HISTOPATHOLOGY OF WHEAT INFECTION

by

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DECLARATION

This thesis contains no material which has been previously submitted for a degree to any other University. The work described in this thesis, except where otherwise acknowledged, was carried out by myself during the period of my candidature.

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The aim of this study was to explain, in histological terms, the differential levels of susceptibility of a group of wheat cultivars (Triticum aestivum L.) to two economically important pathogens - viz. Mycosphaerella graminicola Fckl. (Septoria tritici Rob. apud Desm.) and Leptosphaeria nodorum Mull. (Septoria nodorum Berk.). These two pathogens are the causal agents of the diseases known as 'speckled leaf blotch' and 'glume blotch' respectively.

The four wheat cultivars were affected by the two pathogens to different extents. The susceptibility of the four cultivars differed as shown by the relative extent of foliar symptoms, pycnidia and pycnidiospores produced per unit area, and by the sporulation index of both pathogens. The design of the experiments did not allow direct comparisons between the two pathogens.

Both pathogens were found to elicit similar symptoms on infected leaves. At the end of the incubation period small chlorotic flecks appeared which gradually enlarged and coalesced to form necrotic lesions. The pycnidia of both pathogens were shown to be produced in the substomatal cavities of necrotic tissue.

The pre- and post-penetration events observed with both pathogens were photographically recorded and the life cycles of both pathogens were documented.

It is thought unlikely that histological defence reactions play any role in determining the level of susceptibility of the wheat cultivars.
used to either of the two pathogens. In contrast, barriers of a histological nature were identified in response to attempted infection by two non-pathogens of wheat. The success of the host in countering attempted infections by the non-pathogens in contrast to the lack of success with the pathogens is discussed.