19:B & C and appeared to be approximately 1.5 μm in diameter. The pore itself (plate 4.18:F) was approximately 0.3 μm in diameter but the whole structure, including annulus, was about 0.9 μm in diameter. This compared with a diameter of the conidiogenous region of the conidiophore of about 5 μm . After the conidium matured, the conidiophore regerminated and a second conidium was formed, again apically (plate

Plate 4.18 Surface morphology of conidium ontogeny using the SEM.

A: Conidiophores emerging from between epidermal cells on the abaxial surface of a ryegrass leaf. Bar = 10 μm

B: Conidiophore emerging from cell of abaxial epidermis. Bar = 1 μm

C: Conidiophore apex. Bar = 1 μm

D: Cluster of conidiophores on the surface of the leaf. Bar = 10 μm

E,F: Mature conidiophores where conidia have been shed during tissue preparation. Note prominent pore marking point of conidium attachment. Bar = 1 μm

G: Young conidium produced at apex of conidiophore. Bar = 1 μm

H: Cluster of conidiophores, one of which has a developing conidium. Bar = 10 μm

Abbreviations: Co - conidium, Cp - conidiophore. Arrow indicates pore at tip of conidiophore.

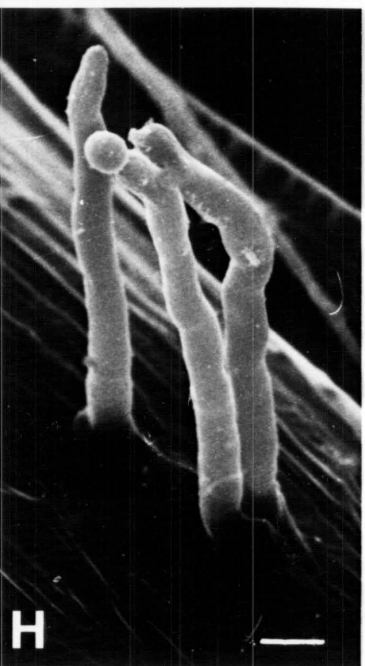
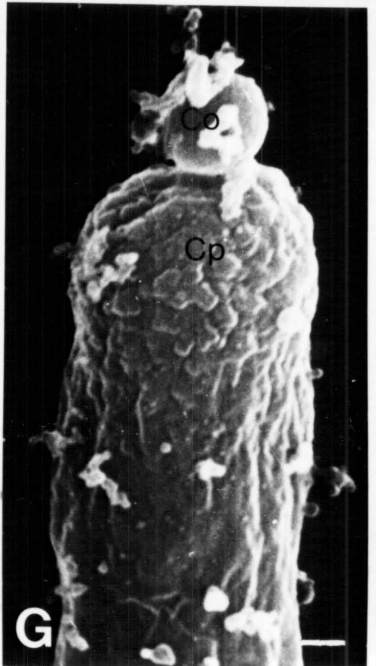
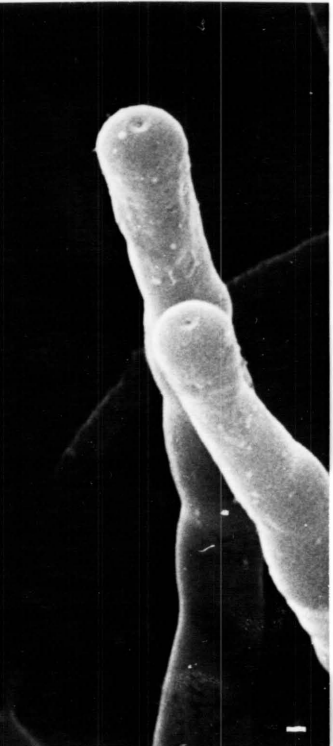
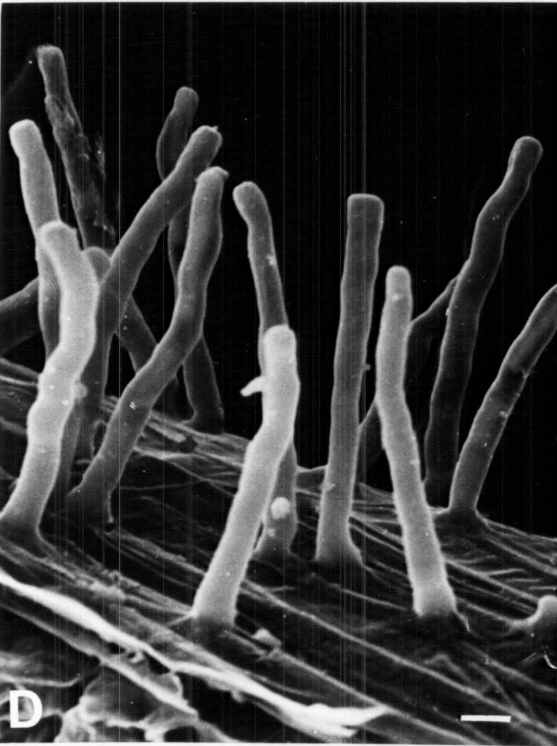
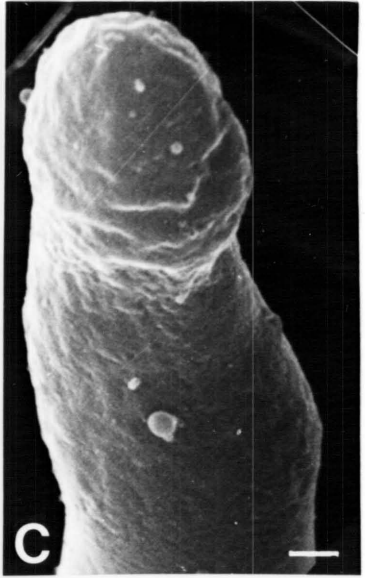
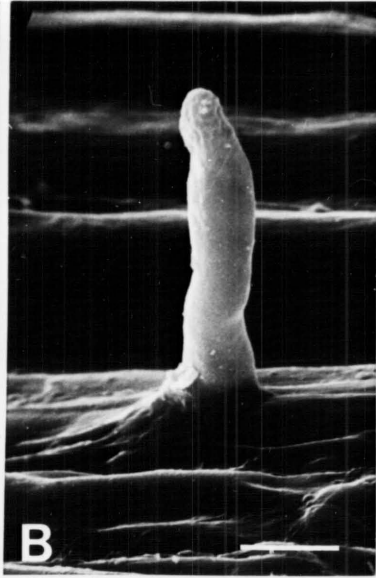
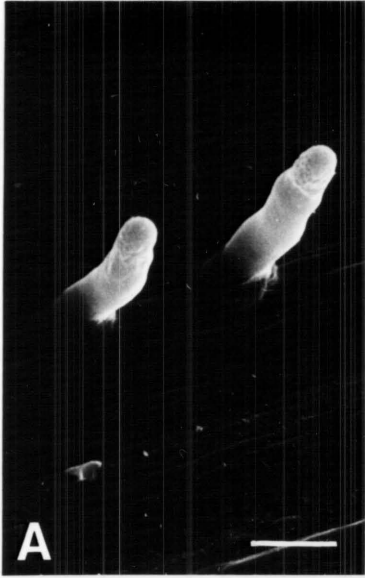
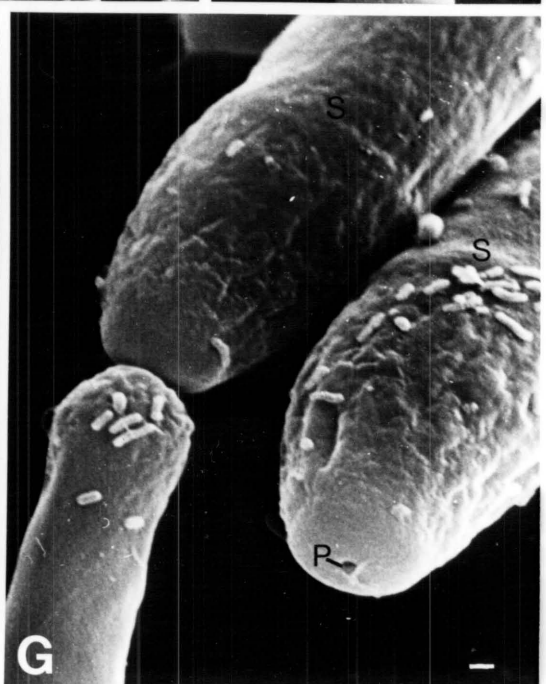
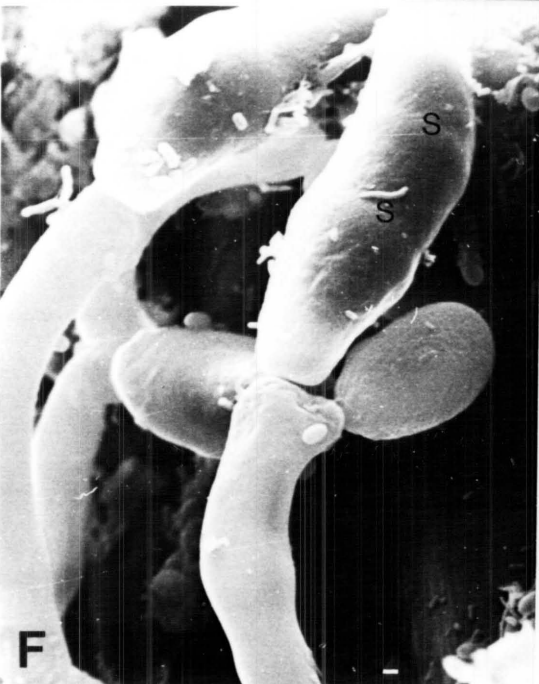
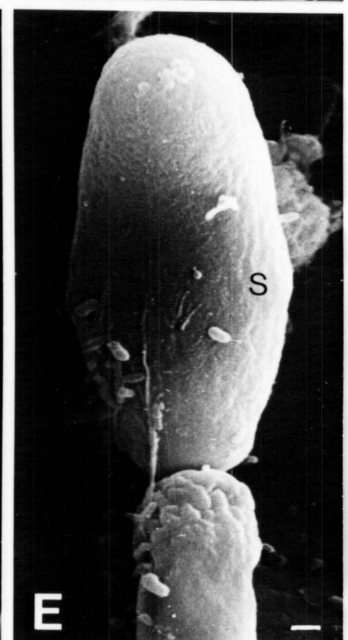
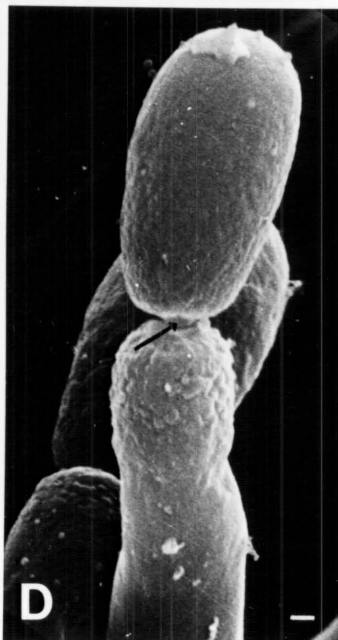
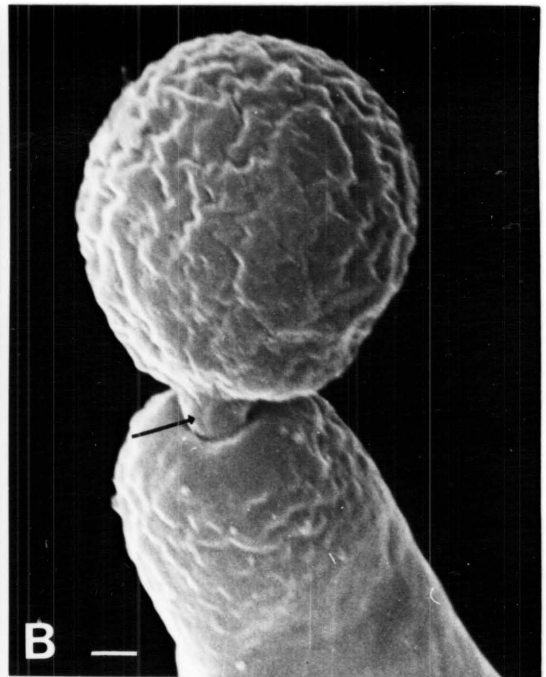


Plate 4.19 Surface morphology of conidium ontogeny
continued.

Bar = 1 μ m

- A - E: Stages in development of conidia at the conidiophore apex. Note rough surface of conidiogenous cell of conidiophore and on young conidium and also the conspicuous connection between conidium and conidiophore in B and C. Note formation of septa in E.
- F: Second conidium produced on conidiophore alongside mature first conidium.
- G: Mature conidium attached to conidiophore with another detached conidium showing prominent pore in hilum.

Abbreviations: P - pore, S - septum



4.19:F). When dislodged, mature conidia showed a prominent pore in the base of the hilum (plate 4.19:G).

The ultrastructure of conidium ontogeny was followed using the TEM. Using the remounting technique (p.19) it was possible to determine the exact plane of a selected section by examining sections before and after the one showing the desired stage. This obviated many of the problems often described in the literature regarding interpretation of results.

Plate 4.20 shows a conidiophore emerging from a stomate on the adaxial leaf surface. The cuticular beak which normally closes the stomata was forced open and was surrounded by fungal wall material (plate 4.20:C). The base of the conidiophore within the substomatal cavity was globose and surrounded by a fibrillar layer, which was not observed on the emerged conidiophore (plate 4.20:B). The conidiophore had two prominent wall layers, an outer electron dense layer and an inner electron transparent layer. Within the inner layer, it was often possible to distinguish a further, lighter, layer (innermost). The conidium grew through a narrow pore in the apex of the conidiophore (plate 4.21:A) and consisted of the usual two wall layers which corresponded closely to the wall layers at the apex of the conidiophore (plate 4.21:D & E). Apical vesicles were present in the conidium and these were sometimes in close proximity to the plasmalemma (plate 4.21:B).

As the conidium matured the electron dense layer at the apex became thicker. When a conidium was dislodged the pore was plugged with a large Woronin body (plate 4.21:G) and dark pigmentation of the hilum was formed. As the first conidium reached maturity, the conidiophore regerminated and later formed a second conidium (plate 4.21:H). A third,

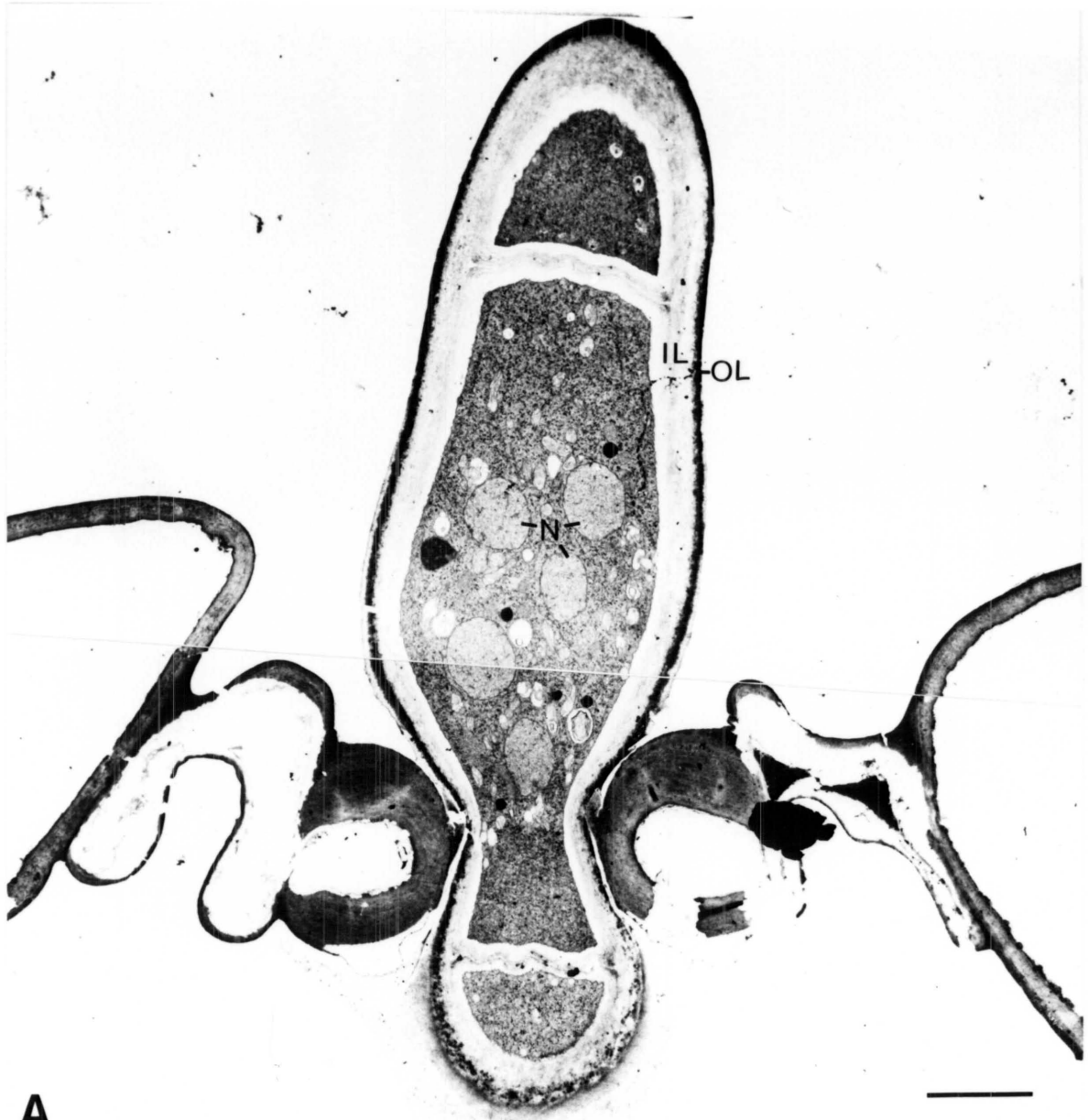
Plate 4.20 Ultrastructure of conidium ontogeny: Emergence of a conidiophore from a stomate on the adaxial surface of a ryegrass leaf.

A: Non-median section of conidiophore, showing relationship of conidiophore to stomatal apparatus. Bar = 2 μ m

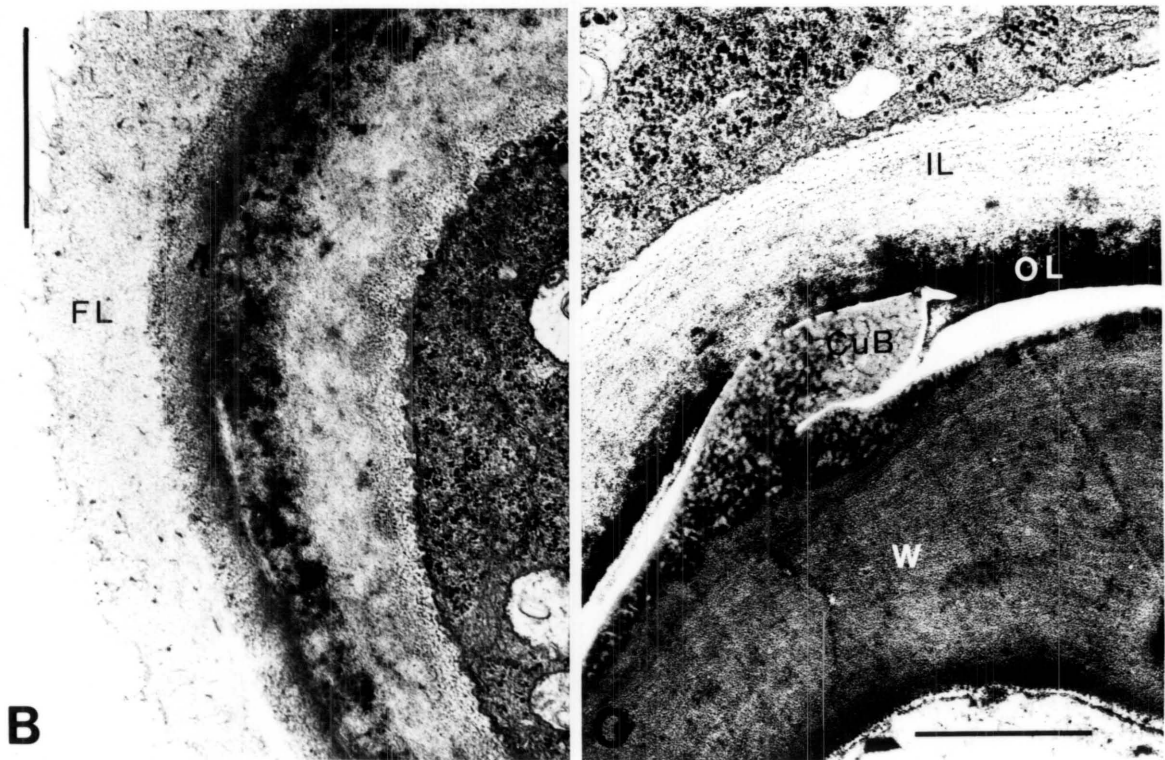
B: Wall structure of conidiophore base in sub-stomatal cavity. Note outer fibrillar layer. Bar = 1 μ m

C: Cuticular beak of the guard cell is forced open by the emerging conidiophore. Bar = 0.5 μ m

Abbreviations: CuB - cuticular beak, FL - fibrillar layer, IL - inner layer of fungal wall, N - nucleus, OL - outer layer of fungal wall, W - wall



A

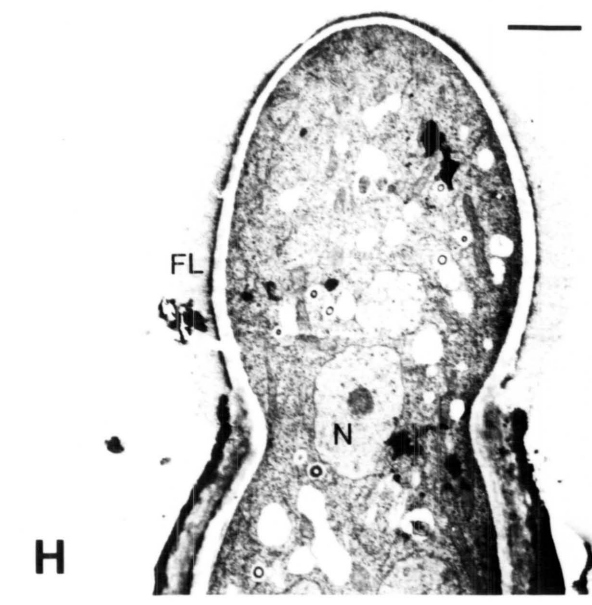
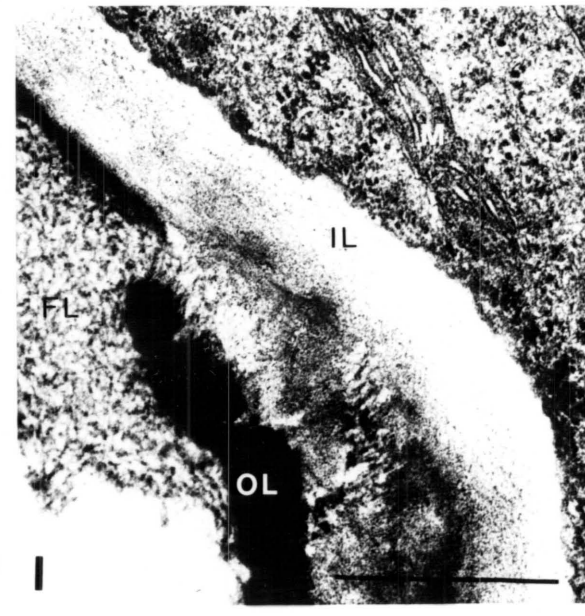
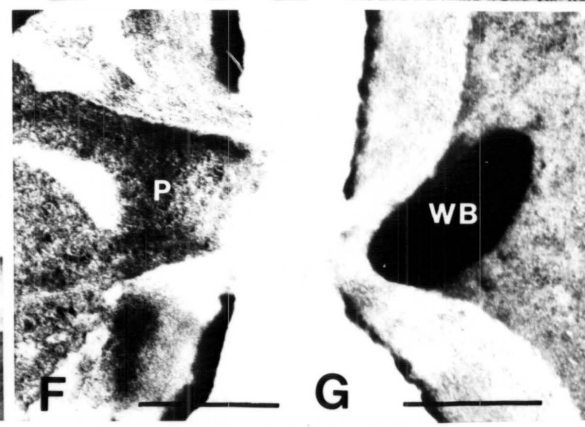
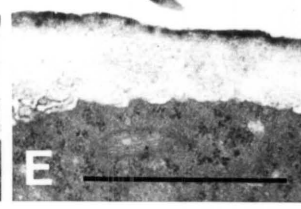
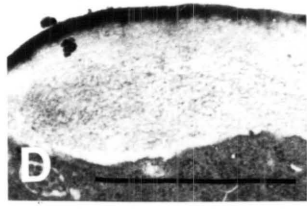
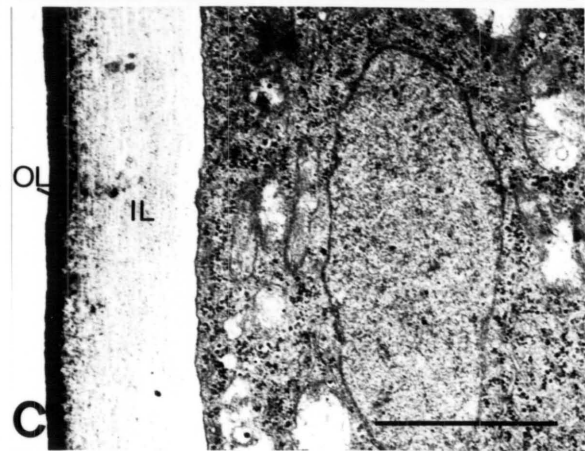
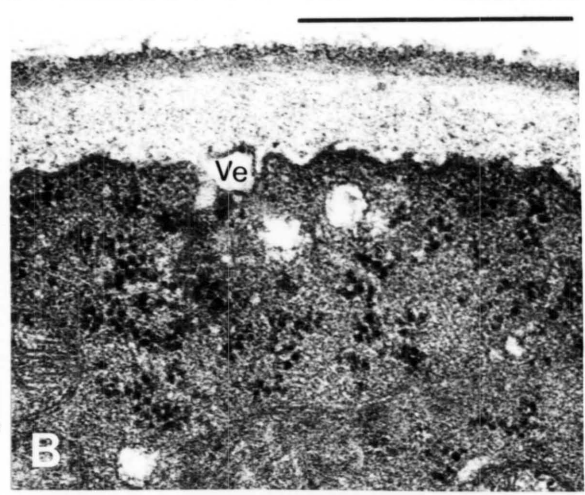
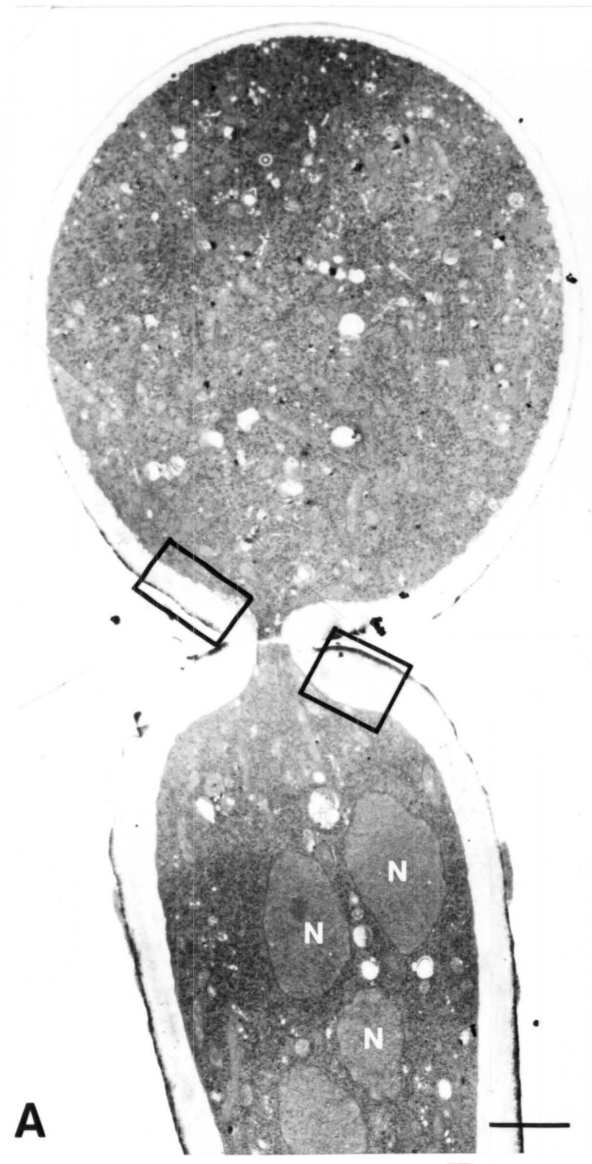


B

Plate 4.21 Ultrastructure of conidium ontogeny: conidium production and reprodiferation of the conidiophore.

- A: Poroconidium at unicellular stage on conidiophore. Bar = 1 μ m
- B: Apical region of conidium showing vesicles. Bar = 0.5 μ m
- C: Conidiophore wall. Bar = 1 μ m
- D: Detail of conidiophore inset. Bar = 1 μ m
- E: Detail of conidium inset. Bar = 1 μ m
- F: Mature conidiophore apex showing pore. Bar = 0.5 μ m
- G: Detached conidium where pore is plugged by a Woronin body. Bar = 0.5 μ m
- H: Reprodiferation of conidiophore following production of a conidium. Bar = 1 μ m
- I: Detail of region of reprodiferation. Note continuity of innermost wall layer of mature conidiophore with young conidiophore. Bar = 0.5 μ m

Abbreviations: FL - fibrillar layer, IL - inner layer of wall, N - nucleus, OL - outer layer of wall, P - pore, Ve - vesicle, WB - Woronin body



innermost, electron transparent layer formed at the conidiophore apex and this was continuous with the regerminated conidiophore (plate 4.21:I). An outer electron dense layer formed almost immediately, but this was not continuous with the outer layer of the original conidiophore. The new conidiophore had an outermost fibrillar layer similar to that of the conidiophore base.

4.3 Discussion

4.3.1 The fungal wall

The fungal cell wall is important, not only in terms of the morphology of stages in the life history, but also in the efficiency of parasitism. Cell wall modifications are responsible for the growth changes in the fungus and hence in enabling successful penetration and fungal ramification within the host tissues.

The cell wall of *D. dictyoides* consists of two layers in its 'typical' form - an outer electron dense layer and an inner electron transparent layer as seen in the TEM. The inner layer is present in young hyphae and the outer layer is usually added a short time later. This two-layered structure is typical of many fungal walls (Hawker & Hendy 1963, Murray & Maxwell 1974). From the basic structure, modifications such as the addition of another layer or the subtraction of a layer - usually the outer layer - are possible. The outer layer probably confers strength but certainly reduces elasticity and appears to increase in thickness with maturation. It is probably this layer which is particularly involved in the reduction in plasticity leading to the formulation of the hypothesis of Madelin (1979) (see p.66). Where fungal structures are fully mature, such as in the conidium or a mature conidiophore prior to germination, a new wall layer may be produced, indicating that the inner wall has also lost its plasticity and must be split or lysed to allow egress of the juvenile hypha below. The outer wall layer appears to reduce contact with the external environment or with the host tissues. Evidence for this is seen in the reduction or absence of the outer wall layer in the region of the penetration peg or in the early stages of contact between intercellular hyphae

and the host mesophyll cells.

To date most studies of the fungal wall have centred on either spore germination or conidium ontogeny and there is a paucity of information on the fungal wall in relation to parasitism, the period between those two processes. A multi-disciplinary study involving the biosynthetic and structural processes of wall formation throughout the fungal life history would be useful to determine cell wall functions in terms of the ability to successfully carry out the parasitic way of life. This was beyond the scope of the present study which merely provides a commentary of changes in the fungal wall in relation to the host pathogen interaction.

4.3.2 Spore germination

The life history of a plant pathogenic organism such as *D. dictyoides* is a continuous process where intervals between stages must normally be minimised in order to optimise the spread of disease. There may be an interruption in growth prior to the production of conidia, usually due to the lack of sufficient humidity or between conidium production and its subsequent germination on a new susceptible leaf surface. The mature conidium provides the best known example of the cessation of fungal wall growth.

The first visible sign of germination of conidia of *D. dictyoides* is the bulging of a limited region of the basal or apical cell. Marchant (1966) interpreted electron micrographs of germinating *Fusarium culmorum* conidia as indicating enzymic breakdown of the outer wall layer at the point of germ tube emergence rather than mechanical rupture. Fletcher (1971) concurred and considered that enzyme action must play a part. Observations in the present study indicate

there was some rupturing of the outer wall layer, but in the absence of a pre-formed germination pore, enzyme action may be involved in the initial softening of the wall in the site of germination.

In the present study, a third, innermost, wall layer was found in the region of the forming germ tube at the time of germination and this was continuous with the germ tube. This is in agreement with the result of Gull & Trinci (1971) for *Botrytis cinerea*, and corresponds to type III of Bartnicki-Garcia (1968). This new layer is not distinct from the normal inner layer of the conidium which suggests that it may not be an 'entirely' new wall and so could equally be interpreted as germination type I. The hypothesis of Madelin (1979) (see p.66), relating to conidium ontogeny, is appropriate here in suggesting that the wall layers of the mature conidium are no longer 'plasticisable', requiring the emergence of a juvenile hyphal wall. This may be a more useful interpretation than one requiring an arbitrary explanation of wall layers in the absence of information on the relationship between the layers seen under the TEM.

The fibrillar layer (also sometimes referred to as a mucilage layer in the literature) probably provides some adherence of the germ tube to the leaf surface as suggested by Hawker & Hendy (1963) and Murray & Maxwell (1974). The observation that conidia of *D. dictyoides* (present study) adhere to glass slides once germinated whilst ungerminated conidia do not, provides evidence for the production of an adhesive substance.

All cells in the multiseptate conidia are involved in germination, although germ tubes normally emerge only from the end cells. The unplugged septal pores allow the utilisation of the contents of all cells.

When the germinated conidium becomes senescent, few recognisable organelles remain, but the large vacuoles present in senescent hyphae of *Pyrenochaeta terrestris* (Gunasekaran *et al.* (1973) are absent and degenerated cytoplasm is dispersed through the cells. This may be because the cells become 'drained' of contents to provide nutrients for the developing hyphae. The septal pore remains unplugged suggesting the removal of compounds from the conidium to the germ tube.

The distance a germ tube grows over the leaf surface varies, although in general, longer germ tubes are produced on leaves inoculated in the laboratory than those found on leaves brought in from the field. This may be related to the duration of leaf wetness. Drops of spore suspension normally remain on the leaf for at least twenty-four hours. When leaves from the field, with natural spore loads, are incubated under high humidity, long germ tubes are again produced.

4.3.3. Appressoria

The formation of appressoria over the anticlinal wall of the epidermis has been reported widely (Preece *et al.* 1979) but there is little agreement on the origin of the stimulus for a germ tube to terminate forward growth and begin to swell (Emmett & Parbery 1975).

Production of appressoria in the depression in the anticlinal wall region, seen most clearly under the SEM, probably allows more efficient contact with the host, because of the increased host area available for adherence. Firm attachment is important if mechanical penetration is involved. There are two different aspects to the stimulus for a germ tube to cease apical growth. The first determines the length of the germ tube (whether it will be long or short) while

the second determines the specific site of appressorium formation. There are probably two sets of conditions, each made up of one or more stimuli, involved.

The stimuli initiating appressorium formation over the anticlinal wall must be largely constant, while the treatment provided can modify the overall length of germ tubes. The observation that germ tubes formed in nature are almost invariably shorter than those produced on inoculated leaves leads to the suggestion that cessation of germ tube growth may be initiated by conditions unfavourable to vegetative growth. Moisture is one possibility. The drying of a leaf surface may provide a stimulus although this could also be compounded by temperature. When leaves collected in the field have water added and are re-incubated, the conidia naturally present produce long germ tubes, which provides evidence for the above hypothesis. The stimuli inducing appressoria to form specifically over anticlinal walls of the host are likely to include thigmotropism or factors inherent in plants as discussed by Emmett and Parbery (1975).

Bacteria were commonly found on, and surrounding, appressoria. This may be indicative of an accumulation of substances around the appressoria, either of fungal origin or leached from the host. Leaf saprophytes were frequently observed growing above the anticlinal walls of the epidermis and this suggests that host leachates may be present in this region. The increased accumulation of bacteria around appressoria suggests, however, that the concentration of substances is greater around the appressorium. Austin *et al.* (1977) found that some bacteria on the phylloplane of *L. perenne* were antagonistic to *D. dictyoides*, but in the current study, bacteria were not observed purely in association with lysed hyphae.

Specific binding substances, as outlined by Edwards & Allen (1970) and Mckeen (1974) were not observed in this study, but appeared to be produced by germ tubes and may have an early role in appressorium adhesion. The increase in density where the outer layer of the appressorium wall abutts the epidermis serves to anchor the appressorium to the host. Production of vesicular material at the site of attempted penetration may serve to dissolve the inner fungal wall layer as well as the host cuticle. The inner fungal wall is absent only in the region of production of vesicular material and this suggests a role similar to that of the 'lomasome-like' structures reported by Politis & Wheeler (1973). A function in wall dissolution is also suggested by the vesicular material at the interface between the penetration peg and host wall. The fungal wall is again absent in this region.

4.3.4 Penetration

Where penetration is direct, a cleanly circumscribed round hole develops through which the penetration peg traverses the host epidermal wall. Apposition material may or may not be produced by the attacked epidermal cell but penetration, once commenced, is seldom limited by such structures. This mode of penetration is similar to that reported by Politis & Wheeler (1973), Knox-Davies (1974) and Van Caesele & Grumbles (1979).

Penetration is not always direct and infection may remain subcuticular for up to forty-eight hours, as reported by Murray & Maxwell (1975) and Wheeler (1977) for other species of *Drechslera*. Penetration of the epidermal wall is halted before the penetration peg has reached the host plasmalemma. The depth of initial penetration varies and does not conform to a particular host wall layer. From this point, the fungus

may ramify over several epidermal cells and this can enable penetration of several epidermal cells from a single appressorium, although most frequently only one or two epidermal cells are penetrated. If the infection is to succeed, and enable a lesion to develop, an epidermal wall must be breached. This is also found in infection of corn by *D. carbonus* (Murray & Maxwell 1975). In the extreme case of this 'subcuticular' penetration, the fungus is not seen to penetrate further than approximately ten percent of the host wall and disease develops no further. This phenomenon was observed equally in field resistant lines and in susceptible lines and so is unlikely to represent a host defence mechanism. Rather, it probably results from a 'faulty' penetration attempt.

Following penetration of an epidermal cell, the infection hyphae ramify through much of the length of the degenerated host cell. Energy resources from this cell may be required for the later intercellular spread of the fungus through the host mesophyll or the hyphae may be unable to 'penetrate out' of the epidermal cell until the nutrient supply is exhausted. An extensive intracellular hypha enables multiple egress of the fungus into the host mesophyll, increasing the likelihood of uninterrupted spread through host tissues.

4.3.5 Lesion development

D. dictyoides does not fall within the necrotrophic category often used for *Drechslera* leaf diseases such as *D. victoriae* (Luke *et al.* 1966), *D. maydis* (White *et al.* 1973) and *D. sacchari* (Strobel *et al.* 1972). Host reaction during the colonisation stage of the life history occurs sometime after host and pathogen are in contact and tissue necrosis is not in advance of the hyphae.

In the early stages of lesion development intracellular hyphae are rare and the fungus remains within the prominent intercellular spaces of the ryegrass mesophyll. Hancock & Huisman (1981) calculated that diffusion rates across host cell walls are too rapid to offer a great nutritional advantage to intracellular growth, in particular considering the energy expenditure required to penetrate the cell wall. The lack of disruption of host tissues in the early stages of colonisation suggests that the fungus can gain sufficient nutrients from the apoplast system of the host to ramify through the mesophyll tissues. The fibrillar material which increases the area of contact between the fungus and host probably increases the absorptive region.

Sugars present in the apoplast may have regulatory effects on the production of cell wall-degrading enzymes. Experimental evidence suggests that these enzymes are inducible and not synthesised until soluble sugars are exhausted in growth media (Cooper & Ward 1975, English *et al.* 1971, Horton & Keen 1966). The relatively high sugar levels initially present in the apoplast (2 - 10 mM according to Hancock & Huisman 1981) may repress enzyme formation. Hancock & Huisman (1981) suggested the possibility that the initial biotrophic phase of hemibiotrophs corresponds to the period when adequate sugar levels are present in the apoplast and that the necrotrophic phase occurs when the nutrients are depleted and production of cell wall-degrading enzymes is triggered. The results of the current study are in accordance with this suggestion. In addition, the low level of interference with the host in the early stages may enable the pathogen to spread without inducing a defensive reaction from the host. Paus & Raa (1973) found that, in cucumber plants resistant to *Cladosporium cucumerinum*, growth of the pathogen soon became arrested

following death of host cells in the vicinity of the infecting hyphae.

It is only when lesion development is virtually complete and tissue senescence is beginning that the fungal hyphae begin to invade host cells to an appreciable extent. These host cells have become senescent and so nutrient flow has probably slowed, possibly inducing the production of wall degrading enzymes. Even at the commencement of sporulation the majority of hyphae in the host mesophyll are intercellular. These results indicate clearly that *D. dictyoides* is a hemibiotrophic pathogen.

Necrotrophic *Drechslera* diseases are probably those that produce host-specific phytotoxins, such as *D. maydis* (White *et al.* 1973), *D. sacchari* (Strobel *et al.* 1972) and *D. victoriae* (Luke *et al.* 1966). In the present study, culture filtrates of *D. dictyoides* were assessed for their toxicity to ryegrass and were found to have a negligible effect (appendix p.231). Ammon (1963) recorded the production of a substance by *D. dictyoides* f. sp. *dictyoides* which was toxic to *Festuca*. If a similar substance is produced by the isolates of *D. dictyoides* f. sp. *perenne* used in this study, it is unlikely that it is involved until a late stage in lesion development in view of the mild initial host reaction. This result is in agreement with Van Caesele & Grumbles (1979) using *D. teres*.

The chlorotic 'blotch' frequently surrounding the net lesion at the late stages in lesion development may be indicative of the action of toxins or fungal enzymes. The blotch is not evident on all lesions and it is possible that only some isolates of *D. dictyoides* produce such substances. The primary lesion is not caused by toxin activity and merely indicates the presence of fungal hyphae in the vicinity. Hyphae

within lesions grow closely adpressed to host mesophyll cells and frequently follow a line of such cells. It is this mode of growth which produces the characteristic fine longitudinal and transverse flecking forming the net lesion.

4.3.6 Intrahyphal hyphae

In the present study, intrahyphal hyphae have been found in senescent leaf lesions and among subcuticular hyphae. Lowry & Sussman (1966), using 'clock' mutants of *Neurospora*, were the first to study the ultrastructure of intrahyphal hyphae, which formed as the result of invasion of moribund hyphal segments. Calonge (1968) suggested a mechanism for the production of intrahyphal hyphae of *Sclerotinia fructigena* involving growth of a new hypha through the plugged septum separating a normal cell from a degenerating one.

It has been suggested that intrahyphal hyphae are produced under adverse conditions such as ageing or injury (Miller & Anderson 1961, Chan & Stephen 1967, Calonge 1968, Brown & Wylie 1970). Brown & Wylie (1970) found intrahyphal hyphae in microsclerotia of *Verticillium albo-atrum* while Calonge (1968) found those of *S. fructigena* in liquid medium but never in parasitised fruit tissues. It was suggested that:

'... some external conditions ... may kill one or more cells of the hypha and stimulate the neighbouring healthy ones to proliferate intrahyphally.'

Calonge (1968)

Toxic metabolic products were assumed to have caused intrahyphal hyphae to be produced in *Neurospora* (Lowry & Sussman 1966).

As far as I am aware, this study represents the first report of intrahyphal hyphae within infected leaf tissues. Hyphae within the senescent lesions showed little sign of senescence themselves, but toxic substances related to the

death of host cells may have been responsible for their production. Space for growth is limited in the subcuticular zone where intrahyphal hyphae were also found and this may have induced the fungus to proliferate intrahyphally within this zone.

Membrane-bound organelles were still visible within the degenerated 'host' fungal cells. Cell walls remained intact, but available nutrients would have been released to the invading hyphae.

4.3.7 Conidium ontogeny

D. dictyoides clearly can be accommodated within the group of fungi producing porospores, the pore being clearly visible both when the conidium is attached to the conidiophore and on the conidiophore after spore release. The pore separating the conidiophore and conidium is about 0.3 μm , approximately twice the diameter of septal pores in hyphae. At maturity the septal pore is plugged and the two structures are separate.

Conidiophores emerge from stomata, epidermal cells or between epidermal cells on either leaf surface. The host epidermal wall presents no barrier to the egress of conidiophores which appears to be largely mechanical. Conidiophores emerge perpendicular to the leaf surface so it is evident that there is no light or gravity stimulus to their growth.

The wall layers of the conidium initial correspond closely to those of the conidiophore and this supports the view that porospores are a type of holoblastic propagule as claimed by Carroll & Carroll (1974) and Brotzman *et al.* (1975). Senescent natural lesions were chosen and incubated under high humidity. Under these conditions conidiophores began to emerge after three hours and the first conidia appeared after

eight hours. The process was continuous and so meets the criteria for holoblastic conidiogenesis outlined by Madelin (1979) (see p.66). A scheme of ontogeny based on wall layers has its limitations where the outer layer is not thick, as is found in juvenile hyphae. Under this situation it is not possible to be certain whether the outer wall layer of the conidiophore is involved in production of the conidium wall, or whether formation of the outer wall commences soon after conidium production. For this reason, the term porospore is of more value in describing this group of fungi than the terms holoblasty and enteroblasty. The distinctions between the two are also based on cellular events which occur rapidly at restricted sites.

The use of the remounting technique (p.19) made it possible to ascertain the plane of the sections obtained and alleviated the problems of interpretation outlined by Carroll & Carroll (1974).

Conidia are spherical when they first emerge from the conidiophore but become elongate with time due to differential growth. Apical vesicles are probably involved with apical growth and the maximum diameter of the conidia is reached during the one-celled stage. A ring of thickened material remains at the apex of the conidiophore after abscission of conidia. Campbell described a similar region in *Alternaria brassicicola* which he termed an annulus and suggested it served as a support for the maturing conidium. Conidia are large at maturity in comparison with conidiophores and may well require support. The annulus could also have a role in spore release, where drying may provide an electrostatic discharge mechanism.

Conidiophores of *D. dictyoides* are capable of produc-

ing several conidia. As conidia are maturing, the conidiophore may regerminate and produce a second conidium some distance further on, again apically. In nature it is extremely rare to see more than one conidium on a conidiophore at one time as conidia are easily dislodged. In humid chambers, however, several conidia may be present at the same time. The germination of the mature conidiophore after production of an initial conidium is clearly by an enteroblastic process. During maturation of the conidiophore, its wall becomes thickened and appears to be split to allow the emergence of a juvenile hypha.

4.3.8 Septa

The hyphae of *D. dictyoides* are septate, septa being formed at regular intervals along the hyphae. The function of septa in areas such as the germ tube or intercellular hyphae is probably to add rigidity to the hyphae (Hunsley & Gooday 1974). Septa in these regions were never observed to be plugged by Woronin bodies and there is apparent movement of organelles between cells. Septa are also produced when the fungus changes from one 'form' to another and the function of these septa is probably to compartmentalise the fungus. Typical examples of this type of septum are those between conidia and germ tubes, appressoria and primary infection hyphae and intercellular and intracellular hyphae. In each of these situations, the pathogen may be required to seal off one section from another, due to the production of adverse conditions in one region. Woronin bodies are used to plug septal pores and these can be on either the younger side of the septum or the older side. During time-course studies, septa were never observed during formation, even when

observations were made every ten minutes. This suggests that formation of septa by *D. dictyoides*, like that of *Neurospora crassa* (Hunsley & Gooday 1974), is rapid.

CHAPTER FIVEFIELD WORK5.1 Introduction5.1.1 Disease prevalence

The *Drechslera* diseases of ryegrasses are prevalent in most regions where ryegrasses are grown, although damage is not usually great. Sampson & Western (1940) considered that *Drechslera* disease was not highly destructive in England and Wales, nevertheless it was sometimes sufficiently abundant to produce a 'brown effect' on the plants. In Wales, Wilkins (1973) found that leaves frequently became infected before they emerged from the surrounding leaf sheaths, with whole tillers being killed under wet conditions. The disease proved especially severe during late summer and autumn. In surveys of ryegrass diseases in England and Wales, Lam (1981) observed that leaf infection was most frequently caused by *Drechslera* species. *Drechslera* species were present in over seventy percent of fields sampled. There was little damage in terms of diseased leaf area except in the oldest leaf age group. Lam (1981) noted that in 1977 a maximum of five percent of the youngest fully expanded leaf was infected, rising to eleven percent in the next leaf and thirty-eight percent in the oldest leaf, which was frequently senescent. MacGarvie (1968) reported that there had been considerable loss in a seed crop of perennial ryegrass in Scotland in 1948 where nodes, leaves and inflorescences were attacked and the seed crop reduced. It was suggested that crop loss may have been widespread in Scotland. In the United States, infection has been serious enough to justify breeding resistance to

D. siccans into many new ryegrass cultivars (Meyer 1982).

The season in which greatest disease severity occurs appears to vary between growing regions. In New Zealand, net blotch (caused by *D. dictyoides*) is reported to be most severe on ryegrass in winter (Latch 1966) whilst in Britain severity is greatest in late summer and autumn (Wilkins 1973, Heard & Roberts 1975, Lam 1981). Drechsler (1923) found infection by *D. siccans* to be most severe in early summer in the United States.

Lam (1981) investigated meteorological conditions in relation to disease outbreaks. She found an association of two factors - a decrease in the amount of rain accompanied by temperatures reaching 20°C (the optimum for spore production) - to be responsible for the start of an epidemic, manifested by the production of numerous minute spots.

While little has been written on the epidemiology of net blotch of ryegrass, a considerable body of literature is available relating to a similar disease, net blotch of barley, caused by *D. teres*. Under field conditions it is prevalent during damp weather, humid conditions for a least ten to thirty hours being necessary for maximum infection (Shipton et al. 1973). According to Jordan (1981), in optimum weather (20°C, 100% relative humidity) net blotch lesions developed and sporulated within five days from inoculation and the pathogen could kill a leaf within fifteen days.

In a glasshouse study, Cook (1975) investigated the effect of levels of *D. siccans* infection on yield of *L. multiflorum*. There was a significant relationship between production loss and percent lesion cover on the second leaf, but yield loss occurred only if there was at least seven percent diseased leaf area. Carr (1975) found *D. dictyoides* did not have a

significant influence on water-soluble carbohydrate content, unlike *Puccinia coronata*, which could cause almost a fifty percent reduction. *D. dictyoides* affected nitrogen content more than did *Puccinia coronata* and it was suggested that where more facultative pathogens kill host tissue in advance of the hyphae, degraded nitrogenous products are respired.

5.1.2 Host resistance

There has been less artificial selection for disease resistance in grasses than in most other crops and thus little of the natural variation has been lost (Wilkins 1975). Cruickshank (1957) found that seventy-two New Zealand strains of ryegrass tested, fell into three distinct classes according to reaction to crown rust (*Puccinia coronata*). Wilkins (1975) found major gene resistance to crown rust on ryegrass (probably due to a single dominant gene) in a few lines, but in the majority, resistance was controlled by a relatively large number of genes.

Resistance of *L. multiflorum* to *Rhynchosporium orthosporium* varied continuously (Wilkins 1975). Some plants had no visible symptoms while others had about sixty-five percent of the leaf area infected and resistance appeared to be entirely under the control of a large number of genes of minor effect.

Wilkins (1973), found resistance of ryegrass to leaf spotting by *D. catenaria* (probably *D. dictyoides* f. sp. *perenne**) in some lines while Frandsen et al. (1981) found a significant heritable variation in resistance of *Festuca* to *D. dictyoides* f. sp. *dictyoides*. In the United States, perennial ryegrass cultivars showing good resistance to *D. siccans* have been released (Bailey et al. 1979, Meyer 1982). Variation
* for taxonomic explanation see p. 24, this thesis.

in the reaction of *Lolium-Festuca* hybrids to *D. dictyoides* between field and greenhouse tests has been reported (Sherwood *et al.* 1973) and this possibility must be taken into account when undertaking experimental work (see chapter seven).

Breese & Hayward (1972) reviewed the genetic basis of breeding methods in forage crops and much of this is applicable to breeding for disease resistance. Some relevant aspects of their study are outlined below. *L. perenne* is an outbreeding plant with a fairly efficient self-incompatibility system. The aim in breeding improved varieties must be to retain an acceptable level of heterozygosity. Generally, ryegrasses are dominant where there is fairly close grazing by animals making regeneration by seed rare and survival depend more on asexual reproduction from vegetative tillers. It appears that the more persistent ryegrass ecotypes are aggregates of genotypically distinct clones. The sexual offspring have maximum genetic heterozygosity so the sexual cycle is adapted to be a colonising phase while stabilisation is achieved through asexual reproduction. Only a small percentage of seeds need have the desirable qualities, so long as they are also competitively superior since seedling elimination under competitive conditions can be massive - reaching ninety percent in sown swards after a few months.

Intraspecific competition allows resistant genotypes to compensate for yield loss in susceptible ones (Carr 1975). Susceptibility of one species to a disease however, can modify the species composition of a pasture which, in turn, could cause a loss in productivity. A pertinent example is the infection of ryegrass in a ryegrass/cocksfoot/red clover ley with *D. siccans* which caused cocksfoot to become the dominant grass (Carr 1975). With the production of higher yielding cultivars or those having more specialised uses such

as turfgrass, there is an increasingly restricted genetic base (Carr 1975).

Carr (1975) summarised the prospects of resistance breeding in the future.

'Provided care is taken to avoid the predominant use of major gene base resistance, which has proved so transitory in other crops, the long term prospects for resistance breeding in the outbred herbage crops appear encouraging.'

(Carr 1975)

In this study, twenty-one ryegrass lines, originating from one field, were selected to include lines likely to show considerable field resistance to *D. dictyoides* as well as lines highly susceptible.

5.2 Experimental design

In May 1981, a field trial to study net blotch infection on ryegrass was set up at the 'Yates Corporate Research Division' farm at Courtenay, Canterbury (fig. 5.0).

Experimental design: Randomised complete block, 21 ryegrass lines X 8 replicates = 168 plots.

Location: Courtenay

Date transplanted: 14 May 1981

Soil type: Barrhill silt loam

Plot size: one spaced plant

Plant spacing: 50 cm

Trial size: 14m X 3 m

Cutting dates: 23 October 1981

15 December 1981

Assessment dates: 21/5/81 16/6/81 30/6/81 14/7/81 28/7/81
 11/8/81 25/8/81 8/9/81 22/9/81 13/10/81
 10/11/81 2/1/82 19/1/82 10/2/82 23/2/82
 17/3/82 30/3/82 14/4/82 28/4/82 12/5/82
 26/5/82 9/6/82 23/6/82 21/7/82 4/8/82
 27/8/82.

Source of ryegrass lines: Perennial ryegrass, originally from Mr T. Ellett's property, Mangere, Auckland. Selection of lines made from Yates trial 80.58.02 at Courtenay.

Ryegrass lines used: 805802/12,16,20,27*,45,56,60,70,72,73,76,83,84,91,98,99,104,125,139,143,150.

* omitted from subsequent analysis since most replicates died during the drought conditions experienced through the trial period.

Note on method of selection of lines

Based on the results of Yates trial 80.58.02 in 1980 (conducted by Mr N. Cameron of 'Yates Corporate Research

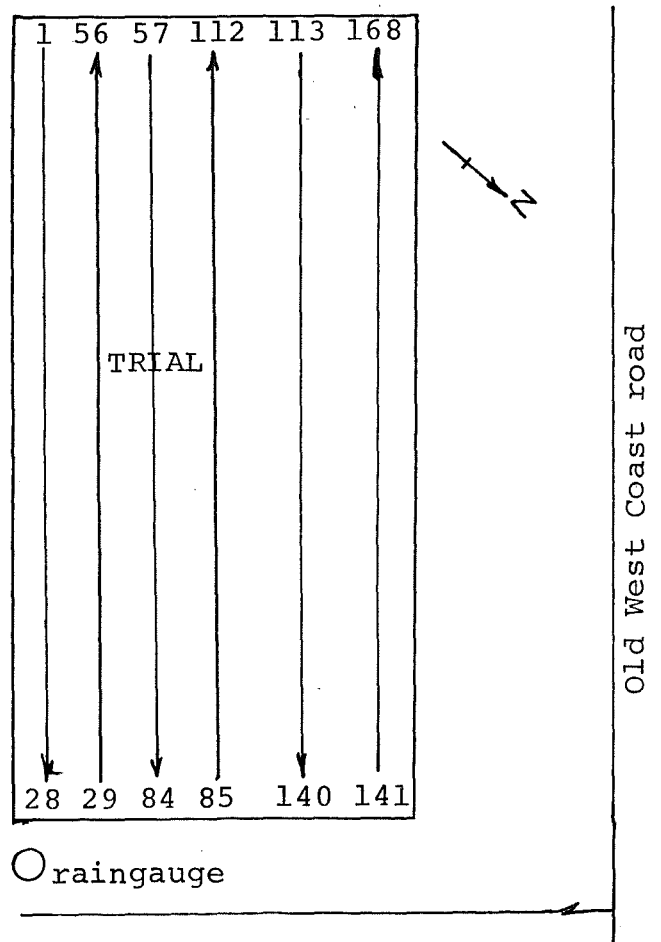
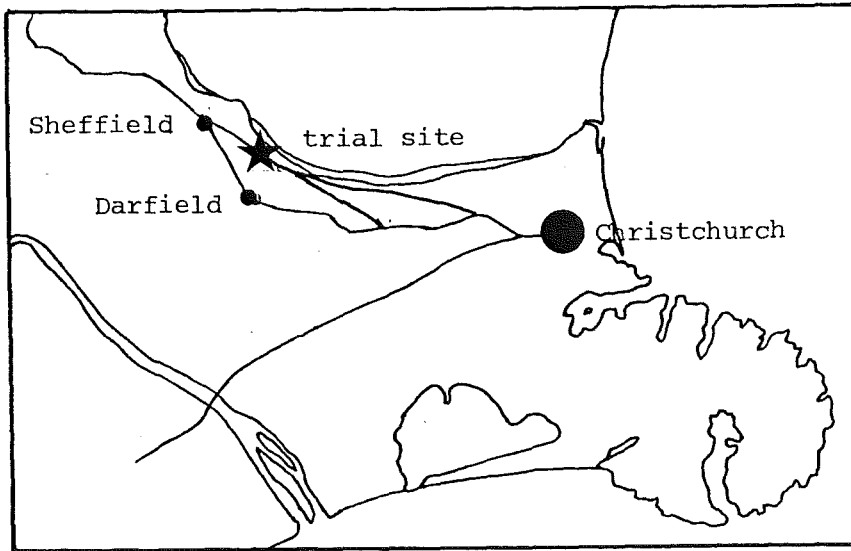


Fig. 5.0 Field trial at Courtenay, Canterbury.

Division') twenty-one lines were selected in order to reflect the range in susceptibility available. Only lines showing good growth characteristics were chosen.

Trial layout

plot ryegrass

no. line no. line no. line no. line no. line no. line

	no.	line	no.	line	no.	line	no.	line	no.	line	no.	line	
Replicate 1	1	91	56	72	57	15	112	12	113	72	168	125	Replicate 8
		56	↑	70		139	↑	70		73	↑	150	
		84		104		27		76		104		84	
		16		125		20		83		20		99	
		99		76		150		56		27		139	
		12		83		45		143		91		16	
		73		60		143		45		60		98	
Replicate 2		91		99		84		99		27		104	Replicate 7
		83		98		16		56		150		143	
		139		73		104		83		91		98	
		20		27		150		139		76		60	
		12		60		76		20		45		16	
		125		70		56		70		125		12	
		143		45		72		73		72		84	
Replicate 3		150		84		45		27		70		45	Replicate 6
		125		139		60		73		84		91	
		73		143		20		20		150		104	
		83		91		76		143		56		60	
		98		12		70		16		98		139	
		72		27		16		72		125		83	
		99		104		56		99		76		12	
Replicate 4		98		45		70		27		70		84	Replicate 5
		76		139		150		125		104		56	
		16		143		84		76		21		139	
		27		99		83		83		72		20	
		56		73		125		12		45		16	
		12		104		60		99		60		73	
	↓	28	72	29	20	84	91	85	143	140	91	141	

Assessment methods

1 Leaf age

In this study the youngest fully expanded leaf is defined as leaf three, with leaf two and leaf four being the next youngest and next oldest respectively (after Lam 1981). Leaf age is dynamic and so a leaf will change status from leaf one to leaf four during the course of its life.

2 Number of plants infected and disease ratings

The disease ratings for each plant were visually assessed according to a rating scale approximating:

- 0 = no net blotch present
- 1 = < 1% leaves infected
- 2 = 1 - 10% leaves infected
- 3 = 10 - 30% leaves infected
- 4 = > 30% leaves infected

Assessments were made of disease on leaves two, three and four. Calculations of the number of plants infected and average disease rating for each ryegrass line, were made.

3 Disease severity

Plants with disease ratings of 0 to 2 had minimal damage in terms of diseased leaf area. Percent diseased leaf area was assessed on all plants with a disease rating of 3 or 4, on all visible leaves (usually leaves two to four) for three randomly selected tillers using the assessment keys of James (1973) as a guide, although there is no net blotch key.

4 Meteorological data

Rainfall data were obtained from a site immediately adjacent to the trial site (courtesy of 'Yates Corporate Research Division') for the period September 1981 to August 1982. Other meteorological data were obtained from Selwyn Plantation Board, Darfield (station H 32412), the

nearest official meteorological station. Data for sunshine hours were obtained from Lincoln (station H 32641).

5 Analysis of results

Data were subjected to analysis of variance (ANOVA) using the statistical computer program 'Teddybear' (University of Otago Computing Centre Technical Report T 5, 1979).

6 Drechslera diseases present

D. dictyoides was the only *Drechslera* species isolated from the trial and so assessments refer to that species. Disease development relied on inoculum available naturally. Disease levels were negligible at the commencement of the trial, although it is possible that some lines had more inoculum present on dead leaves at the plant base, than others.

5.3 Results

5.3.1 Variation in susceptibility

Mean values for each ryegrass line, of percent plants infected, disease rating and diseased leaf area, were calculated to allow for an interline comparison of disease levels (fig. 5.1). Values for percent plants infected were calculated using data from all assessments where at least ten percent of plants in the trial as a whole were infected. Mean disease ratings were calculated for the period from 17/3/82 (prior to this period ratings merely represented percent diseased plants) to the final assessment date on 27/8/82. Mean severity ratings were calculated over the period 17/3/82 - 12/5/82, during the time in which disease severity was relatively high.

With regard to percent plants infected, there was continuous variation between the lines (fig. 5.1:A) although a slight grouping of the nine least diseased lines was observed. The range in infection was considerable. Only five percent of the plants of line 76 were infected in contrast to eighty-three percent of plants of line 16. These differences were consistent between ryegrass lines at the two ends of the scale, where plants were either seldom infected or usually infected.

A semi-logarithmic rating scale was chosen to emphasise the small, but possibly significant, differences between many of the lines where disease was minimal (fig. 5.1:B). Excluding the four most heavily diseased lines, all of the lines had an average disease rating of less than one. This indicates that, on average, lesions were present on less than one percent of leaves. Despite the low levels of disease experienced, there were consistent differences between these

Fig. 5.1 Variation in net blotch infection
between ryegrass lines.

A: Mean percent plants infected of each
ryegrass line.
(17 assessments)

B: Mean disease rating of each ryegrass
line.

(11 assessments: 17/3/82 - 27/8/82)

0 = no disease

1 = < 1% leaves infected

2 = 1 - 10 % leaves infected

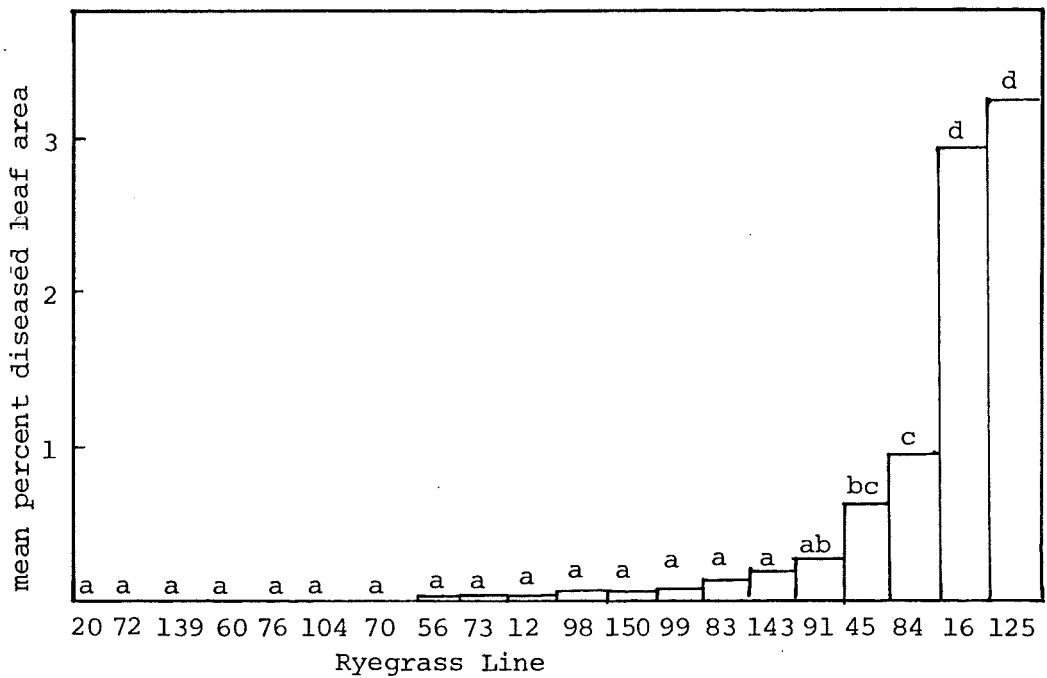
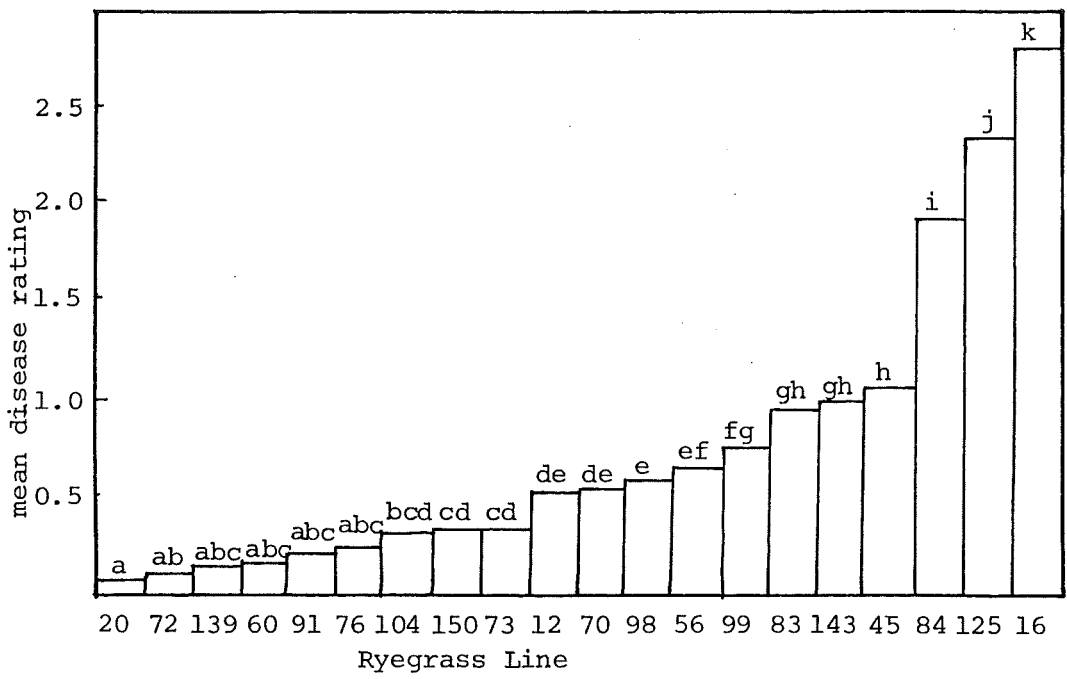
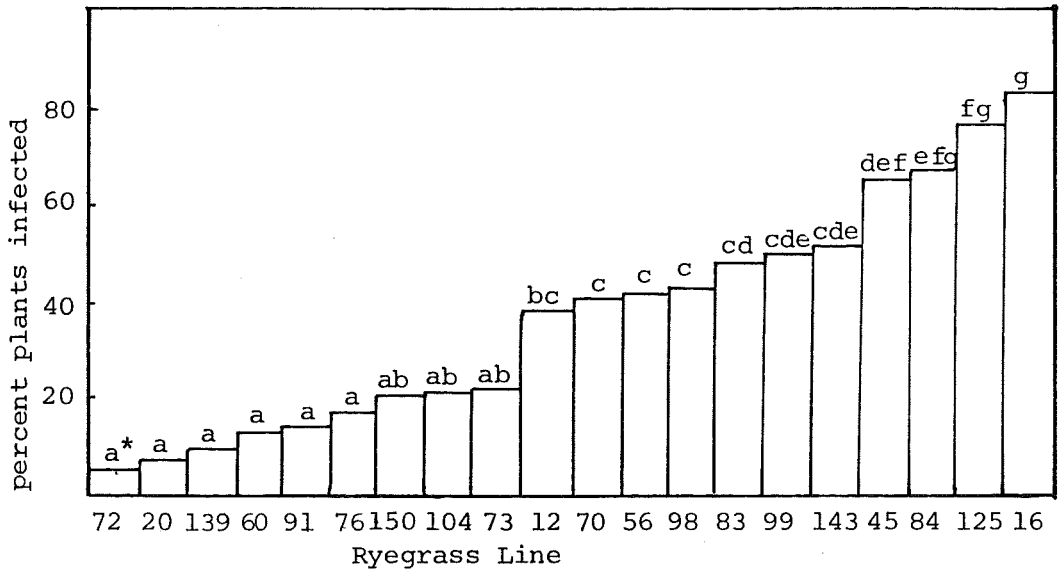
3 = 10 - 30 % leaves infected

4 = > 30% leaves infected

C: Mean percent diseased leaf area on
leaves 2, 3 and 4 of each ryegrass
line.

(5 assessments 17/3/82 - 12/5/82)

* means followed by the same letter are not
significantly different ($P = .05$).
Duncan's multiple range test.



'low disease' lines, which ranged from a rating of 0.1 to almost 1.0 with continuous variation.

In order to further separate these lines with low levels of disease, mean ratings were calculated for the limited period of 17/3/82 - 12/5/82, when disease severity was greatest (fig. 5.2). Variation was again largely continuous between all but the most diseased lines. Negligible disease was consistently recorded on the six lines with the lowest disease ratings, indicating the existence of considerable field resistance among those lines.

The considerable difference in disease between the majority of lines, which showed considerable field resistance and the few heavily diseased lines, is illustrated most clearly using the percent diseased leaf area data (fig. 5.1:C). Severity of disease was insignificant on the majority of ryegrass lines, but use of highly susceptible cultivars could result in considerable crop loss. It should be noted that line 45 showed comparatively high levels of disease on the earlier assessment dates but suffered particularly severely from water stress during the summer drought period. This resulted in a lower mean severity of disease than expected in comparison with the other highly susceptible lines.

Five of the lines - 16, 45, 76, 104 and 125 - were grown outdoors in Christchurch. They were not replicated or randomised and therefore did not represent a legitimate 'trial', but because disease levels varied markedly between lines, two assessments of disease severity were made and the results are shown in appendix 3. In the first assessment, lines 16, 45 and 125, which had shown a high degree of susceptibility in the field trial, recorded 3.3, 15.0 and 0.2% diseased leaf area respectively on leaf three (the

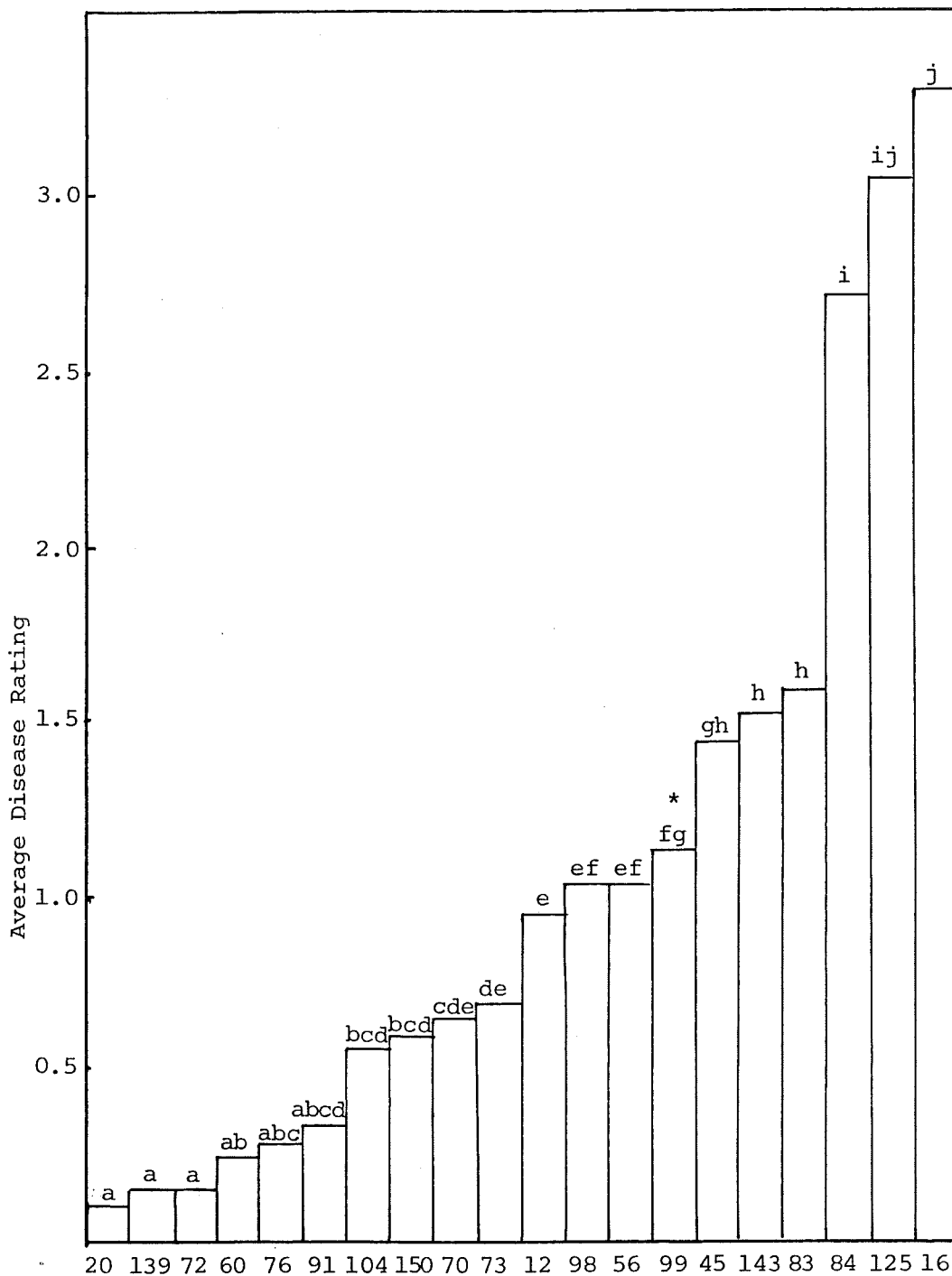


Fig. 5.2. Mean disease rating of each ryegrass line between 17/3/82 and 12/5/82 (5 assessments)

* highly susceptible to drought

youngest fully expanded leaf). Lines 76 and 104, which had shown a consistently low level of disease in the field trial, recorded 0.4 and 0.2% diseased leaf area respectively on leaf three.

The second assessment was made at the commencement of flowering, when disease was again differentially distributed. On line 45 there was a mean of 21% diseased leaf area on the flag leaf and 35% diseased leaf area on the next youngest leaf. Line 104, the least diseased line, had means of 0.2% and 2.0% diseased leaf area for the same two leaves. The differences were highly significant, and are illustrated in plate 5.1.

If the disease loss formula calculated by James *et al.* (1968) for *Rhynchosporium secalis* on barley, is applicable to ryegrass, the loss in seed production on line 45 could have been as high as 16%. Even this would be an under-estimation, as the formula applies at Feekes growth stage 11.1 (Large 1954) while disease severity was assessed at growth stage 10.5, an earlier stage. The production loss estimates of Cook (1975) for *D. siccans* on ryegrass suggest a loss in yield of over 20%.

5.3.2 Rusts

Two rusts have been recorded on ryegrasses in New Zealand, crown rust (caused by *Puccinia coronata*) being the more common one (Latch 1966). While the present study was confined largely to net blotch, crown rust was frequently present on some lines (being particularly severe in January and February) and should not be disregarded. At each assessment date, a record was made of all plants with severe rust infection (pustules on at least 50% of leaves) although no attempt was made to assess the degree of severity with any

Plate 5.1. Disease symptoms of net blotch on two perennial ryegrass lines at the commencement of flowering.

A,B. Line 104 (field resistant). Few disease symptoms are present on either the flag leaf or leaf two.

C,D. Line 45 (highly susceptible). The flag leaf and leaf two are both heavily diseased.



A



C



B



D

more accuracy. Three lines, 98, 84 and 12, most frequently suffered 'severe' rust infection (on over 25% of all possible replicate/assessment date combinations) while lines 16, 56, 60, 72, 83 and 104 were seldom 'severely' infected (less than 5%). There was no correlation between rust infection and average net blotch disease rating for the 21 lines.

5.3.3 Changes in disease with time

Fig. 5.3 shows the change in amount of disease in the field trial with time. There was a slight increase in the number of plants infected from June until August 1981, but disease levels then became negligible until January 1982 when a sharp increase in incidence, although not severity, was noted. This increase was short-lived and disease was limited to a few lesions per plant, as indicated by the average disease ratings given in fig. 5.3:B. In March 1982 there was again a sharp rise in disease levels, at which time disease severity, in the form of percent diseased leaf area, became measurable. Severity of disease dropped markedly again in the latter part of May. There were consistent differences between the group of 'highly susceptible' lines (lines 16, 84 and 125) and the group showing 'field resistance' (lines 72, 76 and 104).

Changes in the pattern of disease were examined in relation to meteorological changes. Figure 5.4 shows the changes in some meteorological factors through the course of the field trial along with changes in disease ratings. In figure 5.5 meteorological conditions were examined more closely over the period of the major disease outbreak. The month prior to the detection of the outbreak was characterised by high sunshine levels, high temperatures, only one day

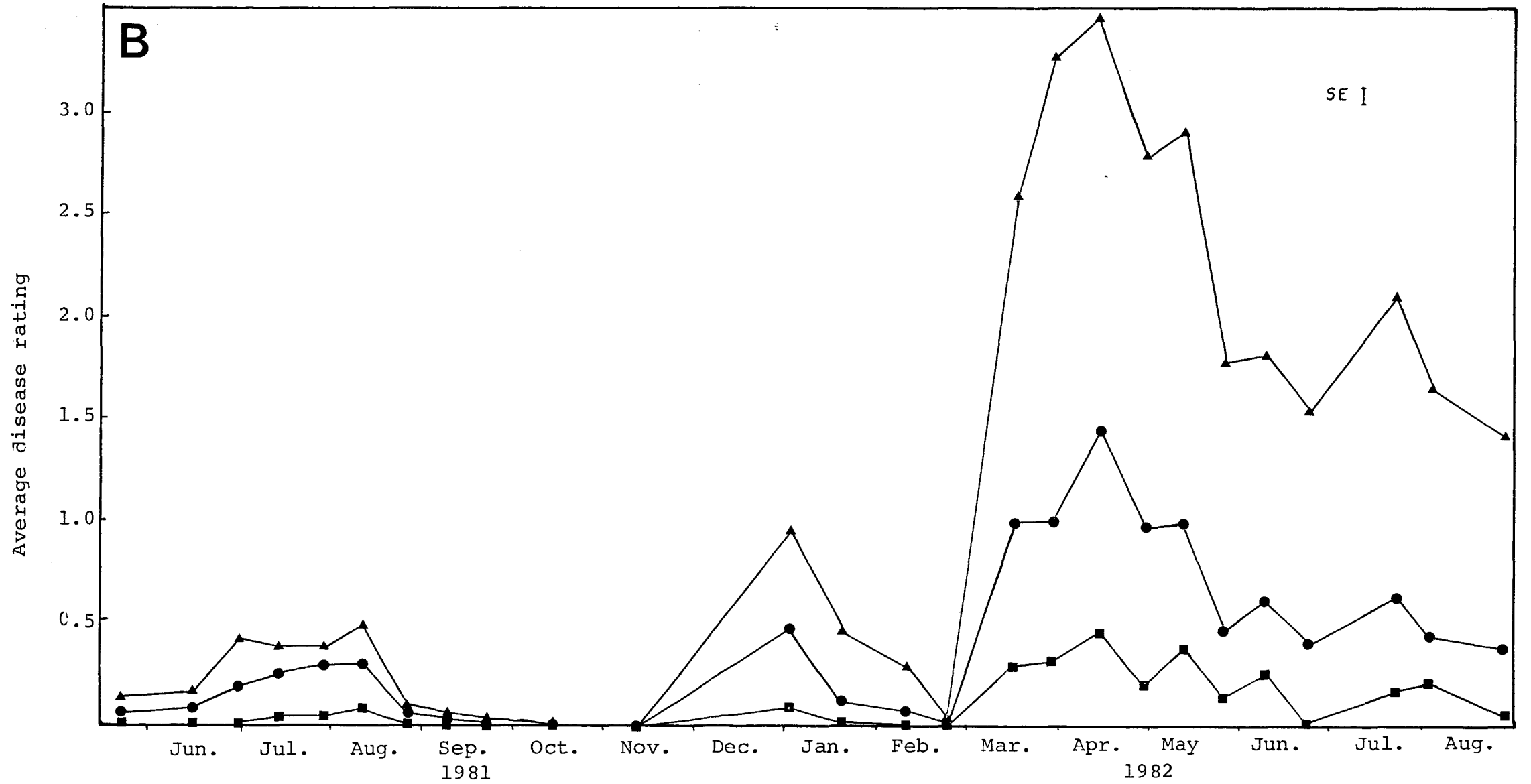
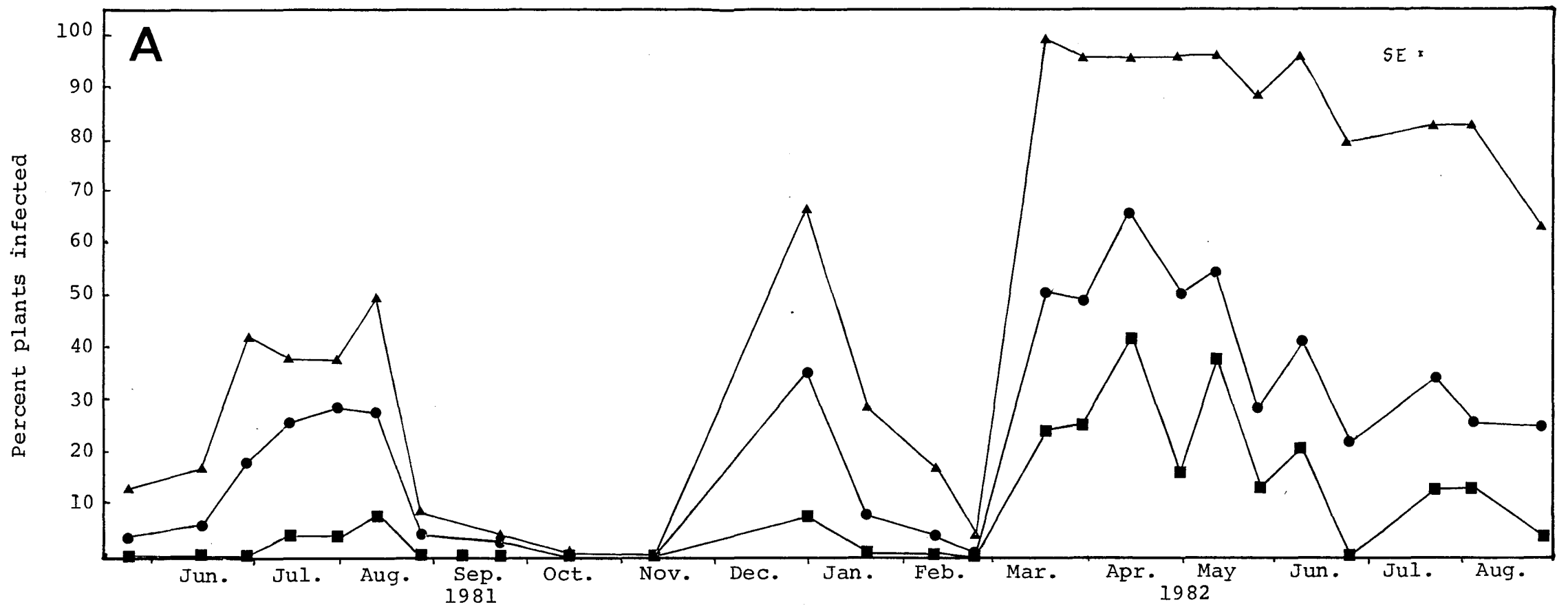


Fig. 5.3. Development of net blotch in the Courtenay field trial from May 1981 to August 1982.

- mean of all 20 lines.
- ▲ mean of lines 16,84 and 125 (highly susceptible)
- mean of lines 72, 76 and 104 (field resistant).

A: Percent plants infected

B: Average disease rating

C: Average percent diseased leaf area

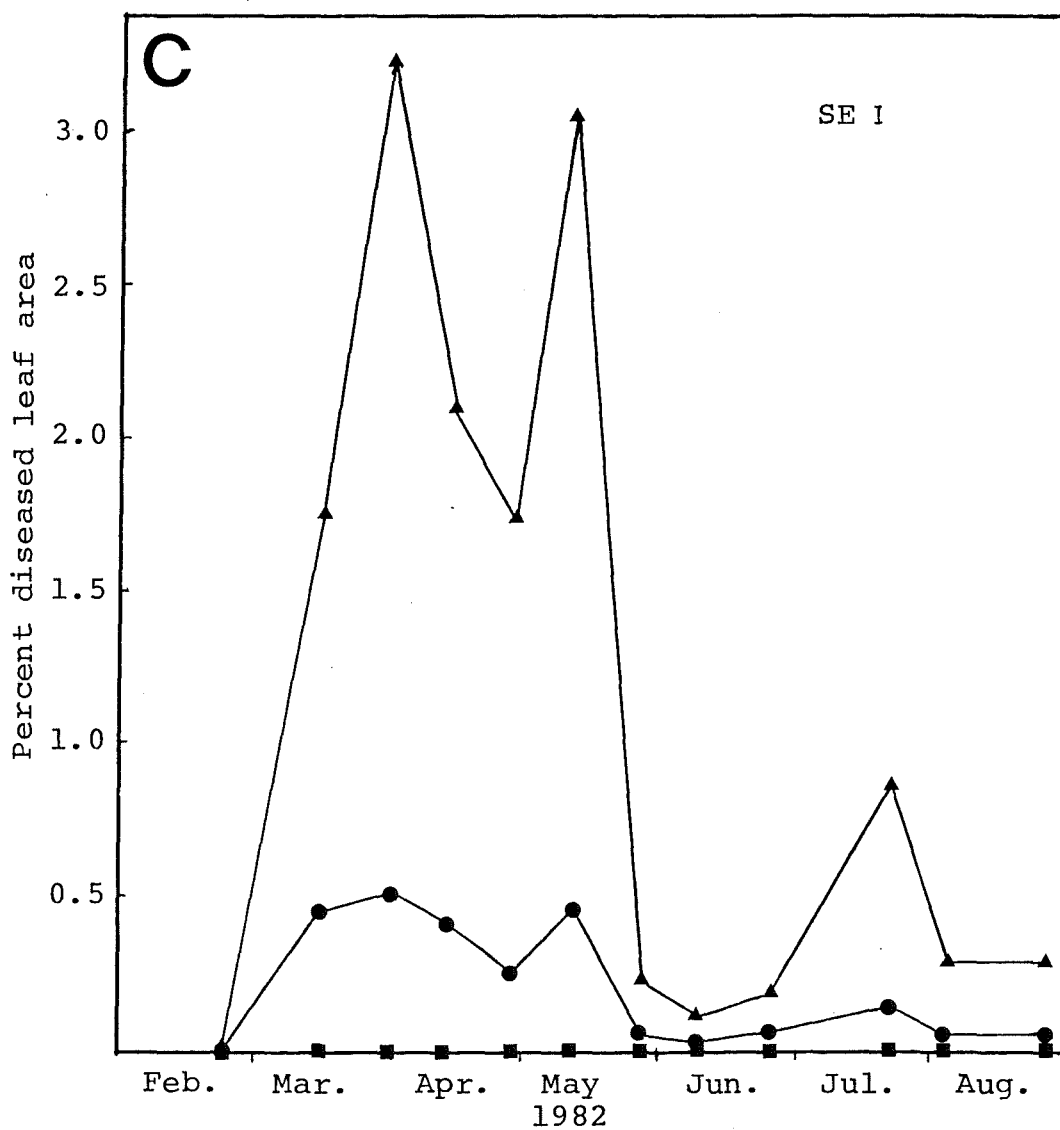


Fig. 5.4. (overleaf) Meteorological changes in relation to levels of net blotch on ryegrass.



A: mean disease ratings (from fig. 5.3:B).

B - G: meteorological data.

B: Monthly total rain-days (0.1 mm or more).

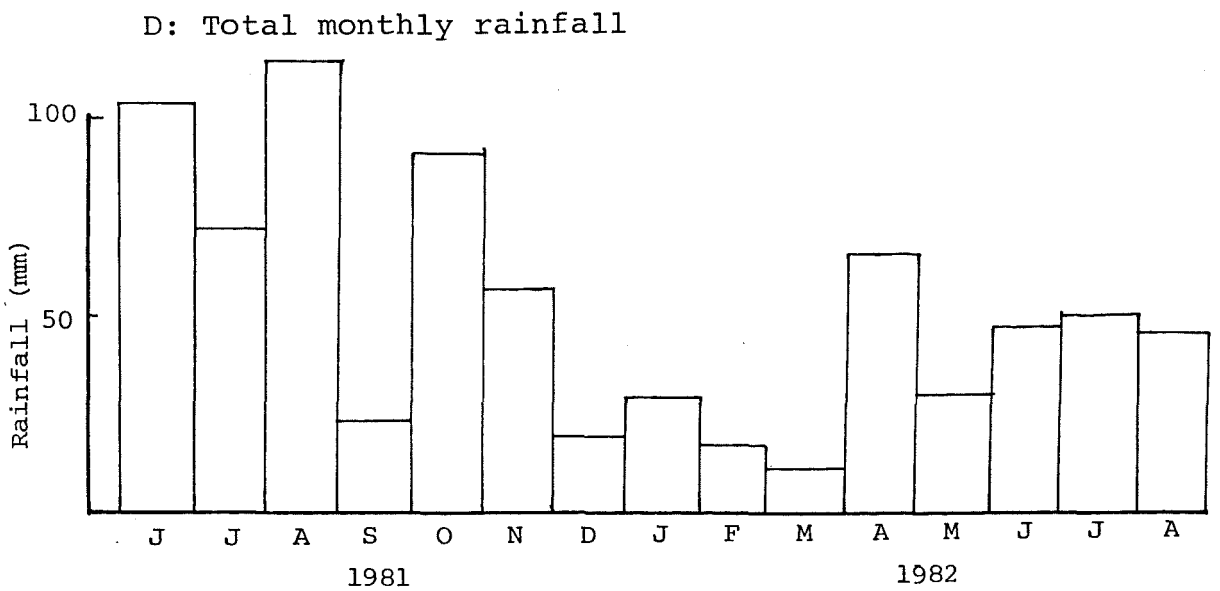
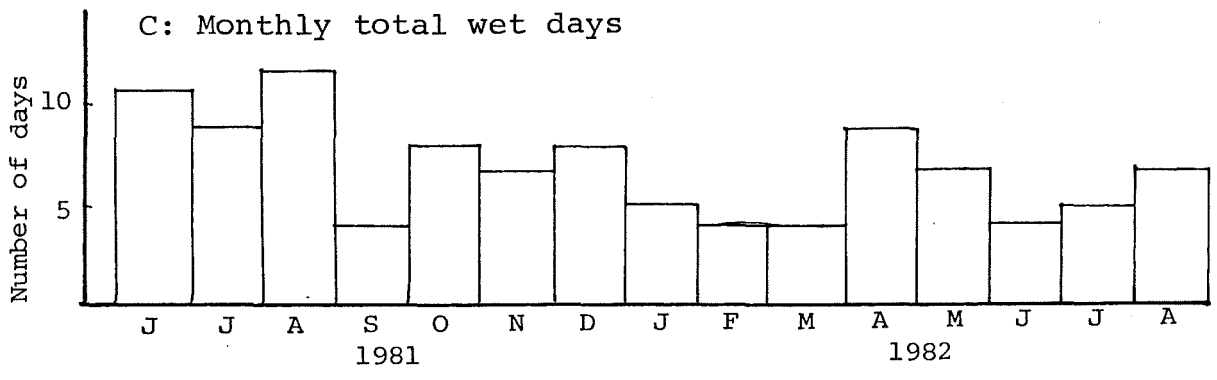
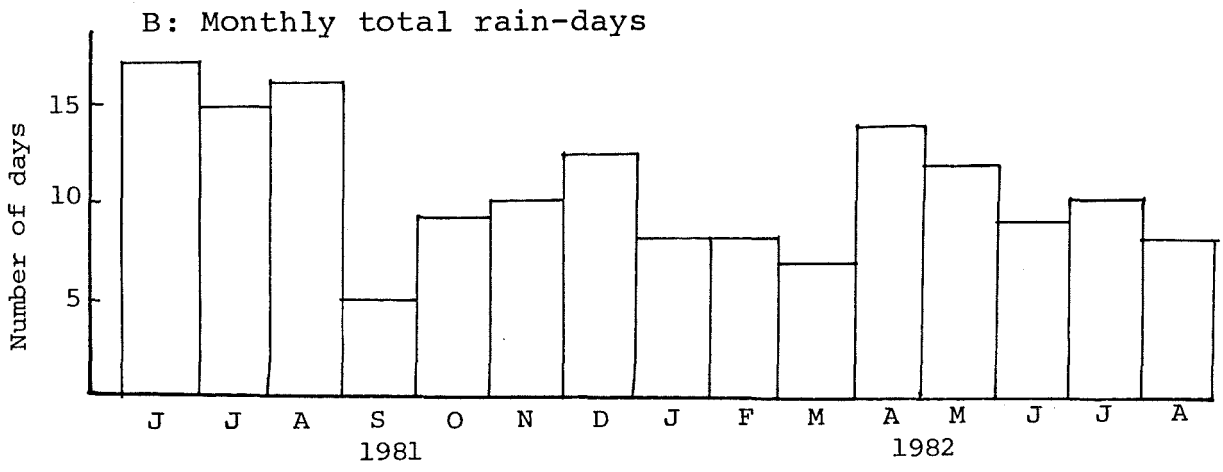
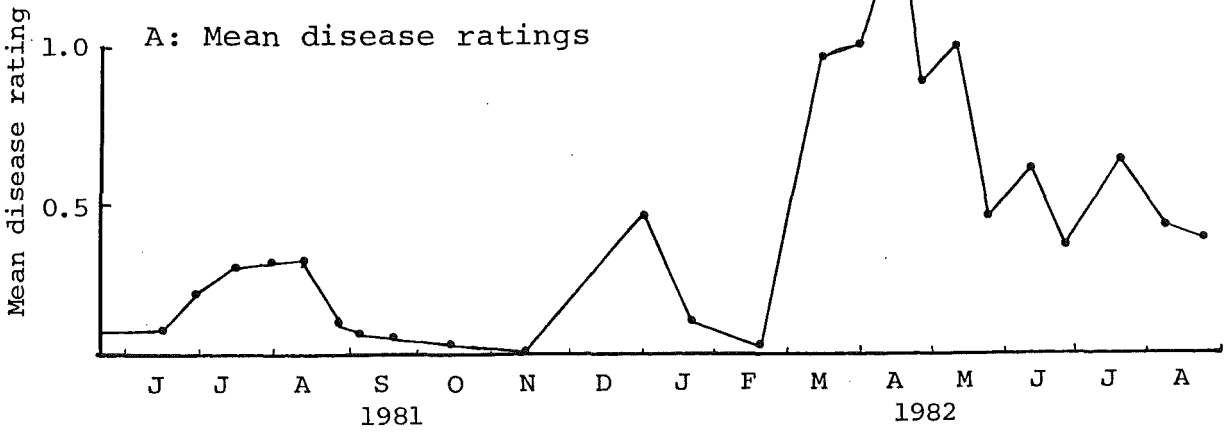
C: Monthly total wet days (1 mm or more).

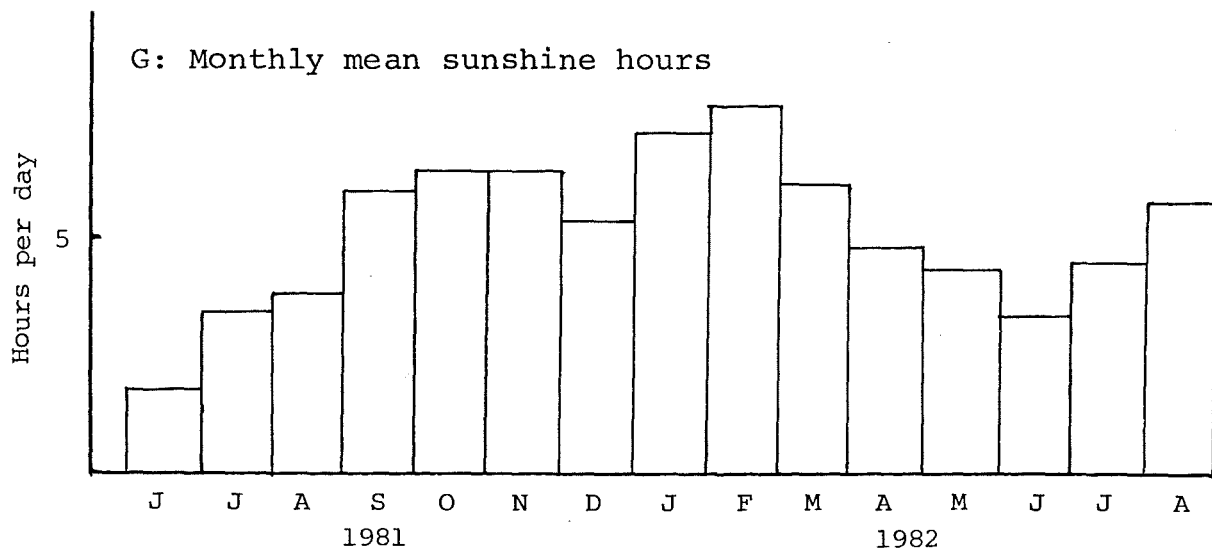
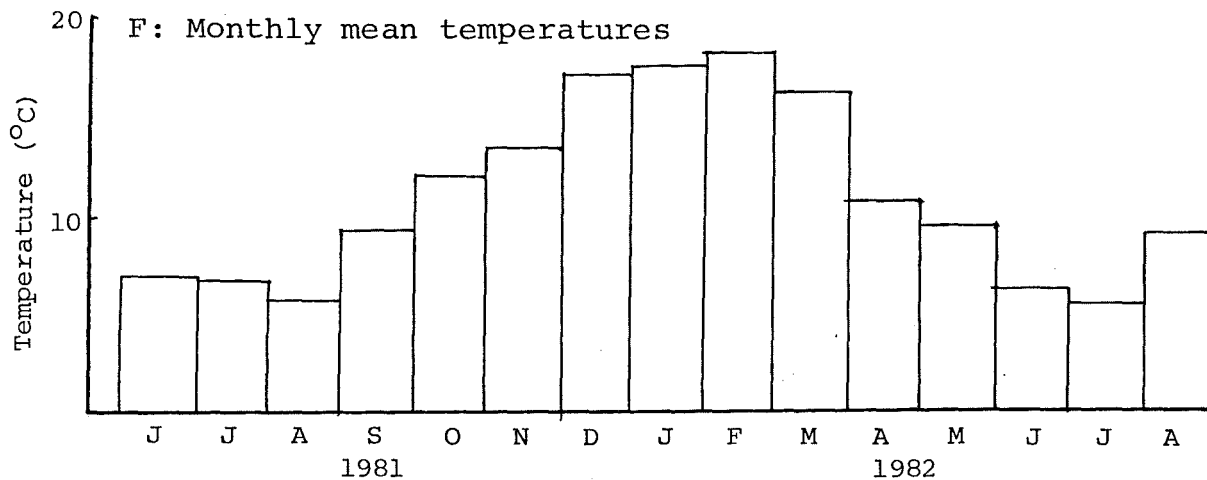
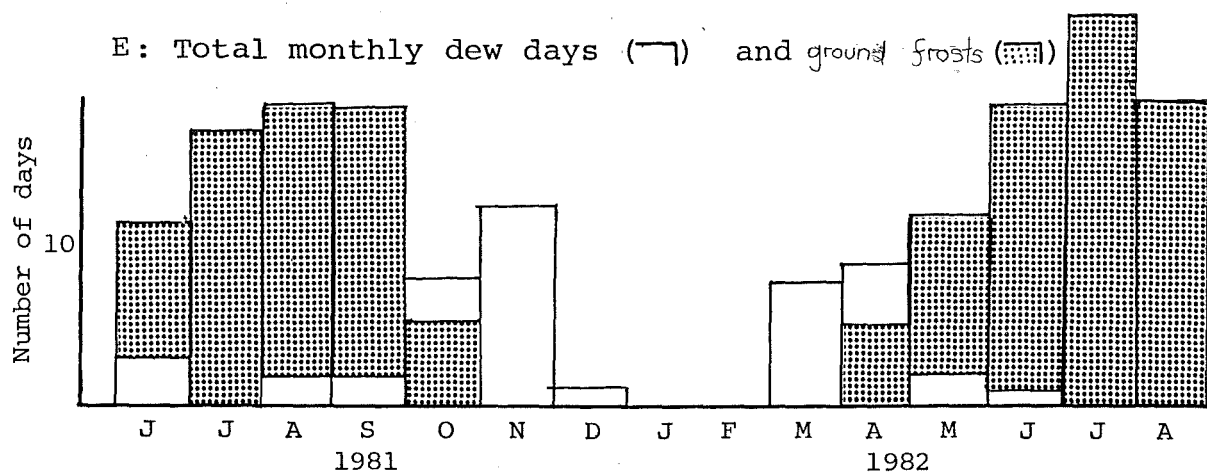
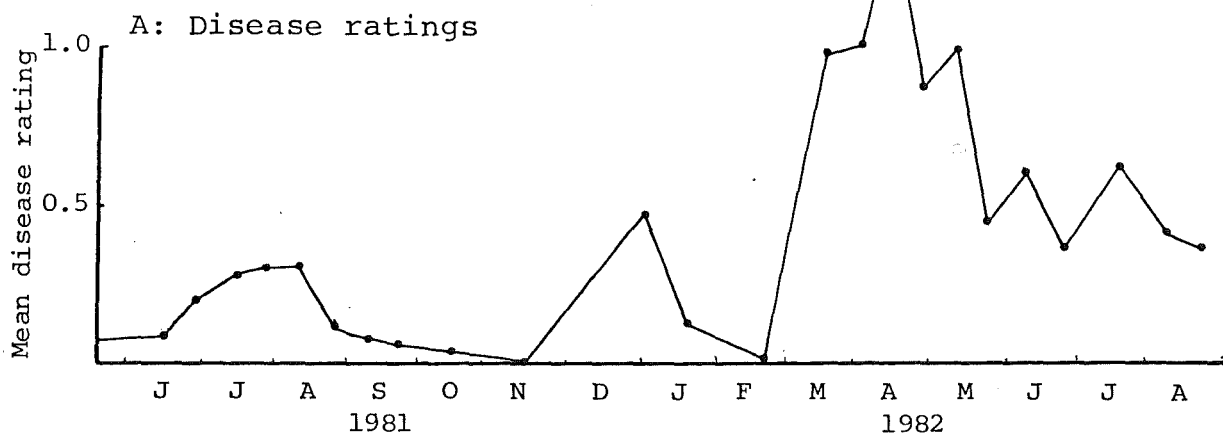
D: Total monthly rainfall.

E: Total monthly dew days  and ground frosts 

F: Monthly mean temperatures

G: Monthly mean sunshine hours





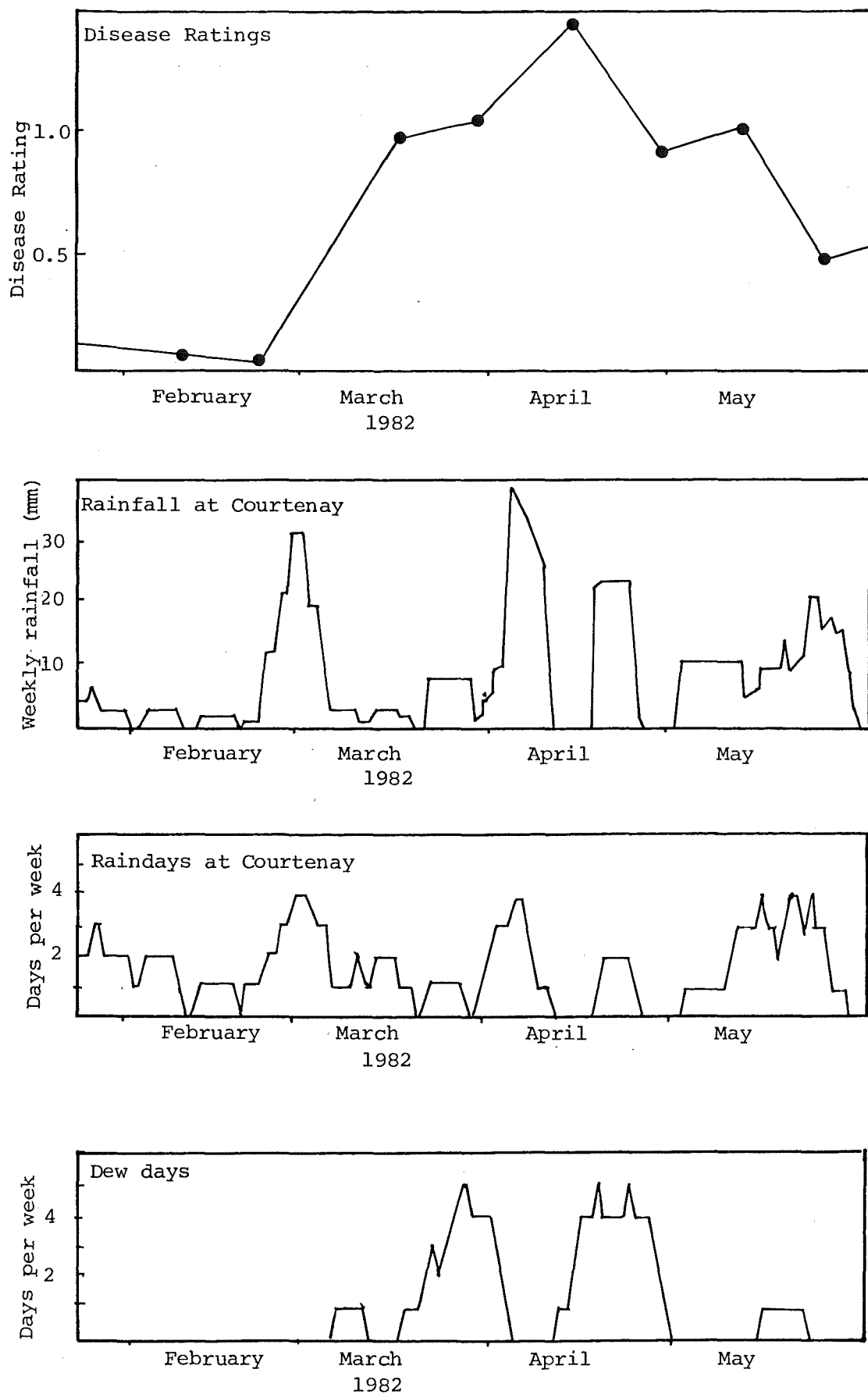


Fig. 5.5. Daily running totals of weekly rainfall, raindays and dew days in relation to disease levels.

recording dew and a low monthly rainfall, although several days of rain were recorded at Courtenay which do not feature in the official Darfield data. The months following detection of the outbreak were characterised by decreasing sunshine hours and temperatures, an increase in the number of days recording dew or rain and an increase in total monthly rainfall. Figure 5.5 shows that rain and dew were quite variable over this time. Disease levels peaked in April and after that time there was a general reduction, although with only minor increases in incidence or severity from time to time. Canterbury suffered from a severe drought from late 1981 through 1982, it being one of the worst on record (NZ Met. service). The effect on the ryegrass plants was readily observable in the form of reduced growth and spread of disease was also probably limited.

5.3.4 Effect of leaf age

The change in diseased leaf area with time in relation to leaf age, is outlined in fig. 5.6. Data represent the means of three highly susceptible lines (lines 16, 84 and 125) as severity of disease was negligible on most other lines. Leaf three (the youngest fully expanded leaf) and leaf four recorded similar diseased leaf areas at the start of the disease outbreak (first noted 17/3/82) but by the next assessment 13 days later, disease had markedly increased on leaf four and noticeably decreased on leaf three. Subsequently disease was consistently more severe on leaf four than on leaf three although increases in disease on leaves of one age group were not always mirrored by increases in the other. Diseased leaf area was always minimal on leaf two, usually the youngest visible leaf.

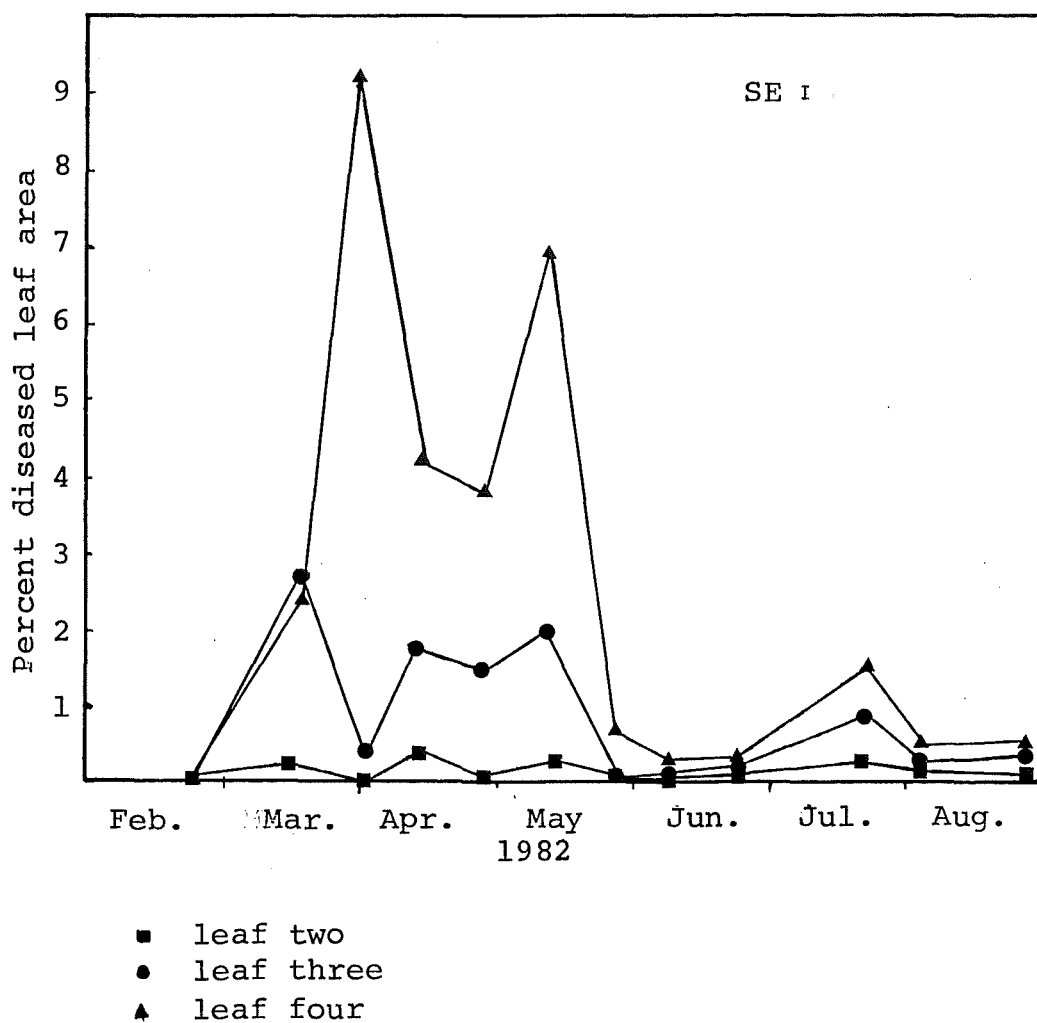


Fig. 5.6 Effect of leaf age on severity of net blotch on ryegrass (mean of three highly susceptible lines: 16, 84, 125).

5.4 Discussion

5.4.1 Disease prevalence

Two separate disease outbreaks occurred during the course of this field trial; the first being a short-lived one of low severity in early January 1982, followed by a second in March 1982. In November 1981 there were thirteen days recording dew (fig. 5.4:E) as compared with eight and one in October and December respectively. In December 1981 thirteen days were recorded with rain, although total rainfall was low. There was sufficient moisture provided by dew and rain during this period to allow sporulation of old lesions and subsequently an increase in infection (fig. 5.3) although damage remained negligible during this time and then virtually disappeared from the trial.

The major disease outbreak began in March 1982 when disease increased rapidly in both incidence and severity, especially on the more susceptible lines. It occurred during the warmest, driest conditions of the Canterbury drought. A close look at both the official Darfield data and that from Courtenay shows that there were three days of heavy rain recorded at Courtenay in January and a concentration of rain-days at the end of February at both sites. In England and Wales infection of ryegrass by *Drechslera* is greatest in late summer or early autumn (Wilkins 1973, Heard & Roberts 1975, Lam 1981). This agrees with the present study but the reason for the increase was probably different. Lam (1981) concluded that a decrease in the amount of rain allowed sporulation to take place, while still retaining sufficient moisture to allow infection to occur. The reverse was probably the case in Canterbury in 1982. With a lack of rain or dew, there would have been insufficient

moisture for infection and probably also sporulation of lesions on senescent leaves. Therefore sporulation and subsequent infection could only take place when sufficient moisture was provided.

Moisture requirements of *D. dictyoides* for infection are probably similar to those of *D. teres* outlined by Shipton *et al.* (1973) and Jordan (1981), where humid conditions are necessary for at least ten to thirty hours for maximum infection.

The uniform and sudden appearance of disease in the ryegrass trial suggests the possibility of a release of spores and infection over one or two short periods of time. *D. dictyoides* can survive on senescent leaves at the base of the plant (Lam 1981), and from there produce an abundant crop of conidia in a short period of time if conditions are suitable. Where development of individual lesions was followed it was found that mature net blotch lesions had formed about twenty days after appearance of an initial fleck lesion in May 1982. In November 1982 the time was reduced to seven days, and lesions were capable of sporulating two weeks after initial appearance of symptoms. If it is assumed that conditions of high humidity are required for sporulation and infection, it is likely that the few days rain at the end of February 1982 were responsible for the disease outbreak, with lesions developing over the next two weeks.

Leaf age must be taken into consideration when disease assessments are made. At each assessment date disease severity was minimal on leaf two and usually greatest on leaf four. This may have been due largely to the rate of leaf emergence; as the ryegrass plant grows, leaf two becomes leaf three and

so forth, therefore in order to retain the same diseased leaf area on leaf three of a growing plant, it is necessary for additional infections to occur. There is also the possibility of increased susceptibility with increased leaf age, probably with senescence-induced susceptibility.

The interaction of the time required for lesion development and the rate of leaf production, probably both determined by environmental conditions such as temperature, is complex. Where the rate of leaf appearance is reduced, senescence of individual leaves is delayed and this could be partly responsible for an increase in the time required for lesion development. The possibilities are examined more closely in chapter seven (p.188). As far as the field situation is concerned, it is clearly essential to take leaf age into account when making disease assessments. The difference in disease severity between leaves two, three and four indicates the value in assessing diseased leaf area on each leaf. If time does not permit this, an assessment of diseased leaf area on leaf three probably gives an adequate view of disease severity.

A well-defined scheme of leaf numbering is required when assessing grasses, where leaves are being continuously renewed. The scheme of Lam (1981) was followed in this study and is based around the youngest fully expanded leaf, defined as leaf 'three'. This leaf is the youngest 'mature' leaf and the term represents a range of leaves of a similar physiological age, although the actual age can vary according to the rate of leaf appearance. Leaves younger than leaf three are juvenile and those older are post-mature, often becoming senescent. With time, therefore, a leaf two becomes leaf three and leaf three becomes leaf four.

The data for severity according to leaf age can give valuable information on the timing of new infections and the expansion of existing ones. Comparison of disease ratings with diseased leaf area according to leaf age indicates that there were in fact three phases of infection, each followed by lesion expansion. The disease outbreak began with the appearance of a large number of new lesions, but this was followed largely by the expansion of existing lesions with few new infections as indicated by the drop in disease severity on leaf three along with a marked increase in diseased leaf area on leaf four. A second period with new infections was recorded on 14/4/82 with an increase in disease ratings. After that time there was an overall drop in disease levels until 21/7/82, when there was an increase in disease on all leaves, indicating some new infections.

Three and five days rain was recorded in the two weeks prior to the first two phases of disease respectively but little rain was recorded prior to the disease increase around 21/7/82. There appears to have been sufficient moisture for these first two increases to have occurred, but the cause of the last slight increase is unknown, although frosts were prevalent in July.

In the present study, maximum diseased leaf area over all twenty ryegrass lines, was 0.5% on leaf three and 1.8% on leaf four. Severity of disease has been recorded in the United Kingdom at as high as ten times that amount (Lam 1981). Levels of disease, therefore, remained relatively low in the Courtenay ryegrass trial, and this is partially attributable to the drought conditions present through much of the trial. Lam's results indicate that the disease has more destructive potential than that indicated by the current

work although it is a disease of high incidence but low severity at present. The mean figures for the three highly susceptible lines (fig 5.6) showed diseased leaf area could be as high as 2.5% on leaf three and 9% on leaf four and this was in a particularly dry area. The use of a highly susceptible cultivar in a wetter region of New Zealand could be disastrous. In New Zealand, leaf damage can also be severe over the winter months (Latch 1966) and while this was not recorded in the field trial, it was observed in Christchurch.

5.4.2 Disease resistance

The susceptibility of line 45 to drought produced spurious results for that line on the later assessment dates. Disease levels on that line were far below those expected from March 1982, and this can be attributed to the small size of the water-stressed plants providing an unsuitable microclimate for infection. In Christchurch, where there was sufficient ground moisture for normal growth, line 45 was consistently the most heavily infected line.

Ryegrass, like most forage grasses, is strongly heterozygous (Carr 1975). It is therefore likely that a large number of genes would confer partial resistance to disease. The continuous nature of the resistance expressed among the lines showing considerable resistance is suggestive of the action of a large number of genes of minor importance. There appear to be few lines with extreme susceptibility and this can be expected in a natural population to which a long term field of ryegrass approaches.

Some ryegrass types, with otherwise suitable growth characteristics, are highly susceptible to net blotch.

Disease assessments must be made when evaluating new ryegrass clones to avoid a loss of disease resistance within new, less heterogenous, cultivars. The following extreme example indicates a possible result of neglecting to take disease resistance into account.

'Not much was heard about *H. turcicum** in the old days of open-pollinated maize. But when hybrid maize was introduced many of the early inbreds were very susceptible; during the process of inbreeding not enough attention was given to disease resistance, and many loci became homozygous for susceptibility where previously open-pollinated crops were heterozygous and heterogenous. In 1942, at the time when hybrid maize was largely replacing the old open-pollinated varieties, there was a destructive epidemic.'

Vanderplank (1982)

Crown rust was not accurately assessed in the field trial, although at times it was a more serious problem than net blotch. There was no apparent correlation of susceptibility between the two diseases.

The abnormal environmental conditions prevailing for the duration of the field trial are unlikely to have been conducive to severe disease, and have probably understressed the importance of net blotch of ryegrass in New Zealand. It is clear that the use of highly susceptible lines in the production of new cultivars could have serious consequences. Lines 45, 84, 125 and 16 showed an unacceptable level of susceptibility to disease whilst lines 20, 72, 139, 60, 91 and 76 showed considerable field resistance under the conditions experienced.

* = *D. turcica*

CHAPTER SIX

THE RYEGRASS EPIDERMIS IN RELATION TO DISEASE

6.1 Introduction

The cuticle and epidermal cell wall serve as the initial barriers to infection by leaf pathogens such as *D. dictyoides*. Spores frequently germinate and often produce appressoria on non-host species hence the epidermis must provide an important early defence mechanism. The structure of the monocotyledonous leaf epidermis is not fully resolved and therefore the role of the various layers in host resistance is not well understood. A diagrammatic representation of the plant cuticle was given by Holloway (1971) who suggested that it consisted of a superficial wax layer external to the cuticular membrane. The cuticular membrane consisted of cutin and cuticular wax, the cuticular wax becoming progressively replaced by cellulose closer to the pectin layer. The cellulosic cell wall was adjacent to the plasmalemma.

6.1.1 Epicuticular waxes

The extent to which the epidermis provides a barrier to invasion has long been debated (Martin 1964). Agrios (1980) suggested a mechanism by which the waxy cuticle could conceivably reduce disease incidence:

'Surface hairs and wax on leaves, stems or fruits repel water and pathogens suspended in it, and keep pathogens from coming into contact with the host surface and from having available moisture to germinate, grow and move. In this way they promote escape from disease under conditions of reduced rainfall and relatively low number of pathogen propagules.'

Agrios (1980)

Leaves of many plants contain epicuticular waxes, although their distribution and occurrence varies within species as well as between them. In *Zea*, the first five or six leaves are glaucous, the rest being non-waxy (Martin & Juniper 1970). In many grasses, the number and size of epicuticular wax projections is markedly reduced or eliminated over the subsidiary cell and guard cells (Martin & Juniper 1970), although in wheat, wax also covers the surfaces of the stomata.

Epicuticular waxes, when present, serve to preserve the water balance of the plant and may also minimise mechanical damage (Eglinton & Hamilton 1967). Waxes are also useful in preventing wetting of some leaves, the wettability being affected by the type and amount of wax (Eglinton & Hamilton 1967). Airborne pathogens appear to have little difficulty in achieving contact with the host surface but as most spores cannot germinate except in a drop of water or film of mist or dew, 'unwettable' leaf surfaces may be less likely to become infected.

Cuticle thickness provides another possible line of defence. Martin (1964) reviewed the role of the cuticle in the host's defence and suggested that plants with thick cuticles could conceivably present a barrier to invasion, but that the soft waxes were unlikely to present a major obstacle. It appeared more likely that a thick cuticle could reduce the rate of infection, giving the host time to mobilize its defences. Martin (1964) summarized the situation by suggesting that the role of the cuticle in the protection of plants was not great.

Royle (1976) reassessed the situation and considered that structural characters could sometimes provide a background level of resistance. He added that:

'In future it should not be surprising to find that structural features may become valuable in augmenting other resistance characters operating at both the individual and plant level and within populations of the host plant.'

Royle (1976)

There is little published information on the epicuticular waxes of *Lolium* although Koziol & Cowling (1981) investigated the effect of pollutants in a SEM study. Leaves were dehydrated in an ethanol and acetone series and while they claim that waxes remained in their natural state, it seems unlikely that they would remain at all. Their results will be discussed in relation to those of the present study.

6.1.2 The epidermal wall

Albersheim *et al.* (1969) put forward the hypothesis that:

'... in many instances ... it is an interaction between the pathogen and the carbohydrates of the host which determines the pathogen's ability to produce enzymes capable of degrading the host's cell wall.'

Albersheim *et al.* (1969)

In other words, the specific nature of the carbohydrate constituents of a susceptible cultivar would initiate the production of an enzyme by the pathogen which could be capable of degrading the host's cell walls. To support this hypothesis they showed that the enzymes produced in growth media were determined by the carbohydrates available. For instance pectinase was only produced in the presence of pectin, however, once produced, such enzymes were equally able to affect cell walls isolated from both resistant and susceptible cultivars (Albersheim *et al.* 1969). Although these enzymes were involved in general pathogenesis, Albersheim

& Anderson-Prouty (1975) considered they were not determinants of varietal specificity. The similarity of primary cell walls between the various monocotyledons provides evidence for this. Modifications to the cell walls during secondary wall formation could inhibit dramatically the ability of enzymes to degrade the cell wall polymers of their hosts (Albersheim & Anderson-Prouty 1975). This provides a possible line of resistance.

Both the abaxial and the adaxial ryegrass epidermis give negative staining reactions for lignin, but ferulic acid, a phenolic acid, is bound to these unlignified cell walls (Harris *et al.* 1980). The same phenolic acids present in ryegrass are also present in wheat, where there is no correlation between the susceptibility of cultivars and their cell wall contents or degradability of their walls (Hartley *et al.* 1978). There is, however, a difference in susceptibility between the 'tips' and 'bases' of the leaf. The cell walls of the more resistant bases are degraded less easily (Hartley *et al.* 1978) and show a more marked colour reaction with diazonium salt, an indicator of phenolic acids. These phenolic acids are attached to carbohydrates and perhaps even to cellulose in walls which are not lignified (Preston 1979). Cell walls richer in these esters are not degraded by cellulase, whereas walls showing only a weak reaction are easily degraded.

6.1.3 Reaction of the cytoplasm of the epidermal cell.

The host epidermis provides more than just a structural barrier to penetration.

'It is important to remember that cells that ... we observe with the electron microscope, and many of those seen with the light microscope, have been killed, fixed and often also stained. But life in the cell is very different. For

in the living cell there is movement of various kinds: Brownian movement; active and often rapid cytoplasmic streaming, effecting the movement of organelles such as plastids and mitochondria ...'

Cutter (1978)

All cells react to invasion, usually before the process is complete. The host cell reaction, or that of the surrounding cells can be responsible for the production of a resistant reaction and even the reaction of a susceptible host can tell us much about host/pathogen interactions.

Sikora (1981) investigated cytoplasmic streaming in *Paramecium* and found that most cytoplasmic particles and organelles flowed along a permanent route in a constant direction. Vogelmann & Miller (1980) found the average velocity of nuclear migration in germinating spores of *Onoclea sensibilis* (a fern) to be $0.256 \mu\text{m min}^{-1}$.

While much is known about the existence and mechanics of cytoplasmic streaming and nuclear migration, less is known of the activity of the cytoplasm and nucleus of the host during infection. Murray & Maxwell (1975) reported that 63 - 83% of appressoria of *D. carbonus* had an epidermal cell nucleus within $10 \mu\text{m}$ in the early stages of infection on both resistant and susceptible corn cultivars.

Contreras & Boothroyd (1975), examining *D. maydis* on corn, found that nuclei of epidermal cells adjacent to the site of penetration moved closer to the penetrated cell simultaneously with penetration. No precise observations were made to determine if the fungus had any minor effects on position of nuclei prior to penetration. In susceptible tissue only nuclei in cells adjacent to the site of penetration were affected but in resistant tissue as many as thirty

epidermal cells were involved. It was suggested that lack of nuclear movement indicated an early adverse effect of the pathogen on the plant cell integrity, leading to subsequent susceptibility. In this situation, then, host resistance did not prevent penetration of a host epidermal cell, but did elicit a response from the nuclei of surrounding cells and this may have been a manifestation of resistance.

Nuclei of host cells of onion bulbs tend to migrate to the face of the cell closest to the wound or infection with *Botrytis allii* or *Aspergillus niger* (Pappelis *et al.* 1974). Forty-eight hours after wounding, approximately fifty percent of nuclei in the first three horizontal rows of cells above the wound had moved downward towards the wound. Kulfinski & Pappelis (1971b) found nuclear size in onion bulbs was increased by wounding and decreased by infection. When inoculated with *B. allii* (Kulfinski & Pappelis 1971a) nuclear area in onion bulbs decreased by 72%.

Heale *et al.* (1982) noted that 68% of nuclei migrated to the site of inoculation of carrot slices with *B. cinerea* as compared with 46% in control slices showing a wound-healing response. It was suggested that the migration may have been related to an altered positioning of cytoplasmic strands as a prelude to various metabolic alterations controlling respiratory activity, cell wall modifications and phytoalexin accumulation.

In a time-course study of the response of nuclei in *Nicotiana* epidermal hairs to wounding, Benda (1959) found that if a neighbouring cell was killed, the nucleus moved to the side closest to that cell.

Few studies of nuclear migration in response to infection have used time-course techniques. One such study is

that of Tomiyama (1956) who compared the hypersensitive and susceptible responses of potato to *Phytophthora infestans*. In resistant varieties, acceleration of cytoplasmic streaming and migration of the nucleus occurred in the early stages of penetration, while in the susceptible varieties, the migration occurred much later. It was suggested that migration of nuclei may have resulted from a stronger motive force of cytoplasmic streaming in the direction of the site of infection than away from it.

In the study of the life history of *D. dictyoides* on ryegrass (chapter four p.89) an association was noted between appressoria and host nuclei but it was not possible to determine the kinetics of this nuclear migration. To resolve the problem and also to observe changes in the pattern of cytoplasmic streaming, a time-lapse study of infection was undertaken. Leaf sheaths were inoculated with a spore suspension of *D. dictyoides* and changes in cytoplasmic flow or nuclear position were monitored in relation to appressorial formation or penetration.

6.2 Results

6.2.1 Cuticular waxes

Plate 6.1 shows the distribution of epicuticular waxes on the two leaf surfaces of *L. perenne*. Leaves were collected from plants in the field to ensure waxes were in their natural state. Leaves were coated fresh to maintain the wax structure. There were no visible differences between the highly susceptible lines (16, 45 and 125) and the field resistant lines (16, 45 and 125) in terms of density or type of epicuticular wax. The major difference was between the adaxial and abaxial leaf surfaces. Wax projections were not observed on the abaxial surface, while the adaxial leaf surface normally had a heavy coating of wax (plate 6.1:E). The wax was not evenly distributed on the leaf surface and some areas showed little wax while adjacent regions were densely covered (plate 6.1:F). The regions with a reduced density of wax were randomly distributed over the leaf surface and did not conform to a pattern. Wax projections were diminished over the guard cells of the stomata and were almost absent in the vicinity of the pore, although the subsidiary cells had a density similar to that of the rest of the leaf (plate 6.1:D).

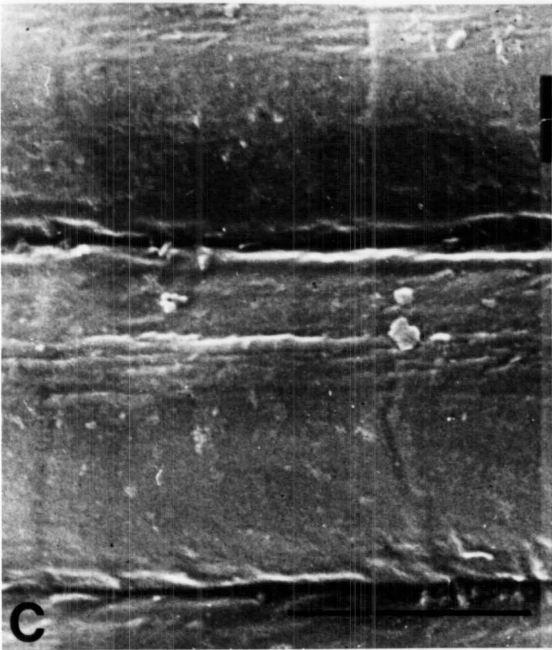
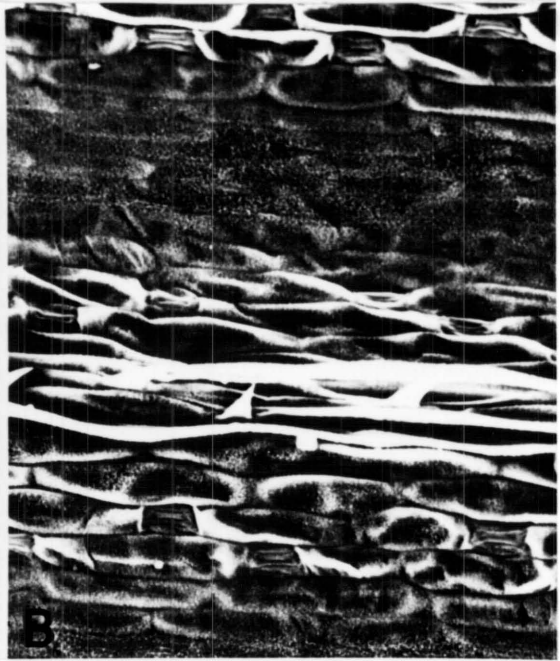
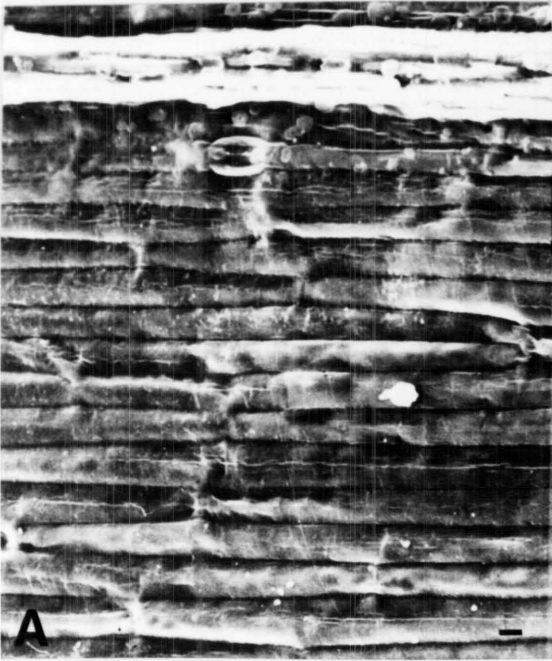
6.2.2 The epidermal wall

The ryegrass epidermal wall was investigated with the use of optical microscopy and the TEM. Freeze microtome sections were stained with toluidine-blue, phloroglucinol or diazonium hydrazine and examined under an optical microscope. Control sections were unstained. For TEM, sections were prepared according to the standard method (p. 18) or using the PA-TCH-AgPr method (p. 21).

Plate 6.1. Distribution of epicuticular waxes on leaves
of *Lolium perenne* (fresh-coated material)

Bar = 10 μ m

- A: General appearance of abaxial leaf surface .
- B: General appearance of adaxial leaf surface.
- C: Detail of abaxial leaf surface. Note the
the total absence of epicuticular wax
projections.
- D: Distribution of epicuticular wax projections
around a stomate on the adaxial leaf
surface.
- E: Detail of epicuticular wax projections on
adaxial leaf surface.
- F: Detail of adaxial leaf surface showing the
uneven wax distribution.



Light microscopy

Lignin stains blue-green with toluidine-blue and pink with phoroglucinol. In both cases the xylem of silicated epidermal cells gave a positive staining reaction while the remainder of the epidermal cells did not. With diazonium hydrazine , lignin stains red, while unligified walls containing ferulic acid often give a weaker staining reaction. In this study the xylem and silicated epidermal cell walls stained red, while the remainder of the epidermal cell walls (the ones through which penetration most often took place) stained orange. Two layers could be distinguished in the epidermal wall but both were stained orange. There was no visible difference between line 45 (highly susceptible) and line 76 (field resistant).

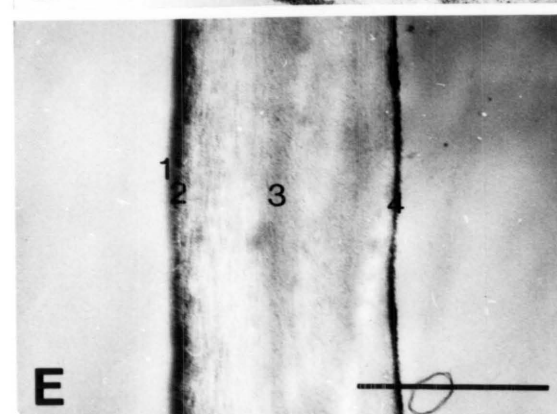
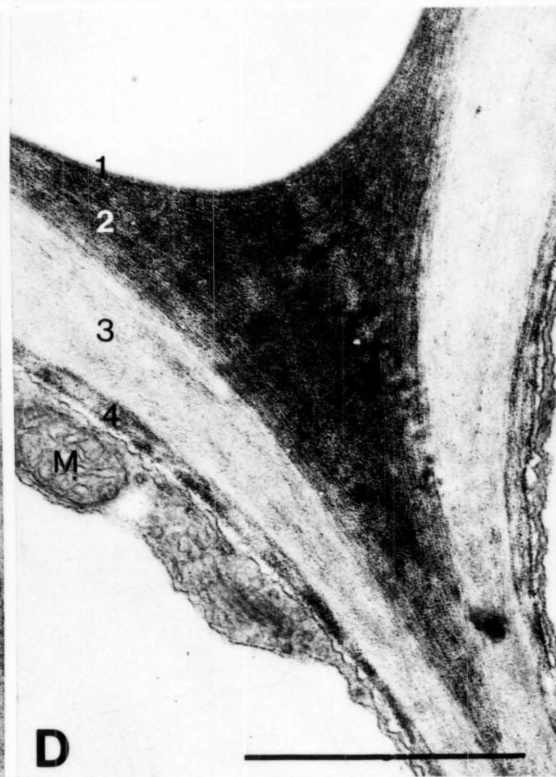
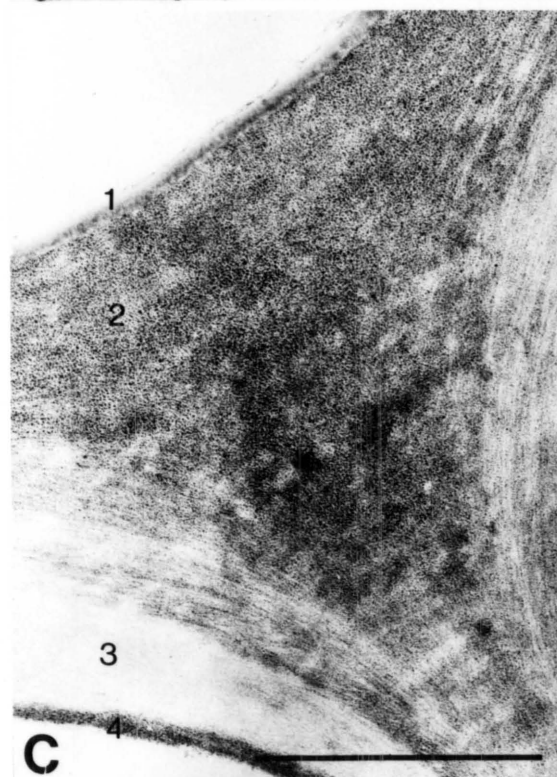
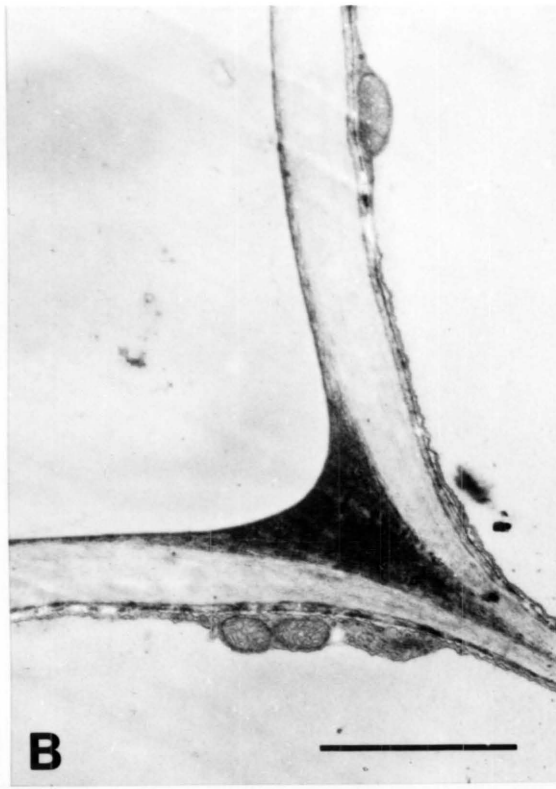
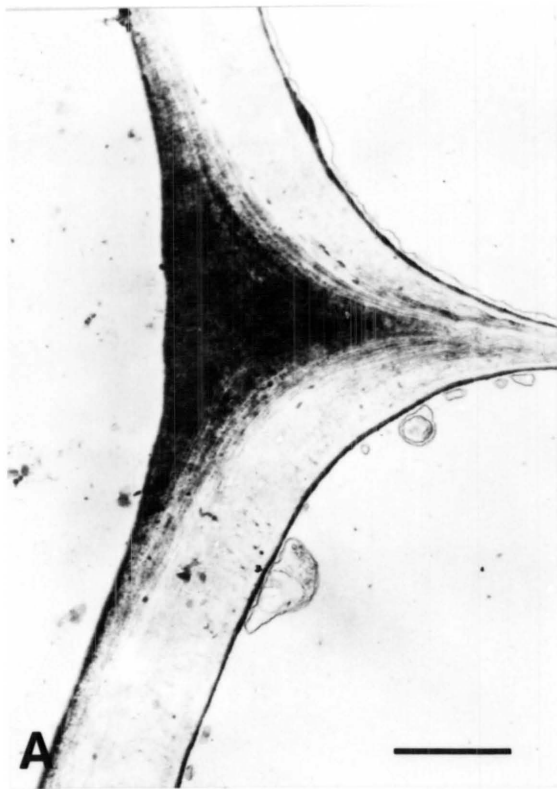
TEM

The PA-TCH-AgPr test for polysaccharide was used to elucidate the structure of the ryegrass epidermal cell wall. Using this stain (plate 6.2) four distinct layers could be seen in the epidermal wall. The first was a narrow outer non-staining region also seen using uranyl-acetate and lead citrate, which probably represented the cuticle proper. Layer two was a densely staining region, forming a thick layer at the longitudinal junctions of epidermal cells but only a narrow layer between. Layer three was a less darkly stained layer, similar in thickness both at the longitudinal junctions and between. The fourth, innermost, layer was a densely staining layer abutting the plasmalemma. The four layers were also present when sections were stained with lead citrate and uranyl acetate, but were not as distinct. The layers were equally distinct on both abaxial and adaxial epidermises but the abaxial epidermal wall was approximately twice as

Plate 6.2. Abaxial and adaxial epidermal cell walls of
Lolium perenne. (PA-TCH-AgPr stained).

Bar = 1 μ m

- A: Anticlinal wall region of abaxial epidermis of leaf.
- B: Anticlinal wall region of adaxial epidermis of leaf.
- C: Detail of wall layers of abaxial epidermis in anticlinal wall region. Note four distinct layers; an outer unstained region, a second heavily stained region, a third lightly stained region and an inner heavily stained region.
- D: Detail of wall layers on adaxial epidermis in anticlinal wall region. The four layers are again present.
- E: Detail of wall layers of abaxial epidermis mid-way between anticlinal walls. Note the four layers are still present, but layer two is markedly reduced in thickness.
- F: Detail of wall layers of adaxial epidermis midway between anticlinal walls. Note that the adaxial epidermis is approximately half as thick as the abaxial epidermis. Epicuticular wax projections have been dissolved during tissue preparation.



thick as that of the adaxial epidermis.

6.2.3 Effect of leaf surface on infection

6.2.3.1 Spore distribution

The difference in surface structure of the two ryegrass leaf surfaces suggests the possibility of differential spore deposition and infection. To assess the relative numbers of *Drechslera* conidia on the two leaf surfaces, eighty-seven three centimetre leaf pieces (using only leaf three on each tiller) of *L. perenne* were collected from the field. They were cleared and the number of *Drechslera* conidia per mm² on both leaf surfaces was assessed microscopically. As an indication of the numbers of conidia involved, a mean figure of 0.98 ± 0.10 conidia per mm² was obtained for the abaxial leaf surface. The actual numbers on each leaf were converted to the percentage on each leaf surface, and this resulted in an estimate of 94.9% ± 0.8% of the total conidia being present on the abaxial leaf surface.

A second assessment was of the distribution of conidia on the leaf surfaces of four ryegrass plants growing outdoors and showing a range in disease levels. Each plant corresponded to a different ryegrass line, the lines being 16, 45, 76 and 104 (from the field trial, see chapter five) and had 5%, 90%, 0% and 5% of tillers infected respectively, at the time of assessment. Two three centimetre segments were removed from the middle portion of leaf three of five replicate tillers. The leaf pieces were cleared and the number of conidia on each leaf surface counted using an optical microscope. The results are presented in table 6.1. Significantly more conidia were present on the abaxial leaf surface than on the adaxial surface and there were significantly more conidia on the leaves of the most heavily infected plant (line 45) than on

Table 6.1. Distribution of conidia on the abaxial and adaxial leaf surfaces of four ryegrass lines with different disease levels.

ryegrass line	% diseased tillers	<i>Drechslera</i> conidia cm ²		
		abaxial	adaxial	mean
16	5	0.6 a/a*	0.0 a/a	0.3 a/-
45	90	6.2 b/b	0.5 b/a	3.4 b/-
76	0	0.0 a/a	0.0 a/a	0.0 a/-
104	5	0.4 a/a	0.0 a/a	0.2 a/-
		1.8 -/b	0.1 -/a	

* means for different ryegrass lines (down a/) or different leaf surfaces (across /a) followed by the same letter are not significantly different ($P = 0.05$). Duncans multiple range test.

the other plants. This plant also had the only conidia which had penetrated the leaf, although germination of conidia was similar on the three plants on which conidia were found. No developed lesions were present on the leaves sampled, but the leaves on line 45 were adjacent to lower diseased, and possibly sporulating, leaves.

In a preliminary experiment to determine if there was any difference in spore deposition or infection between field resistant lines and highly susceptible lines, trap plants were placed, in the field, close to heavily diseased plants. After two weeks, leaves were removed and spore distribution was assessed. There was a greater accumulation of conidia on leaves of the highly susceptible line than on leaves of the field resistant line. The investigation was continued.

Three replicate plants of each of lines 45 (highly

susceptible) and 72 (field resistant) were taken from the glasshouse and placed randomly in an area of uncut ryegrass with a moderate level of net blotch present. Control leaves were removed and spore distribution assessed to indicate initial levels of conidia on the leaves. After three weeks, disease symptoms appeared but no sporulation had occurred to compound the differences. Eight leaf pieces from leaves three of each plant were removed. Distribution of *Drechslera* conidia on the abaxial leaf surface was assessed.

Results are illustrated in table 6.2. Significantly more conidia were present on leaves of line 45 (susceptible)

Table 6.2. Presence of *Drechslera* conidia on trap plants of ryegrass lines highly susceptible or field resistant to *D. dictyoides* three weeks after being placed in the field.

	line 45 (susceptible)	line 72 (field resistant)
Total conidia per cm ²	12.4 b*	1.9 a
Percent germinated	47 b	26 a
germ tubes	8.5 b	0.6 a
per appressoria	7.1 b	0.4 a
cm ² penetrations	1.9 b	0.2 a
necroses	1.2 b	0.1 a
germ tubes	1.2 a	1.2 a
per appressoria	1.2 a	0.8 a
germinated conidium penetrations	0.3 a	0.4 a
necroses	0.2 a	0.2 a
percent tillers diseased	100 b	17 a
CONTROL (conidia per cm ²)	0.4	0.0

* means followed by the same letter are not significantly different ($P = 0.05$) T - test.

than on line 72 (field resistant) after three weeks. Percent germination of conidia was also higher on the susceptible line but there were similar numbers of germ tubes, appressoria and penetrations per germinated conidium. The major difference between the field resistant and susceptible plants was related to the number of conidia present on the leaf surface and their germination. This led to an increased number of penetrations and consequently more necrosis on the susceptible line than on the resistant line. Penetrated conidia induced necrosis equally on the two lines.

Percent diseased leaf area on leaves two, three and four of ten randomly selected tillers per plant, was assessed approximately two months after the plants were placed in the field. Mean figures for each leaf category on each plant were calculated and used to compare the ryegrass lines in terms of severity of disease (table 6.3). There were highly significant differences in percent diseased leaf area between the two ryegrass lines on all three leaves; line 45 (susceptible) having the greatest severity. Disease severity increased with increasing leaf age on both lines.

Table 6.3. Severity of net blotch on trap plants of two ryegrass lines differing in field susceptibility to *D. dictyoides* two months after being placed in the field.

	mean percent diseased leaf area	
	Line 45 (susceptible)	Line 72 (field resistant)
leaf 2	2 b*	0.1 a
leaf 3	27 b	1 a
leaf 4	47 b	6 a

* means followed by the same letter are not significantly different ($P = 0.01$) T - test.

6.2.3.2 Infection of the abaxial and adaxial epidermis

Fewer conidia of *D. dictyoides* are found on the adaxial surface of a ryegrass leaf than on the abaxial (lower) surface. This does not mean, however, that those conidia which do reach the adaxial surface are incapable of infecting, given appropriate conditions.

To assess the relative susceptibility of the two leaf surfaces, detached leaves (leaf three was used) were inoculated with a spore suspension on either the abaxial or adaxial leaf surface. It was necessary to double the usual concentration of 'Tween 80', a wetting agent (see p. 229) to enable the inoculation drop to remain in position on the adaxial surface. Four leaves of each surface were sampled at each time interval (10h, 15h, 20h, 24h), cleared, and infection was assessed microscopically. The experiment was carried out twice and the results combined.

There were no significant differences in germination or germ tube production between leaf surfaces. Results of appressorium production and penetration are given in table 6.4.

Table 6.4. Effect of leaf surface on the infection of *L. perenne* with *D. dictyoides* on inoculated leaves.

time after inoculation (hours)	appressoria per germinated conidium		penetrations per germinated conidium	
	adaxial	abaxial	adaxial	abaxial
10	0.17 a*	0.12 a	0.00 a	0.01 a
15	0.23 a	0.45 b	0.03 a	0.03 a
20	0.49 a	0.58 a	0.19 b	0.02 a
24	0.70 a	0.88 b	0.30 b	0.13 a

* means for different leaf surfaces followed by the same letter are not significantly different ($P = 0.05$) T - test.

More appressoria were produced on the abaxial leaf surface than on the adaxial surface but this was significant only at some time intervals. There were few penetrations on either leaf surface fifteen hours after inoculation, but after twenty hours there were 0.19 penetrations per germinated conidium on the adaxial leaf surface, significantly more than on the abaxial surface, where only 0.02 penetrations were recorded. After twenty-four hours the number of penetrations had increased on both surfaces, but there were still significantly more penetrations on the adaxial than on the abaxial leaf surface.

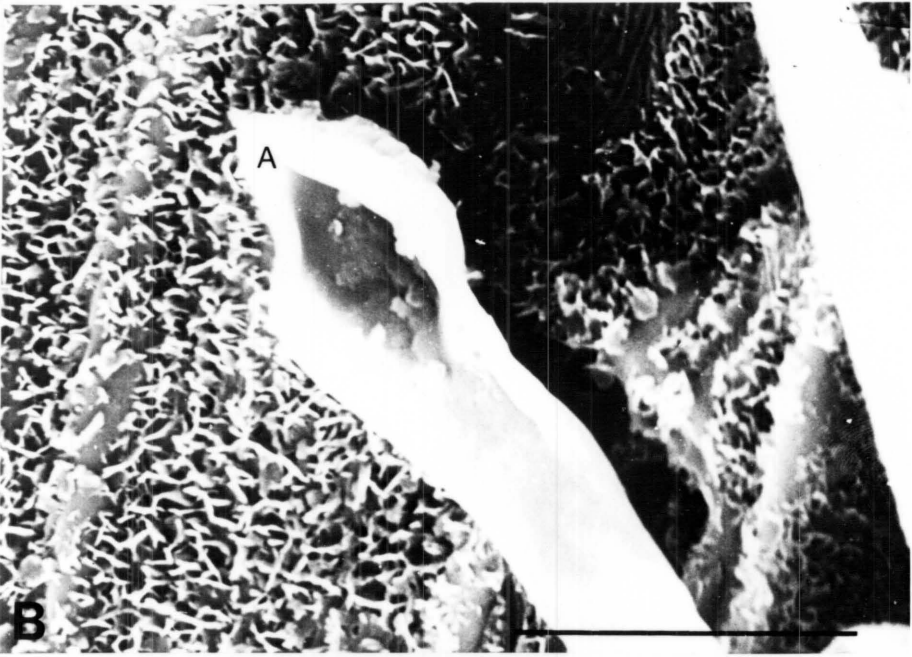
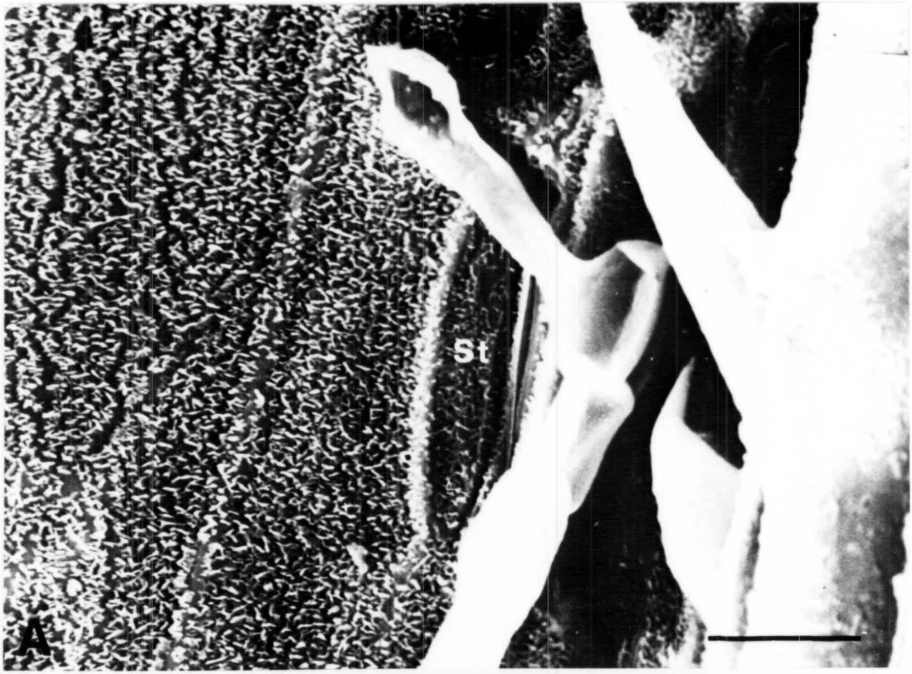
The infection process was followed on the two leaf surfaces using the SEM, with fresh-coated leaves to retain the wax structure of the adaxial surface intact (plate 6.3).

Conidia germinated in the same manner on both leaf surfaces. The germ tubes did not appear to adhere strongly to the host and there was no indication of dissolution of the epicuticular waxes. Adherence was probably greatest in the raised regions of the leaf, where host and fungus were in contact but there was no sign of conidia becoming dislodged. There was no indication of dissolution of surface waxes around the appressoria, but appressoria were fixed firmly to the host surface. Stomata were more common on the adaxial leaf surface than on the abaxial surface, but penetrations through stomata were rare, even on the adaxial surface, and germ tubes frequently grew over them (plate 6.3:A) and produced appressoria later.

Plate 6.3. Infection of the adaxial and abaxial surfaces
of the *L. perenne* leaf by *D. dictyoides*.
(fresh-coated material)

- A: Germ tube growing over a stomate and producing an appressorium on the subsidiary cell beyond (adaxial leaf surface).
- B: Detail of appressorium and surrounding region. Note lack of wax dissolution around germ tube and appressorium (adaxial leaf surface).
- C: Infection on abaxial leaf surface. Note the absence of epicuticular wax projections.

(bar = 10 μ m)

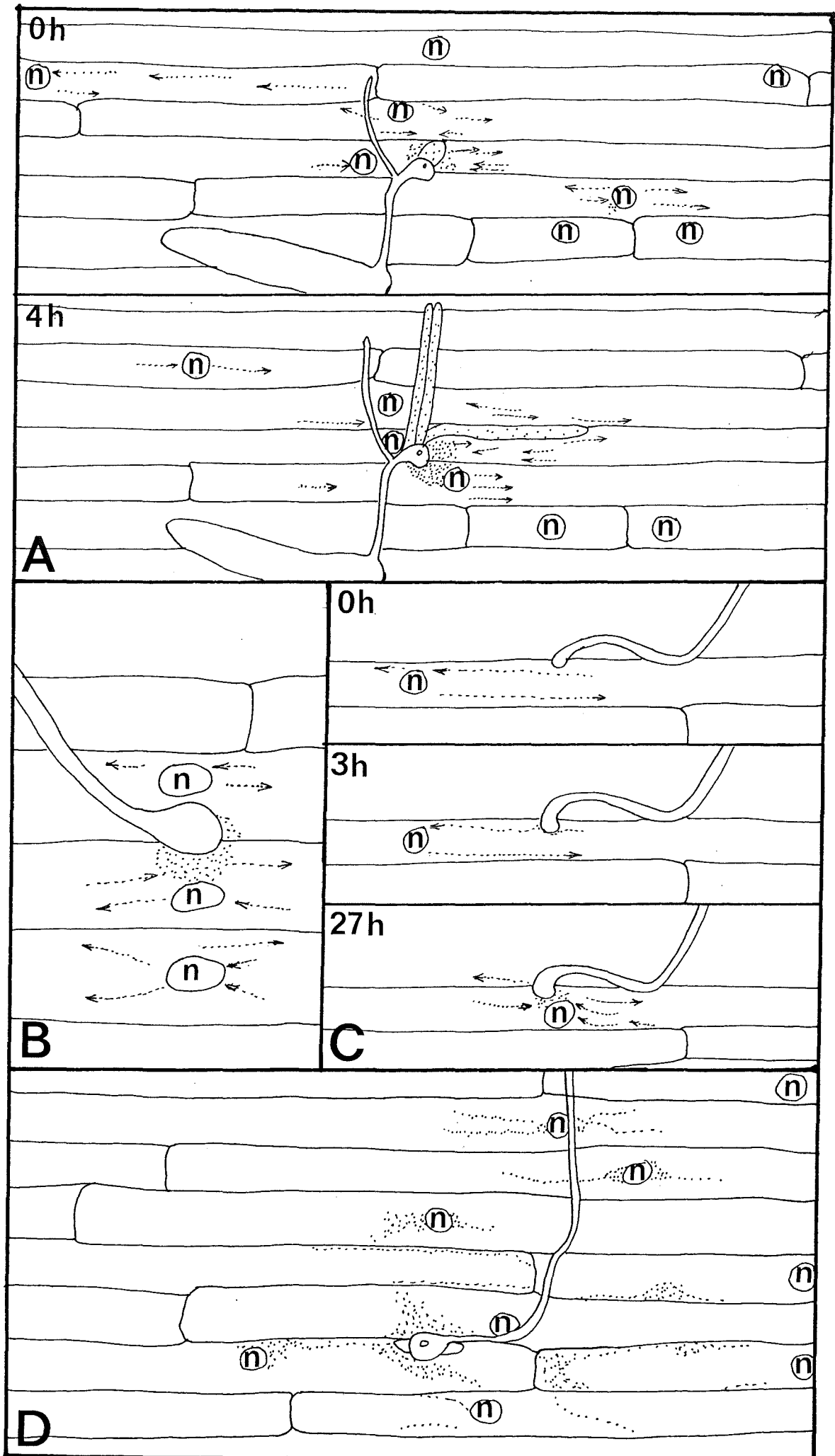


6.2.4 The cytoplasm and nucleus of the epidermal cell

There was a frequent association between host nuclei and appressoria. By following selected appressoria over a period of time, it was possible to determine the changes in cytoplasmic flow and nuclear position in the host in relation to stages in the infection process. The dynamics of this association are illustrated in fig. 6.1. Fig. 6.1:A shows a subcuticular infection just after penetration, and again after four hours when the fungal hyphae had ramified through the subcuticular zone of the host tissues. Over that four hour period, some nuclei had moved closer to the appressorium. Host cytoplasm accumulated beneath the appressorium and patterns of cytoplasmic flow altered, although flow was not unidirectional and cytoplasm continued to flow throughout the cell. These three changes; nuclear position, cytoplasmic accumulation and cytoplasmic streaming, were characteristic of the host reaction to penetration attempts by *D. dictyoides*.

The nucleus seldom began to migrate until the appressorium was fully formed. Figure 6.1:C shows a germ tube just after it has begun to swell into an appressorium. There were two major cytoplasmic strands in this region of the cell. Three hours later the appressorium was recognisable but the host cell showed no reaction. After twenty-seven hours the host nucleus had moved adjacent to the appressorium and the cytoplasmic streaming was more intense leading to an accumulation of cytoplasm beneath the appressorium.

Figure 6.1:D shows a leaf sheath which was fixed in glutaraldehyde, leaving the cytoplasm and nuclei fixed at a time just after penetration of an epidermal cell. Cytoplasm had accumulated around the infection site and the nuclei of host cells some distance away had migrated closer to the



appressorium.

The rate of flow of cytoplasm was assessed in host cells a range of distances from appressoria using a calibrated eyepiece on a Lietz ortholux microscope. Flow rate varied from $3.0 \mu\text{m sec}^{-1}$ to $17.5 \mu\text{m sec}^{-1}$ with no consistent difference detected between cells close to the appressoria and those further away. Nuclear movement was slower, the fastest rate observed in the leaf sheath being $0.2 \mu\text{m min}^{-1}$.

The leaf sheath was used to assess the dynamics of cytoplasmic streaming and nuclear migration, but the tissue most often infected by *D. dictyoides* was the leaf. To assess the effects of *D. dictyoides* on nuclear position in ryegrass leaves, detached leaves were inoculated with the fungus and cleared after incubation. It was possible to distinguish between those appressoria which had not penetrated and those which had produced penetration pegs, but it was not possible to determine the exact time at which nuclear migration occurred. The sequence of events was, however, probably similar to that on the leaf sheath. Figure 6.2 illustrates the frequency of nuclear migration in response to appressorium formation on the leaf. The mean epidermal cell length was $665 \mu\text{m}$, but 83% of host nuclei were found within $25 \mu\text{m}$ of the appressorium where penetration had not taken place. This provides clear evidence that nuclear migration occurred before penetration was complete. Nuclear migration also occurred in cells surrounding the cell beneath the appressorium, and this occurred irrespective of whether that cell had been penetrated or not.

Plate 6.4 summarises the results obtained using leaf sheaths and leaves, in photographic form.

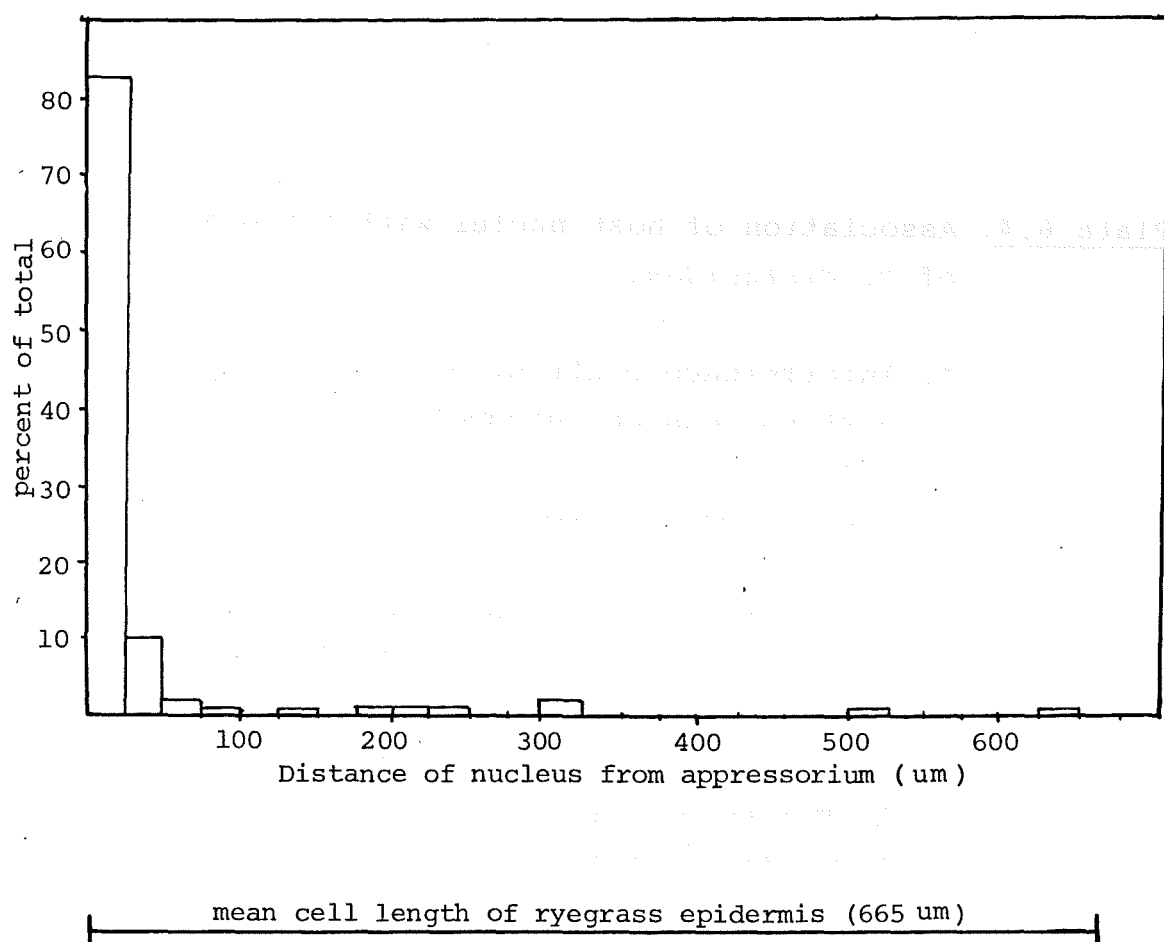
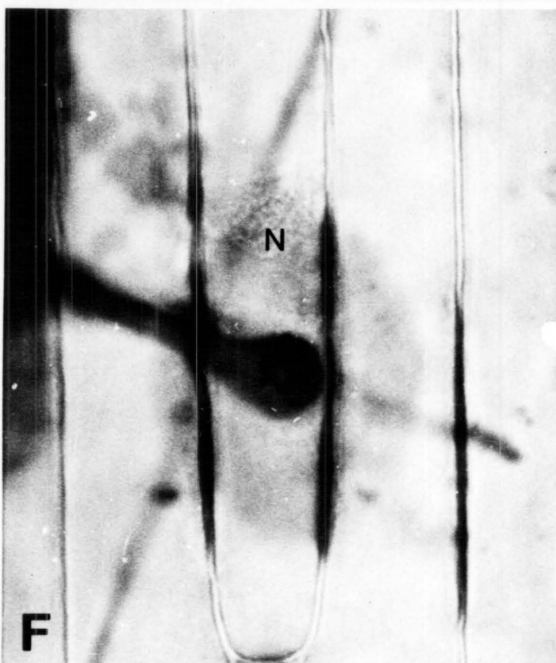
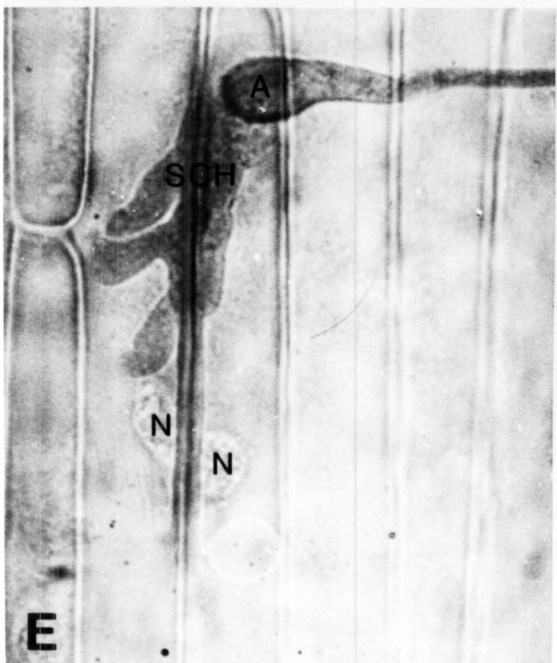
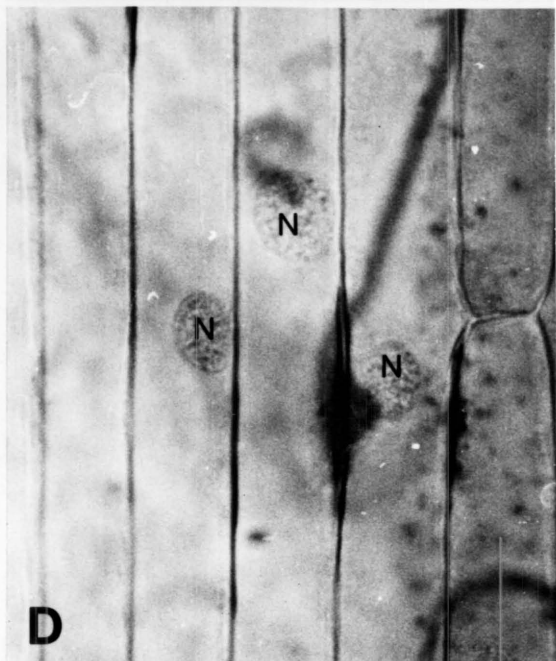
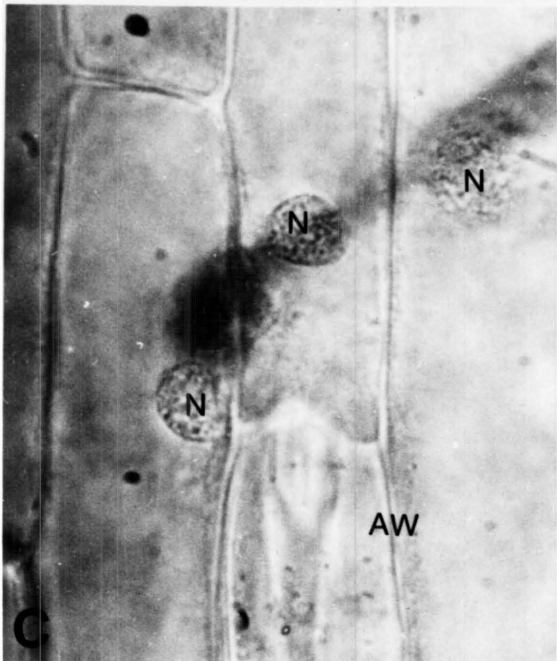
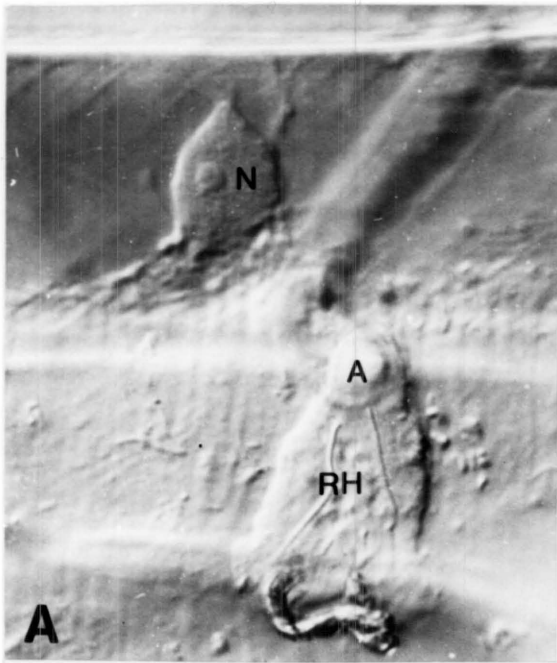


Fig. 6.2. Frequency distribution of host nuclei in unpenetrated cells below a fungal appressorium (twenty-four hours after inoculation of the leaf). Based on 113 measurements.

Plate 6.4. Association of host nuclei with appressoria of *D. dictyoides*.

- A. Interference contrast micrograph showing host nucleus and accumulation of cytoplasm near the appressorium in a cell of the leaf sheath adjacent to an infected cell.
- B. Association of host nucleus and fungal appressorium in the leaf sheath.
- C & D. Association of host nuclei and fungal germ tube and appressorium in an abaxial ryegrass epidermis.
- E. Association of host nuclei with subcuticular hyphae of *D. dictyoides*.
- F. Nucleus and 'halo' surrounding penetration attempt by *D. dictyoides*.



6.3 Discussion

Surface structure provided the greatest difference between the two ryegrass epidermises. Epicuticular wax projections are abundant on the adaxial (upper) leaf surface but absent on the abaxial (lower) leaf surface. In this respect the present study is at variance with the work of Koziol & Cowling (1981) who considered that the opposite was the case. Careful investigation of their photomicrographs leads to the opinion that they were not observing epicuticular wax projections. The amorphous clumps of material are quite unlike the regular wax patterns found in the present study and it seems likely that the epicuticular waxes were dissolved during the severe dehydration with ethanol and acetone.

The relative distribution of conidia on the two ryegrass leaf surfaces is determined by their surface structures. Few conidia were found on the adaxial leaf surface and the glaucous nature of that surface appears to be largely responsible. The possibility of electrostatic repulsion of spore and leaf surface due to like charge as outlined by Campbell *et al.* (1980) was not investigated, but the wax projections are unlikely to provide a suitable surface for adherence and air-borne conidia which land on the leaf surface are probably easily dislodged. The adaxial surface is mostly free of spores, hyphae, bacteria and dirt - a feature of the abaxial surface - and this indicates that the surface is not conducive to particle lodgement. Infection studies usually add a wetting agent, such as 'Tween 80', to the spore suspension to enable infection drops to remain on the leaf. This may negate the importance of waxes.

Those conidia which are present on the adaxial leaf surface must contend with a reduced water supply in comparison with the abaxial surface. The abaxial leaf surface was ob-

served to remain wet longer than the adaxial surface and therefore spores on the adaxial surface may not have moisture for a sufficient period of time to complete the infection process. The epicuticular waxes are unlikely to provide a physical barrier to infection as penetrations were not restricted to areas with diminished densities of wax. Wax density also had no bearing on the positioning of appressoria.

The PA-TCH-AgPr staining method for TEM was used to determine if there were any inherent differences in carbohydrate structure between the two leaf surfaces and also between leaves of lines highly susceptible to net blotch and those of lines showing field resistance. The method depends on the oxidation of 1:2-glycol linkages of certain carbohydrates to aldehydes. Thiocarbohydrazide (TCH) reacts with such aldehyde groups, but at the same time retains its own capacity to reduce silver proteinate. Treatment of the sections with silver proteinate solution releases silver at the sites of the original glycol linkages (Hayat 1975). Therefore if ferulic acid is bound to the cellulose, this reaction will not proceed. Wheat and ryegrass are similar in their phenolic constituents (Harris *et al.* 1980), both having ferulic acid in the epidermal wall. In ryegrass, it is likely that the phenolic constituents are concentrated in the non-TCH reacting region of the wall, although no qualitative difference was observed using light microscopy. Subcuticular penetration is not limited to the TCH staining region of the epidermal cell wall and this suggests that layer three, the non-TCH staining region, does not confer resistance to infection. The outer electron-transparent layer is interpreted as the cuticle-proper, the staining region next to it as the cuticular layer as suggested by Holloway (1971). The third layer is part of the

epidermal cell wall, the explanation for the lack of a staining reaction probably being the presence of ferulic acid masking the cellulose. The inner layer (layer 4) is the cell wall proper and appears to be predominantly cellulosic.

Hartley *et al.* (1978) found that there was a difference in phenolic content of the epidermal walls of the two surfaces in wheat, the greater content in the abaxial epidermis leading to greater resistance of that surface. In ryegrass, the abaxial epidermal wall is approximately twice as thick as the adaxial wall and this provides an explanation for the more rapid penetration of the adaxial surface, although a quantitative difference in phenolic content cannot be ruled out. No difference in wall structure was observed between highly susceptible lines and those showing field resistance and it is unlikely that structural features play a major role in the differential disease reaction seen in the field.

As well as a difference between the abaxial and adaxial leaf surfaces, there was a difference in spore accumulation between line 45 (highly susceptible) and line 72 (field resistant). This is probably more a reflection of the rate of germination than of initial deposition. Ungerminated conidia can wash or blow off the leaf, whilst germinated conidia strongly adhere to the leaf surface. Most ungerminated conidia present on leaf surfaces were 'young' in term of their staining reactions and ungerminated conidia easily become dislodged from the leaf. It is possible that conidia are deposited more easily on line 45 than on line 72 but as there were no detected differences in leaf surface morphology, this is unlikely.

Expressed as a proportion of germinated conidia present, there were no significant differences in appressorium formation or penetration between the two lines. Therefore these results

suggest that there is little difference in resistance to penetration by germinated conidia of *D. dictyoides* between the lines.

In this investigation, host nuclei were found to become associated with appressoria or infection hyphae of *D. dictyoides*. This association appears to be common among host/pathogen interactions (Contreras & Boothroyd 1975, Tomiyama 1956, Pappelis *et al.* 1974), but most previous studies have relied on the observation of fixed tissues. The processes of nuclear migration and cytoplasmic streaming are ones of change in response to stimuli and are best examined in living tissues.

The use of time-lapse techniques in the present study has enabled the demonstration that nuclear migration and changes in the patterns of cytoplasmic streaming do not normally commence until the appressorium is fully formed. It is postulated that nuclear migration does not begin until a penetration attempt is made, but the exact stimulus to nuclear migration is not known. Contreras & Boothroyd (1975) suggested that nuclear movement took place simultaneously with penetration and formation of a primary infection hypha. The present study clearly demonstrates that successful penetration is not a prerequisite of nuclear migration. Several previous workers have suggested that nuclei can migrate as a wound-healing response (Pappelis *et al.* 1974, Benda 1959) but Heale *et al.* (1982) found that nuclear migration in carrot slices was greater in response to a pathogen than to wounding. The current investigation demonstrates that nuclear migration occurs even where penetration does not and it is unlikely that nuclear migration is purely in response to tissue damage.

Tomiyama (1956), using time-lapse techniques, found that migration of the nucleus occurred in the early stages

of penetration and not necessarily after penetration had occurred. He also observed an acceleration of cytoplasmic streaming. The rate of cytoplasmic flow did not increase in response to infection in the present study, but the volume of cytoplasm moving in the region of the appressorium did, resulting in a net accumulation of cytoplasm at the site of attempted penetration. Nuclear migration in response to *D. dictyoides* was markedly slower than the rate of cytoplasmic streaming but was consistent with results found by Vogelmann & Miller (1980) in germinating fern spores.

In the laboratory at 20°C, it was observed that few penetrations occur more than forty-eight hours after inoculation of leaves with a spore suspension. Appressoria which have not produced intracellular infection pegs by this time, seldom succeed in bringing about infection. Rapid nuclear migration may aid, or be an affect of, resistance in these situations. There was no difference between the lines varying in susceptibility to *D. dictyoides* and so the nuclear response is unlikely to be a cause of the differential disease among ryegrass lines in the field.

CHAPTER SEVENAN INVESTIGATION OF SOME FACTORS CONCERNED WITH INFECTION7.1 Introduction

There are innumerable factors of host, pathogen and environmental origin, which are important in the disease cycle. Host factors can determine the level of host resistance while pathogen factors can determine the level of fungal pathogenicity. Environmental factors can modify either the ability of the host to resist infection or of the pathogen to gain entrance to the host.

7.1.1 Host resistance

Disease resistance can be defined as:

' ... the ability of a plant to remain relatively unaffected by a disease because of its inherent genetic and physiological or structural characteristics.'

Roberts & Boothroyd (1972)

Host plants vary in resistance to disease; some plants being severely attacked and some mildly, with many gradations between. Two fundamental forms of resistance occur; vertical resistance where a host is more resistant to some races of a pathogen than to others and horizontal resistance where resistance is evenly spread against all races. Robinson (1976) suggested that:

'... all the evidence indicates quite clearly that... horizontal resistance does indeed occur in every plant, against every parasite, even if, in many cultivars, it currently occurs at an agriculturally inadequate level.'

Horizontal resistance is usually conferred by a large

number of mechanisms, which reduce the rates of host infection, colonisation and reproduction of the pathogens and remains constant and permanent. Vertical resistance, however, is more transient and frequently overcome with the appearance of new races of the pathogen.

Ever since the terms 'horizontal' and 'vertical' resistance were coined, there has been controversy regarding their use. It is not proposed to make any contribution to the controversy here, but merely to clarify the terms used in this study. Kiyosawa (1982) was concerned that, while it is common to equate field resistance, nonspecific resistance, horizontal resistance and minor gene resistance, they are not necessarily the same. He suggested that it was preferable to use the terms 'true' and 'field' resistance to mean high and low levels respectively. In this study, lines with consistently low levels of disease in the field were defined as being 'field resistant'. No attempt was made to assess the reactions to different races of *D. dictyoides*, if in fact such races exist.

The mechanisms by which resistance is manifest are legion and a full review of the topic is beyond the scope of this study. Some studies pertinent to the resistance of hosts to *Drechslera* species will, however, be discussed.

Resistance of non-host grasses to infection by species of *Drechslera* is often manifest by the production of papillae or wall appositions (Sherwood & Vance 1976, 1980). Resistant varieties of host species do not, however, appear to produce these sufficiently frequently to account for the resistance. In the case of *D. turcica* on corn, resistance is expressed only after the fungus gains entry to the host xylem (Hilu & Hooker 1963, 1964). Necrosis is delayed by two to seven

days in resistant tissue and sporulation is delayed and reduced in comparison with susceptible tissue. The differential response of corn tissue resistant or susceptible to *D. maydis* (Smith & Toth 1982) and to *D. carbonus* (Comstock & Scheffer 1973) also occurred only after penetration.

Keeling & Bantarri (1975) found smaller and fewer lesions were produced on barley resistant to *D. teres* than on susceptible tissue and that sporulation was less on resistant tissue. Resistance was expressed after penetration and the sap of resistant tissue was found to be inhibitory to fungal growth. Unlike resistance to *D. teres* barley plants resistant to *D. sorokiniana* had reduced germ tube growth and appressorial formation, fewer and smaller lesions and less sporulation (Mumford 1966).

According to Mishra & Prasad (1964), rice cultivars resistant to *D. oryzae* had a thicker epidermis and cuticle and a greater number of silicated epidermal cells than did susceptible cultivars. More appressoria were produced on susceptible than on resistant tissue (Hau & Rush 1982) and this may have been attributable to the fungitoxic substance produced by the host (Trivedi & Sinha 1978). Induction of resistance was possibly by pre-inoculation with germination fluids of the pathogen (Sinha & Das 1972) and this may have been related to the same fungitoxic substance.

Wilkins (1973) investigated the nature of resistance of *Lolium* to *D. catenaria* (probably = *D. dictyoides*, see p.24 of this thesis) and *D. siccans*. Germ tube growth of *D. catenaria* was measured in sap of resistant and susceptible ryegrass plants. There was no significant difference between the two except where plants had been previously inoculated with *D. catenaria*. Wilkins suggested the possibility of

differential formation of phytoalexins among different genotypes of the host to explain this. Infection of ryegrass with *D. siccans* took place indiscriminately in the glasshouse, although differential resistance was evident in the field.

Resistance of host species to *Drechslera* infection can be manifest either before or after penetration has occurred and there appears to be no common resistance mechanism.

7.1.2 Leaf age

Disease severity of net blotch in the field increases with increasing leaf age, and in the United Kingdom leaf four can have five times more infection than leaf three, the youngest fully expanded leaf (GRI Annual Report 1976, Lam 1981). The situation was the same in the field chapter outlined in chapter five (p.142).

There are two possible explanations for this age-related difference in disease. It may be a reflection of the length of time the leaves have been subjected to the pathogen (old leaves having been exposed to the environment for longer than young leaves) or old leaves may be intrinsically more susceptible than young leaves.

A relationship of leaf age with susceptibility has been reported for leaf infection of *Poa pratensis* with *D. sorokiniana*. Disease development was greatest on the oldest (postmature) leaf followed by the youngest (premature) leaf, with low levels of disease on the two intermediate (mature) leaves (Nilsen & Hodges 1980). A direct relationship was found between leaf senescence and pathogenesis. The use of auxin-like herbicides increased severity of symptoms, suggesting an enhancement of the senescent-induced disease inter-

action (Hodges 1980).

Resistance to disease increases with leaf age in the case of *Coccomyces hiemalis* on Montmorency cherry (Eisensmith *et al.* 1982). In that case, penetration was via stomata, unlike the direct penetration of *L. perenne* by *D. dictyoides*. No suggestion was made of the mechanism of the reduction in infection. The longevity of cherry leaves, which normally live until autumn, is greater than grass leaves. Any senescence induced susceptibility of cherry leaves would not occur until the autumn.

Spedding & Diekmahns (1972) gave a resumé of leaf senescence in grasses, some points of which are outlined below. The number of leaves on a tiller appears to be constant under constant conditions, so leaf senescence must occur at the same rate as leaf production. Until fully emerged, a leaf imports assimilates as well as retaining all it produces, but once mature, assimilates are translocated to expanding aerial parts and increasingly to the root system. Compounds containing nitrogen and phosphorous are withdrawn from the leaf and utilised elsewhere.

Hunt (1965) recorded the appearance of new leaves of *L. multiflorum* every six to eight days in summer, implying that leaf one can become leaf four in as few as eighteen days.

A laboratory study was undertaken to determine if there is a differential susceptibility to disease according to the age of ryegrass leaves.

7.1.3 The *Lolium* endophyte

It has long been known that *L. perenne* frequently carries an endophytic fungus. McLennan (1920) concluded that

all plants of the species carried the endophyte, which has been found in seeds, leaf sheaths, leaves, flowering stems and seeds (Sampson 1935, 1937, Neill 1940, 1941). Fletcher & Harvey (1981) recently established an association between the endophyte and ryegrass staggers in sheep, while Gallagher *et al.* (1981) found plants from staggers-producing pastures to contain two potent neurotoxins likely to be responsible for the symptoms. More recently, the *Lolium* endophyte has been shown to be associated with ryegrass resistance to Argentine stem weevil (Annual report PHDS, Lincoln, NZ, 1981 - 82).

The present study was initiated to assess the presence of the *Lolium* endophyte in ryegrass plants showing field resistance or susceptibility to *D. dictyoides*. In the course of the ultrastructural study in chapter four, the endophyte was found to be present within a leaf lesion caused by *D. dictyoides*. This provided a unique opportunity to assess the association and relationship, if any, between the two fungi *in vivo*.

7.1.4 Effects of temperature on spore germination and leaf penetration.

Many fungal spores germinate over a wide temperature range, often from just over freezing point to 30°C and the optimum for germination may differ from the optimum for vegetative growth (Tarr 1972). Hawker (1950) noted two phases of germination, probably a physical one (for instance intake of water) and a chemical one (involving enzyme action), which could have different temperature optima.

Hodges (1972) found conidia of *D. sorokiniana* germinated at a faster rate at 22°C than at 10°C, but above 22°C, the percent germination declined. Bidari & Govindu (1976) found

the maximum germination of conidia of the same fungus occurred at temperatures of 25⁰C to 35⁰C, according to isolate. The difference in optimum temperature between the two authors may be a reflection of the adaptation of the fungus to different climates. Rate of conidium germination can be more important than the ability of a conidium to germinate eventually. Favourable environmental conditions for germination may not continue indefinitely as, for instance, with the evaporation of a drop of dew rendering the moisture level insufficient for germination.

Temperature can also be important in infection. Secondary infection of barley with *D. teres* is reported to occur at 8⁰C to 33⁰C, with an optimum temperature of around 25⁰C (Singh 1963). The situation of *Colletotrichum graminicola* on barley appears to be more complex. Skoropad (1967) found that appressoria were formed at 15⁰C to 35⁰C, but penetration could occur only at 25⁰C to 30⁰C. Appressoria which formed at 15⁰C to 20⁰C remained dormant until temperatures of 25⁰C to 30⁰C were reached.

7.2 Results

7.2.1 Differential host reaction

To assess the expression of field resistance in the laboratory six ryegrass lines, showing either a consistently high or consistently low level of disease in the field trial, were inoculated with *D. dictyoides* and the infection process followed. The lines used were lines 16, 45 and 125 (heavily diseased in the field trial) and 72, 76 and 104 (relatively lightly diseased in the field trial).

In four experiments, detached leaves of *L. perenne* were inoculated with *D. dictyoides* and after an incubation period cleared and examined microscopically to assess spore germination, appressorium formation and penetration of the leaf. There was no difference in germination between the lines and seldom any difference in appressorium production, therefore only the results of leaf penetrations are given in table 7.1.

Table 7.1. Penetration by *D. dictyoides* of *L. perenne* lines showing high or low levels of field resistance.

Line	mean penetrations per germinated conidium				
	expt 1 (4 reps) 24 h	expt 2 (3 reps) 24h 48h		expt 3 (4 reps) 36 h	expt 4 (9 reps) 24 h
16 (S) #	0.33	0.16	0.30	0.86	0.47
45 (S)	0.48	0.07	0.34	0.57	0.51
72 (R)	0.36	0.01	0.14	0.06	0.37
76 (R)	0.40	0.01	0.39	0.03	0.09
104 (R)	0.33	0.00	0.05	0.03	0.14
125 (S)	0.66	0.50	1.11	0.48	0.66
L.S.D. (5%)	0.21	0.18	0.25	0.15	0.23

(S) = susceptible, (R) = resistant

The results were not consistent between experiments and for this reason are treated separately. In experiment 1 line 125 had the greatest number of penetrations and this was significantly greater than two of the three field resistant lines. In experiment 2, there were few penetrations in any lines other than line 125 after 24 hours but after 48 hours the three highly susceptible lines had been penetrated more often than two of the three field resistant lines. Experiment 3 gave a clear distinction between the field resistant lines and the susceptible lines, while in experiment 4 the highly susceptible lines were penetrated significantly more often than two of the three resistant lines. Papillae and wall appositions were seldom observed and did not appear to be any more common on the field resistant lines than on the susceptible lines. While differences were not consistent overall, there was some distinction between the set of field resistant lines and the set of susceptible lines. Caution must be taken, however, in the interpretation of results because of the variability between experiments.

To compare lesion development on the same six ryegrass lines, detached leaves were inoculated with a dilute spore suspension ($5,000$ spores ml^{-1}) of *D. dictyoides*. Eight isolates of *D. dictyoides* were used in an attempt to cover the pathogenic range of the fungus and three replicate leaves were inoculated with each isolate. Host reaction was assessed according to a scale of 1 - 4 (see p.13) after four days and degree of sporulation on the leaves was assessed after seven days again on a scale of 0 - 4 (see p.13). Results are presented in table 7.2. Mean reactions are listed for each isolate but results for all twenty-four leaves per ryegrass line were combined for statistical analysis.

Table 7.2. Development of lesions and sporulation of eight isolates of *D. dictyoides* after inoculation onto the abaxial leaf surfaces of six lines of *L. perenne* varying in susceptibility to disease.

		Mean host reaction after four days*								mean
		<i>D. dictyoides</i> isolate								
		1	2	3	4	5	6	7	8	
16 (S) #		1.3	2.7	3.0	1.7	2.7	4.0	4.0	3.7	3.0
45 (S)		2.3	2.3	3.0	3.0	2.0	2.0	3.0	3.0	2.4
72 (R)		1.3	0.7	1.3	1.0	2.0	3.0	2.0	3.3	1.9
76 (R)		2.3	1.7	1.3	1.0	1.0	2.0	2.3	2.3	1.8
104 (R)		0.7	1.0	1.7	1.3	1.0	2.0	2.0	2.7	1.5
125 (S)		1.7	1.7	2.0	2.3	1.7	2.7	2.3	3.0	2.2
									L.S.D.	0.7
		Mean degree of sporulation after seven days**								mean
		<i>D. dictyoides</i> isolate								
		1	2	3	4	5	6	7	8	
16 (S)		0.3	1.7	1.0	1.3	1.0	2.0	1.0	1.3	1.2
45 (S)		0.7	0.7	1.7	1.0	0.3	1.3	1.3	3.0	1.3
72 (R)		0.3	0.7	0.7	0.7	0.3	0.3	1.0	1.3	0.7
76 (R)		2.3	1.7	0.3	1.7	0.0	1.3	1.7	2.0	1.4
104 (R)		0.3	0.3	0.3	0.3	0.0	0.7	0.7	1.7	0.5
125 (S)		1.0	1.7	1.0	1.7	1.3	1.7	1.0	1.7	1.3
									L.S.D.	0.6

* lesion development assessed on a scale of 0 - 4 (see p.13)

** sporulation assessed on a scale of 0 - 4 (see p.13)

(S) = susceptible, (R) = field resistant

While results varied between fungal isolates, there was a general pattern of a slightly more moderate host reaction from the three lines showing field resistance in comparison with those showing a high degree of susceptibility in the field. Overall, line 16, a highly susceptible line, showed significantly more disease than the three field resistant

lines (72, 76 and 104). Line 45 showed a significantly greater disease reaction than two of the field resistant lines.

Two of the three field resistant lines showed significantly less sporulation than the highly susceptible lines. One field resistant line (line 76), however, showed a greater degree of sporulation than the three highly susceptible lines.

When whole plants of the same six lines, were sprayed with *D. dictyoides* and incubated under high humidity, more lesions of greater severity were produced on line 45, but no differences were observed among the other lines. The results were particularly variable.

7.2.2 Leaf age

To assess whether older leaves were inherently more susceptible to *D. dictyoides* infection than young leaves, leaves two, three (the youngest fully expanded leaf) and four, were inoculated with a spore suspension. The infection process was divided into three stages: prepenetration to penetration, lesion development and sporulation.

Three experiments were conducted with four, six and eight replicates respectively to assess the effect of leaf age on initial infection. Results were consistent between experiments and only the combined results are given (table 7.3). There were no significant differences in percent germination, germ tube formation, appressorium formation or penetration of the leaf. The slightly lower level of appressorium formation and penetration on leaf three was well within the natural variation of the experiment.

During lesion development the differential effect of leaf age first became evident. In experiments where development

Table 7.3. Effect of leaf age on spore germination, appressoria formation and penetration of the abaxial epidermis of the leaf of *L. perenne* by *D. dictyoides* (18 reps).

Leaf	germination	number per germinated conidium		
	(%)	germ tubes	appressoria	penetrations
2	94	1.20	0.82	0.33
3	93	1.23	0.71	0.24
4	98	1.17	0.86	0.35
L.S.D. (5%)	11	0.25	0.29	0.16

of visual lesions was followed according to leaf age, there were consistent increases in lesion severity with increasing leaf age. Four days after inoculation with 10 μ l of a 5,000 spores ml^{-1} suspension of *D. dictyoides* conidia, small discrete net blotch lesions were produced on leaf two with, at most, limited chlorosis. Each lesion was restricted to the area of the inoculation drop and was made up of tiny fleck lesions at the sites of penetration. On leaf three, lesions were more severe but still largely restricted to the area of the inoculum drop, although some chlorosis was often present surrounding the lesion. On leaf four, necrosis extended well past the bounds of the inoculum drop, the remainder of the leaf being chlorotic.

Sporulation appears to be largely a reflection of the induced senescence of the leaf due to disease. During the course of this study sporulation was only observed where leaves were in advanced stages of senescence with widespread chlorosis. In two of the experiments where visual development of lesions was followed, the subsequent sporulation was assessed seven days after inoculation. Results are given in table 7.4. There were consistent differences between leaves

Table 7.4. Sporulation of *D. dictyoides* on detached leaves of *L. perenne* according to leaf age seven days after inoculation of the abaxial leaf surface.

Leaf	Sporulation [#]		
	Experiment 1 (6 reps.)	Experiment 2 (10 reps.)	Mean of combined results (16 reps)
2	1.2	0.1	0.5
3	3.2	0.4	1.5
4	3.3	2.4	2.7
L.S.D. (5%)	1.2	0.7	0.8

[#] based on a rating scale of 0 - 4 (see p. 13).

two and four, although results for leaf three varied between experiments. The terms leaf 'two', 'three' and 'four' do not refer to absolute leaf ages - leaf three remains the youngest fully expanded leaf from the time it first becomes fully expanded until another leaf expands fully. The rate of production of new leaves will also affect the actual age of these leaves.

Leaf senescence is artificially delayed when leaves are floated on a solution of kinetin. This method was used to further investigate the effect of senescence on lesion development and sporulation. Leaves with natural lesions were collected and grouped into pairs; each pair comprising leaves of a similar age, with the same extent of lesion development. One leaf was floated on distilled water, while the other was floated on an aqueous solution of kinetin ($0.2 \mu\text{g ml}^{-1}$). Development of lesions was followed. Small lesions on young leaves developed at a similar rate on water and kinetin, with little chlorosis of the leaf. Lesions on older leaves floated on water became chlorotic three to four days earlier than did those floated on kinetin and hence

sporulation also commenced earlier on leaves on water.

In nature, there was an evident link between the rate of lesion development and the rate of appearance of new leaves. In July 1982, when lesions on young leaves were tagged at the initial fleck stage and followed to maturity, lesion development took approximately twenty days. During that time, two new leaves had frequently emerged and so the fully mature lesion was present on leaf four. In October 1982 lesions developed more quickly, often reaching maturity after as few as seven days. The rate of appearance of new leaves however, was also faster. For this reason, lesions capable of sporulating if given suitable environmental conditions, were never found on leaf two and only on the 'withered tips' of leaf three, the tips representing the oldest portion of the leaf. The situation on fertile tillers was a little different due to a greater possible longevity of the leaves but sporulation still occurred most frequently on the older leaves.

7.2.3 *Lolium* endophyte

Presence or absence of the *Lolium* endophyte was assessed in three highly susceptible ryegrass lines (lines 16, 45 and 125) and in three lines showing field resistance (lines 72, 76 and 104). The endophyte was found to be present in all lines, although only a trace was found in line 125.

The endophytic fungus described ultrastructurally by Fineran *et al.* (1983) was found in one mature net blotch lesion on *L. perenne* (line 45). It was closely adpressed to a host mesophyll cell and contained the crystalloid inclusions and aggregated tubules characteristic of the fungus in some ryegrass tissues. Of particular interest was the close association with *D. dictyoides* in the lesion (plate 7.1).

Plate 7.1. Association of the *Lolium* endophyte
with *D. dictyoides* within a leaf
lesion.

A: Association of the ryegrass host, *D. dictyoides*
and the *Lolium* endophyte within a leaf
lesion just prior to sporulation by
D. dictyoides. Bar = 1 μ m

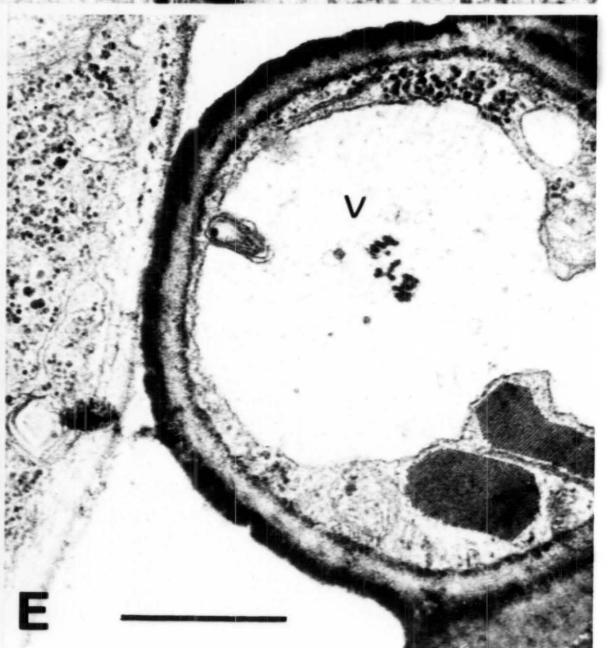
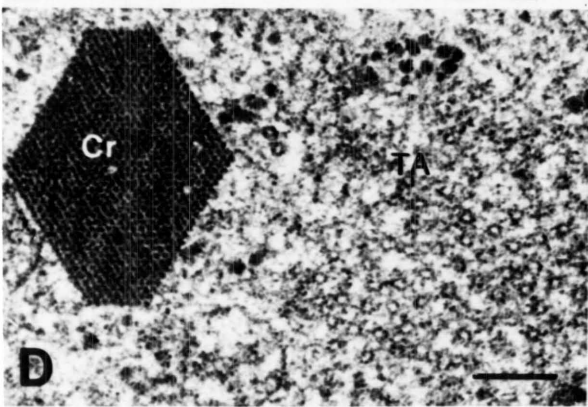
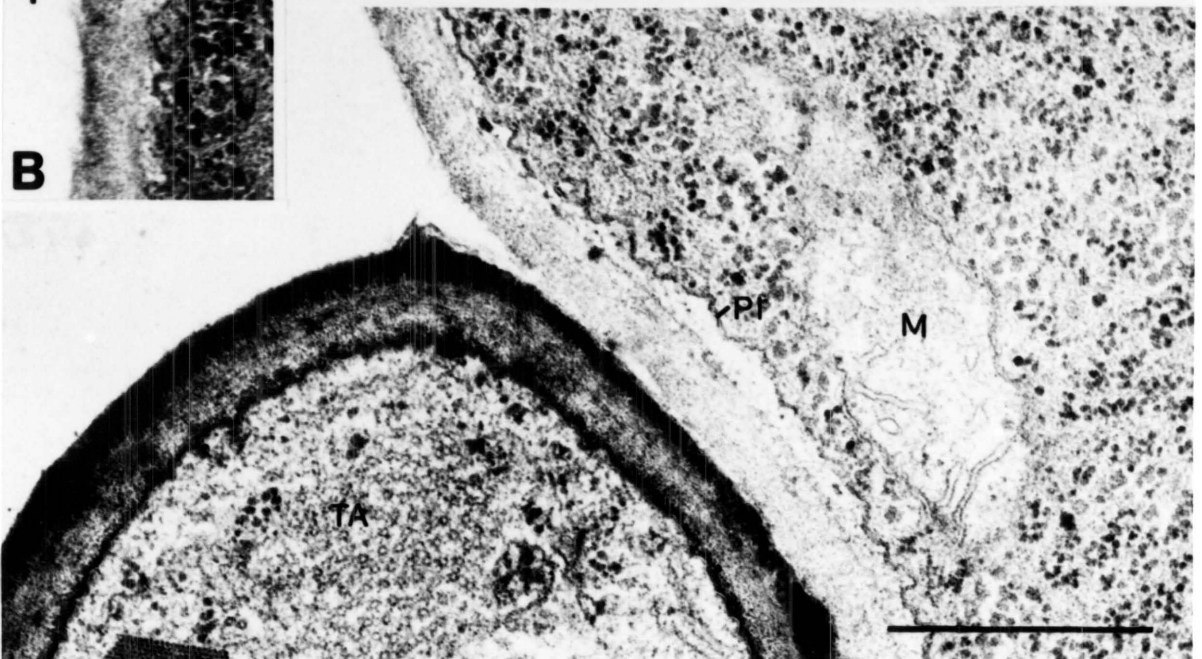
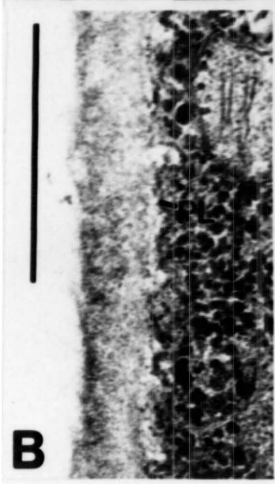
B: Detail of *D. dictyoides* wall opposite the
site of contact with the endophyte. Bar = 1 μ m

C: Contact between the *Lolium* endophyte (below)
and *D. dictyoides*, showing crystalloid
inclusions and tubular aggregate within the
endophytic hypha. Bar = 0.5 μ m

D: Detail of crystalloid inclusions and
tubular aggregate within the endophytic
hypha. Bar = 0.1 μ m

E: Endophytic hypha with large vacuole and
crystalloid inclusions in contact with
D. dictyoides. Bar = 0.5 μ m

Abbreviations: Cr - crystalloid inclusion, M -
mitochondrion, Mb - microbody, PL - plasma-
lemma, TA - tubular aggregate.



There was little apparent reaction by either fungus, although the cell wall of *D. dictyoides* was depressed inwards at the region of contact with the endophyte. The two fungal walls were closely adpressed, but without any observable structural modification to either wall. The endophytic cell wall was more electron-dense than that of *D. dictyoides*. The plasmalemma of *D. dictyoides* was slightly separated from the cell wall in the region of contact with the endophyte, but maintained its integrity. Microbodies were particularly common in *D. dictyoides* in this region. Some areas of the endophyte hypha were highly vacuolate.

7.2.4 Effects of temperature

Germination of two isolates of *D. dictyoides* on glass at temperatures of -5°C to 40°C in the dark, was assessed at intervals up to twenty-four hours. Results are presented in fig. 7.1 and indicate that germination is possible over a wide temperature range. There was an early increase in germination at 15°C , but by six hours, germination was optimal at 20°C . Germination was close to 100% at 20°C after sixteen hours and by twenty-four hours, germination was complete at temperatures of 10°C to 30°C . Over 50% germination had occurred at 5°C and 35°C while limited germination had also occurred at 0°C . Germination did not take place at -5°C or 40°C . By following percent germination at various time intervals, there was no need to measure germ tube length, which is a mixture of the rates of germination and germ tube growth. As the hyphal growth optimum may differ from the optimum for germination, a measure purely of germination is of more value.

To assess the effect of temperature on the penetration

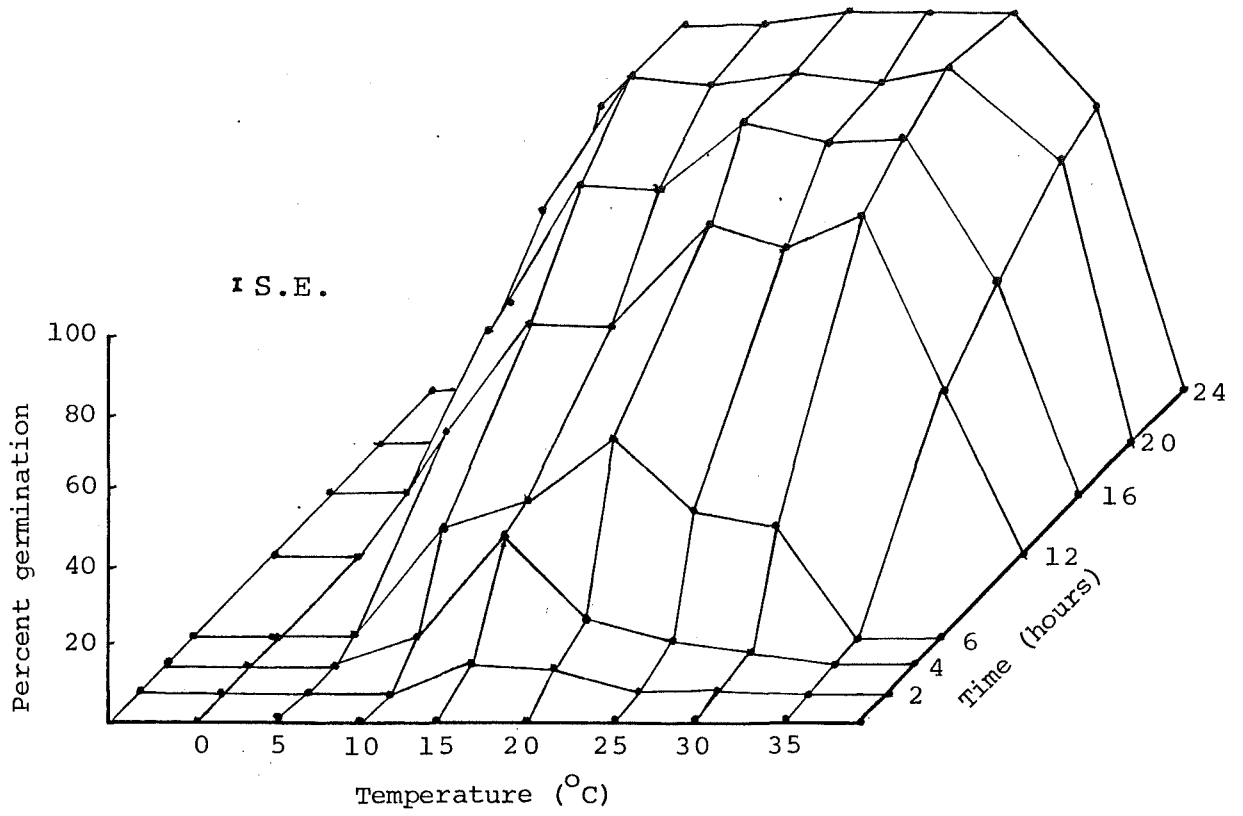


Fig. 7.1. Effect of temperature on spore germination of *D. dictyoides*.

process, detached leaves were inoculated with two isolates of *D. dictyoides* (three replicates per isolate) and incubated at 10°C, 20°C and 30°C in the dark for 24 hours. Results were consistent between isolates and therefore were combined for analysis (table 7.5). After twenty-four hours, germination

Table 7.5. Effect of temperature on spore germination, appressorium formation and penetration of the abaxial leaf surface of *L. perenne* by *D. dictyoides* twenty-four hours after inoculation. (6 reps.).

	germination (%)	number per germinated conidium		
		germ tubes	appressoria	penetrations
10°C	89	1.20	0.72	0.03
20°C	97	1.29	1.14	0.30
30°C	87	1.27	0.93	0.11
L.S.D. (5%)	30	0.36	0.27	0.17

was largely complete at all temperatures, as indicated also by germination on glass. Germ tube production was similar at the different temperatures. More appressoria had formed and more penetrations occurred at the optimum temperature of 20°C than at the other two temperatures. The major effect of temperature appeared to be on the rate at which the infection process proceeded. After twenty-four hours at 20°C, the penetration process was nearing completion with 0.30 penetrations per germinated conidium. At 30°C, appressorium formation was nearly complete (0.93 per germinated conidium) but penetrations were in progress (0.11 per germinated conidium). At 10°C, appressorium formation was still incomplete (0.72 per germinated conidium) and the process of penetration was being initiated.

7.3 Discussion

Field resistance of ryegrass to *D. dictyoides* appears to be expressed in terms of a subtle reduction in infection, disease development and possibly sporulation. Caution must, however, be taken in the use of laboratory results to explain a field phenomenon. None of the field resistant lines showed a defined resistant response such as hypersensitivity or papilla production. Resistance probably relies on the operation of a large number of genes, each of minor effect. Field results indicate that adequate resistance is available to *D. dictyoides* naturally.

The results of laboratory inoculations indicate that there is little reduction in spore germination on resistant tissue and this is in agreement with the findings of Hilu & Hooker (1963), Smith & Toth (1982) and Comstock & Scheffer (1973), using other species of *Drechslera*. Penetrations were sometimes reduced on resistant lines in comparison with susceptible lines, but results were not consistent and should be treated with caution. Differences in spore distribution and infection in the field do not indicate a reduction in infection from germinated conidia on resistant lines (chapter six, p.163). Many previous studies have found resistance to species of *Drechslera* to occur after penetration (Hilu & Hooker 1963, Smith & Toth 1982, Comstock & Scheffer 1973) but other studies have found the opposite (Mumford 1966, Mishra & Prasad 1964, Hau & Rush 1982). There is no reason to suppose that there is a common mechanism in grasses and cereals for resistance to species of *Drechslera* and several different mechanisms may operate in the various lines of a single host species.

Papilla production or hypersensitivity has not been reported in the response of host species to *Drechslera*

infection although papillae are commonly produced by non-host grasses in response to *Drechslera* species (Sherwood & Vance 1976 1980). Likewise, in the present study, papillae were only rarely produced and there was no correlation with field resistance.

The observed increase in disease severity with leaf age is partly attributable to an increase in susceptibility to *D. dictyoides*. Wheeler (1977) suggested that breaks in the cuticle of maize were responsible for infection with *D. maydis*, but this is unlikely to explain the increase in susceptibility of ryegrass with leaf age as there was no difference in number of penetrations of leaves of different ages.

Once penetration has occurred, lesion development and hence also sporulation, is more rapid in older leaves. This supports the hypothesis of Hodges (1980) that there is a senescence-induced increase in susceptibility to disease with increasing leaf age. Infection with *Drechslera* species has been found to increase peroxidase activity (Birecka *et al.* 1975, Vance *et al.* 1976). Thomas & Stoddart (1980) suggested that since this increase is similar to the natural increase during senescence in leaves, that control of senescence may be co-ordinated by the fungus. Lloyd (1980) found that sucrose was translocated rapidly from mature leaves of *L. temulentum*. It is possible that old leaves have less available soluble sugars and that this induces the production of cell wall-degrading enzymes by the fungus which, in turn, leads to a more marked host reaction to invasion.

On young leaves inoculated in the laboratory, lesion development is restricted to the region of inoculation while necrosis and chlorosis spread more rapidly in older leaves.

Sporulation can only occur on senescent tissue, probably after the supply of soluble sugars is largely exhausted and the rapid senescence of old leaves allows earlier sporulation. Where leaves with lesions were floated on kinetin, rate of necrotic lesion development was only slightly slower than in leaves floated on water. In other words, intercellular spread of the fungus was not greatly affected. What kinetin did was to delay the disease-induced senescence of the leaf. As senescence was delayed, so too was fungal sporulation. This provides further evidence for the interaction of the pathogen and senescence of the host leaves.

The explanation for the observation in the field that disease is usually more severe on older leaves, is complex.

Three factors are involved:

- 1) the time a leaf has been subjected to fungal propagules (usually the time since leaf emergence)
- 2) the rate of leaf production and hence the rate of leaf senescence
- 3) the differential susceptibility of leaves of different ages.

The end result is a combination of the above factors. There are also two components to the differential severity of disease according to leaf age. One is the total diseased leaf area while the other is the maturity of individual lesions on leaves of different ages. In the field trial only total diseased leaf area was measured. Mature lesions are seldom found on young leaves as a leaf two, for instance, is unlikely to remain a leaf two for long enough for a lesion to reach maturity. There is a close parallel between the rate of lesion development and the rate of the appearance of new leaves and it is difficult to extract the relative importance of each factor in disease.

Detailed information on the changes in the rate of leaf production along with changes in the rate of lesion development and availability of fungal inoculum would be required to assess the exact contribution of each of the three factors to differential disease levels according to leaf age. The work of Hunt (1965) is useful as an indication of leaf production and senescence in ryegrass. The rate of production and the total number of green leaves present are determined by numerous environmental and other factors specific to each population of plants and must be determined for each investigation.

The investigation of the *Lolium* endophyte in relation to net blotch clearly demonstrates that the endophyte has no role in the disease. The presence of the *Lolium* endophyte in all ryegrass lines assessed, whether field resistant or highly susceptible, indicates that there is no qualitative effect of the endophyte on development of net blotch lesions. The close association of the two fungal species within a single lesion implies that the endophyte is neither toxic nor antagonistic to *D. dictyoides*, at least within a leaf lesion. In addition, both species are able to derive sufficient nutrients within the lesion and *D. dictyoides* was able to sporulate from the lesion. Only one lesion was found with the endophyte present in this close association so the endophyte is unlikely to be required for successful infection by *D. dictyoides*.

Conidia of *D. dictyoides* can germinate over a wide range of temperatures; from about 5°C to around 35°C. Germination is most rapid at 10°C to 30°C, with an optimum

temperature of about 20°C. This temperature is similar to that found for *D. sorokiniana* in the United States (Hodges 1973) but considerably lower than that found in India (Bidari & Govindu 1976). Regional ecotypes of plant pathogenic fungi are probably present and allow maximum use of the temperatures encountered. Temperature optima of Indian isolates can be as high as 35°C but as temperatures in New Zealand seldom reach those levels, such isolates would be selected against here.

There appears to be no difference in the temperature optima of appressoria production and penetration by *D. dictyoides* on ryegrass unlike the situation of *Colletotrichum graminicola* on barley (Skoropad 1967). The optimum for spore germination is slightly lower than that for growth on agar media (p. 46) and this has been recorded among other fungi (Tarr 1972). The optimum temperature for penetration is also close to 20°C, although penetrations do occur at temperatures of 10°C and 30°C. The rate of penetration is slower at 10°C, but at that temperature surface moisture may remain for a sufficient time for the infection process to be completed. At 30°C, the infection process is again slow and evaporation of surface moisture may prevent infection in the field at this temperature.

CHAPTER EIGHTCONCLUDING DISCUSSION

New Zealand pastures are dominated by ryegrass and white clover (Langer 1973). Several diseases have been recorded on ryegrass, the two most important being crown rust (*Puccinia coronata*) and net blotch (caused by *Drechslera dictyoides*). A small scale study of the taxonomy and distribution of the various species of *Drechslera* on ryegrasses, especially perennial ryegrass, was undertaken to reassess their relative importance in the light of suggestions of past mis-identifications (Scharif 1963, Morrison 1980, Lam 1981).

Three species of *Drechslera*, *D. dictyoides*, *D. siccans* and *D. nobleae* were found on leaf lesions on ryegrass, *D. Dictyoides* being responsible for almost all the *Drechslera* lesions on perennial ryegrass. Typically, *D. dictyoides* forms a net or net blotch lesion on ryegrass but the 'net' symptom may become obscured when multiple lesions are present on the same leaf. The net lesion is formed by a network of longitudinal and transverse linear necrotic streaks, representing the presence of fungal hyphae nearby. Frequently, in the later stages of disease development, the necrotic lesion is surrounded by chlorosis, forming the 'blotch'. Net or net blotch lesions were invariably caused by *D. dictyoides* and this lesion type was diagnostic of the species. The reverse was not true as *D. dictyoides* could also be isolated from spot lesions or from withered tips of leaves.

The three species of *Drechslera* produced fructifications which were easily distinguishable under the stereomicroscope and it was seldom necessary to resort to other means of

identification. Confirmation of identification could be made by a slide germination test. If the majority of conidia produced were tapered and germ tubes were produced from the apical cell and the second cell from the base, the species was *D. nobleae*. Tapered conidia germinating from the basal and apical cells were of *D. dictyoides* while straight conidia were of *D. siccans*.

The predominance of *D. dictyoides* on perennial ryegrass is in agreement with previous work in New Zealand (Latch 1966) and it is unlikely that *D. nobleae* will become a problem on either perennial or Italian ryegrass. Scharif (1963) and Lam (1981) suggested that *D. dictyoides* had, on occasions, been misidentified as *D. catenaria* or *D. siccans*. This may have led to reports indicating the latter two species were important on perennial ryegrass. *D. catenaria* was never isolated from ryegrass in this study and *D. siccans* was not common on perennial ryegrass.

The host ranges of the three *Drechslera* species appear to be largely restricted to the ryegrasses, although *D. siccans* is able to infect oats. The inability of *D. siccans* to sporulate on oats suggests that the species is not synonymous with *D. avenae* as suggested by Ibrahim & Threlfall (1966).

D. dictyoides accounted for over ninety percent of *Drechslera* lesions on *L. perenne* in a survey of Hagley Park, Christchurch. The disease is endemic on ryegrass in New Zealand and can usually be found wherever ryegrass is growing. Fungal inoculum is always present and disease can spread rapidly when conditions are suitable. Infection from seed is unlikely to be of significance in the epidemiology of the disease. Incidence of the disease is high but severity is usually low in terms of the diseased leaf area.

While heavy infection with crown rust reduces the palatability of ryegrass to sheep (Cruickshank 1957), there are no such reports for net blotch-infected ryegrass. Therefore, the economic importance of the disease relies on yield loss; loss in dry weight of leaves during vegetative growth which reduces the availability of food to stock and during seed production when disease can reduced the quantity and quality of seed.

Ryegrass is an outbreeding crop, hence cultivars which may be phenotypically uniform, usually possess wide genotypic diversity (Carr 1975). This is valuable in presenting a wide variation in resistance to pathogens. Cultivars are now being bred with an increasingly restricted genetic base and this presents the possibility of future disease problems similar to those caused on corn by *D. maydis* race-T and on 'Victoria' oats by *D. victoriae*.

A field trial was set up with two principle aims. These were: to record changes in disease levels with time and attempt to related them to environmental changes and to assess the differential host reaction to disease. Twenty-one ryegrass lines, all originating from a single long-term pasture, were selected and disease assessed at intervals. During much of the trial period, Canterbury experienced a drought and in addition the trial site was exposed to winds and leaves of the spaced ryegrass plants seldom remained wet for long. For these reasons, disease never reached the levels experienced in other, more sheltered regions. Even with the generally low levels of disease experienced, there was a marked differential host reaction. The majority of lines showed considerable resistance to disease in comparison with the few heavily diseased lines. Disease assessments in Christchurch give an indication of the levels of disease

possible where moisture is sufficient. Disease levels in the field trial increased after rain, but results were not conclusive as the trial was conducted only over a sixteen month period.

To understand the disease situation it is necessary to obtain information on and the interaction of pathogen, host and environment. This study set out to examine some of these factors.

The environment can affect the pathogen directly or via its affect on the host. The environment affects the pathogen between sporulation and infection. For instance, the infection process is optimal at around 20°C. Moisture is necessary for sporulation and infection and during a drought period this can be a limiting factor. This field was not investigated in depth and could be worthy of further work. The effect of the environment on the pathogen via the host can be more subtle. Any factor which can effect the growth or physiological state of the ryegrass plants can have an effect on disease. For instance severity of net blotch increases with nitrogen application (Lam & Lewis 1982).

The terms 'facultative' and 'obligate' parasitism and the terms 'biotroph' and necrotroph' should perhaps be re-examined in the light of results presented. Facultative parasites are often equated with necrotrophs, which grow only on dead cells of the host plant (Tarr 1972). Obligate parasites are usually considered to be highly specialised organisms which live biotrophically and are usually unable to grow in pure culture. *D. dictyoides* is a facultative parasite in that it can be easily cultured on many laboratory growth media.

Hosts and their parasites have co-evolved over a

long period of time (Nelson 1978). The fact that the vast majority of pathogens are extremely host specific suggests a precise interaction between host and parasite. The gross necrotrophic form of parasitism where tissue is killed in advance of hyphae is probably limited to such non-specific pathogens as *Botrytis cinerea* or those, such as *D. maydis* race-T or *D. victoriae*, which produce host-specific phytotoxins. *D. dictyoides* is not a 'necrotrophic' pathogen and the term itself is of limited value. Biotrophs and necrotrophs are but two extremes of a continuum and most pathogens lie somewhere between.

Current knowledge of nutrient flow within the host and senescence of host tissue provide an explanation for the ability of the fungus to grow intercellularly with little immediate reaction from the host. Intercellular pathogens appear to be able to gain sufficient nutrients without the necessity to penetrate the host cells (Hancock & Huisman 1981). This reduces both the required energy input by the fungus and the possibility of a defensive host reaction. In the late stages of lesion development, *D. dictyoides* becomes intracellular to a limited extent and the necessary synthesis of wall-degrading enzymes may be triggered by the reduction in nutrient flow due to host senescence.

The origin and function of the various layers of the fungal wall observed in the TEM are still not clearly understood and because of this they are of limited value in the classification of fungi. Madelin's hypothesis concerning the plasticity of the outer wall layer (p.66) was useful in explaining changes during development of *D. dictyoides*. The outer layer of the wall appears to provide protection to structures such as conidiophores and conidia which may

remain exposed to the external environment for some time. These outer layers apparently become split (and possibly also lysed) to allow the emergence of a 'juvenile' hypha. Where close interaction with the host is required - for instance during penetration, the outer wall layer is frequently absent.

There is still controversy regarding the stimuli or conducive conditions inducing a germ tube to cease apical growth and swell to form an appressorium (Emmett & Parbery 1975). Germ tubes of *D. dictyoides* can be 'long' or 'short' according to variations in the environment (although they are usually short in nature). In addition they are almost invariably formed over the longitudinal junction of epidermal cells. This constancy suggests that the factor(s) inducing appressorium formation specifically are also constant and probably host orientated.

The host epidermis forms the first line of defence, usually preventing infection of non-host species by pathogens. Its contribution to the reduction or prevention of infection of hosts by pathogens may also be important. The leaf surface itself may provide a susceptible host with a disease escape mechanism. The striking difference in concentration of conidia of *D. dictyoides* between the two surfaces of the *L. perenne* leaf is a case in point. As few as two percent of the conidia present on the leaf as a whole are found on the adaxial surface. This is clearly related to the difference in epicuticular waxes. The adaxial leaf surface has a dense cover of wax platelets, while these are absent on the abaxial surface. The adaxial leaf surface is more susceptible to penetration than the abaxial surface and so this difference in waxes provides the leaf with some disease escape.

There were no qualitative differences in epidermal

structure, between highly susceptible and field resistant lines, observed. In spite of this, there were consistent differences in spore accumulation. Fewer spores germinated on the field resistant leaves and so the displacement of ungerminated conidia may have been responsible for the observed differences in spore accumulation.

There are problems inherent in the assessment of field resistance in the laboratory. Where specific resistance structures such as papillae or reactions such as hypersensitivity are not present, results can be inconclusive. Nelson (1978) discussed the 'co-evolution and co-existence of host and parasite in natural ecological niches'. It was suggested that genetic changes in the parasitic population may result in subtle improvements in fitness attributes, for instance to reduce the latent period by a day or two. Alternatively changes in the host may extend the latent period by a day or two. Natural populations of plants and their pathogens would, therefore 'have co-evolved to co-existence by reaching a genetic equilibrium based on the combined effects of many genes in each system.'

In ryegrass, there are probably many genes contributing to field resistance, each contributing a slight reduction in disease and compensating for the virulence genes in the pathogen. In the laboratory, pathogens are provided with an ideal environment for infection and differences between lines may not become apparent. Field results indicate that ryegrass lines are available with adequate levels of field resistance to net blotch and disease resistance should be considered in the production of new cultivars.

The disease cycle of net blotch on ryegrass is a continuous process. Longfellow, in 'The Potter's Song' said:

Turn, turn my wheel, All life is brief;
What now is bud will soon be leaf,
What now is leaf will soon decay ...

The life histories of host and pathogen have evolved together and function inseparably in the disease cycle. The fungus has evolved to take advantage of the continuing cycle of life and death of the host and needs to expend only minimal energy in the provision of senescent material on which to sporulate. The host is continuously producing new leaves which must be penetrated to carry on the disease cycle.

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APPENDIX ONE: Media used in experimental work.

Malt Extract Agar (MEA)

20g 'Difco' malt extract
 20g Agar
 1 l distilled water

Sucrose Proline Agar (SPA) (see Shoemaker 1962)

6.0g sucrose
 2.7g L-proline
 1.3g K_2HPO_4
 1.0g KH_2PO_4
 0.5g KCL
 0.5g $MgSO_4$
 10mg $FeSO_4 \cdot 7H_2O$
 2 mg $ZnSO_4 \cdot 7H_2O$
 1.6mg $MnCl_2 \cdot H_2O$
 20g Agar
 1 l distilled water

Tap Water Agar (TWA)

1 l tap water
 12g agar

Potato Dextrose Agar (PDA)

'Difco' PDA used

Coons Agar (CA)

0.25g asparagine
 3.5g maltose
 0.5g $MgSO_4$
 1.25g KH_2PO_4
 12g agar
 1 l distilled water

 V_8 -juice Agar (V_8 A) (Miller 1955)

200ml 'Campbell's' V_8 -juice
 3.0g $CaCO_3$
 800ml distilled water
 15g agar

All media autoclaved for 20 minutes at 103 kPa.

APPENDIX TWO: Use of 'Tween 80' and detached leaves.1. 'Tween 80'

'Tween 80' was used as a surfactant in spore suspensions both to produce an even distribution of spores and to enable inoculation drops to remain on the leaf surface. To assess the effect of addition of 'Tween 80' to a spore suspension, a spore suspension ($35,000$ conidia ml^{-1}) was made up and $0.5 \mu\text{l ml}^{-1}$ 'Tween 80' was added to half. Germination and average germ tube number were assessed on glass as was germination on the abaxial leaf surface. Results are given below.

	percent germination on glass	percent germination on leaf	Average no. germ tubes per germin- ated conidium.
+ 'Tween 80'	99	99	1.9
- 'Tween 80'	98	99	1.8

There was no difference in spore germination or germ tube production whether 'Tween 80' was added or not.

2. Detached leaves

Detached leaves placed on a solution of Kinetin of benzimidazole to stop senescence are frequently used to assess disease development and the presence of physiological races (Hooker & Yarwood 1966, Sherwood 1957, Ward 1959). The method saves time and space and allows for increased replication. Two experiments were conducted to assess the validity of the use of detached leaves. In the first, *D. dictyoides* was grown on PDA with or without kinetin added at the rate of 0.02 g l^{-1} . Linear spread of mycelium was measured after five days and found to be 15% slower where kinetin was added. No morphological differences were noted and it is unlikely that placement of leaves on a solution of kinetin has any major effect on *D. dictyoides* on the leaf surface. In the other experiment, infection of detached and attached leaves was compared and there was no difference up to four days after inoculation. Therefore it was concluded that detached leaves are of use, at least in the early stages of infection.

APPENDIX THREE: Disease on spaced ryegrass plants in Christchurch.

Plants of perennial ryegrass lines 16, 45, 76, 104 and 125, were planted outdoors in Christchurch. They were spaced 0.5 m apart, but were not replicated or randomised.

Assessment One:

Percent diseased leaf area on each leaf for ten randomly chosen tillers, was assessed on 8/8/82. Results are presented below.

	mean percent diseased leaf area for 10 tillers			
	leaf 2	leaf 3	leaf 4	
line 16	0.3 a,a*	3.3 b,a	18.2 b,b	8.8 b
line 45	0.2 a,a	15.0 c,b	42.0 c,c	19.1 b
line 76	0.1 a,a	0.4 a,a	1.1 a,a	0.5 a
line 104	0.0 a,a	0.2 a,a	3.7 a,b	1.3 a
line 125	0.0 a,a	0.2 a,a	5.5 a,b	1.9 a

*means for different lines (down a,) or for different aged leaves (across ,a) followed by the same letter are not significantly different ($P = 0.05$) Duncans multiple range test.

Assessment Two:

Percent diseased leaf area on each leaf for twenty-five randomly chosen tillers, was assessed on 14/11/82 at the commencement of flowering (Feeke's growth stage 10.5). Leaf numbering was redefined for this assessment as: flag leaf = leaf one, next youngest = leaf two, next leaf = leaf three. Results are presented below.

	Incidence of net blotch(% leaves infected)				
	16	45	76	104	125
percent tillers inf.	92	100	20	44	48
% leaf 1 infected	40	96	4	9	28
% leaf 2 infected	76	100	16	24	40
% leaf 3 infected	95	100	14	40	44

	Severity of net blotch (% dis. leaf area)				
leaf one	4.3 b*	21.4 c	0.1 a	0.2 a	2.4 ab
leaf two	14.7 b	35.0 c	0.7 a	2.0 a	2.7 a
leaf three	38.4 c	27.5 [#] c	1.7 a	3.7 ab	8.0 b

*means for different lines followed by the same letter are not significantly different ($P = 0.05$) Duncans MRT.

most leaves dead and therefore not assessed.

RESULTS

		% reduction in elongation over control			
		ryegrass		clover	
		1:10	1:100	1:10	1:100
25 ^o C	0.1 % Y.E.	85	31	50	0
"	1.0 % Y.E.	46	31	50	17
20 ^o C	0.1 % Y.E.	54	23	50	17
"	1.0 % Y.E.	54	54	50	13
21 ^o C	continuous light	76	0	50	44

The results show that a substance toxic to ryegrass was produced by culture filtrates of *D. dictyoides*. The effect was less marked on clover roots so the toxic substance shows some specificity. At a dilution of only 1:100, there was less than a 50% reduction in root elongation. Filtrates showed little toxicity when compared with that of the previously recorded host-specific phytotoxins and is unlikely to play a major role in lesion expression.

ERRATA

p	l		p	l		p	l		
6	1	Zealnad	read	Zealand	201	15	<i>D.Dictyoides</i>	read	<i>D.dictyoides</i>
7	29	distinguised	""	distinguished	204	17	effect	""	affect
11	6	tween	""	Tween	205	13	provide	""	provides
16	6	students	""	Student's	209	11	assisstance	""	assistance
18	11	glutaldehyde	""	glutaraldehyde	209	18	assisstance	""	assistance
23	17	Drehsler	""	Drechsler	210	43	omega	""	Omega
61	23	satisfactorally	""	satisfactorily	211	32	941	""	942
64	6	stiking	""	striking	213	25	29	""	25
66	6	electon	""	electron	213	25	305	""	306
76	1	mitochodria	""	mitochondria	213	38	structural	""	structure
83	19	dissapeared	""	disappeared	216	16	272	""	273
99	6	the	""	in	218	35	notrogen	""	nitrogen
118	4	coniophore	""	conidiophore	218	36	<i>Drechsera</i>	""	<i>Drechslera</i>
123	4	experimantal	""	experimental	218	43	of	""	in
123	16	genotypicaaly	""	genotypically	219	45	299	""	301
145	13	on	""	in	228	11	KCL	""	KCl
170	4	Lietz	""	Leitz	228	14	ZnSo ₄	""	ZnSO ₄
197	26	availabe	""	available	231	21	Whatmann	""	Whatman

SPECIES AUTHORITIES

Botrytis cinerea Pers.ex Pers.
Cladosporium cucumerinum Ell. & Arth.
Colletotrichum graminicola (Ces.) Wilson
Drechslera avenae (Eidam) Sharif
D.graminea (Rabenh. ex Schlect.) Shoemaker
D.maydis (Nisikado) Subram. & Jain
D.oryzae (Breda de Haan) Subram & Jain
D.poeae (Baudys) Shoemaker
D.sorokiniana (Sacc.) Subram & Jain
D.teres (Sacc.) Shoemaker
D.turcica (pass.) Subram & Jain
D.victoriae (Meehan & Mumphy) Subram & Jain
Phytophthora infestans (Mont.) de Bary
Puccinia coronata Corda
P.graminis Pers.
Pyrenophora dictyoides Paul & Parberry
P.teres Drechsler
Rynchosporium orthosporium Caldwell
Sclerotinia fructigena (Winter) Rehm
Stemphyllium botryosum Wallroth
Ulocladium atrum Preuss
Verticillium albo-atrum Reinke & Berthold

Agrostis palustris Huds.
Anthoxanthum odoratum L.
Arrhenatherum elatius (L.) Beauv. ex J. & C. Presl.
Bromus mollis L.
Cinna arundinacea L.
Cynosurus cristatus L.
Dactylis glomerata L.
Festuca arundinacea Schreb.
F.pratensis Huds.
F.rubra L.
Holcus lanatus L.
Hordeum vulgare L.
Phleum pratense L.
Triticum aestivum L.
Vulpia myurus L.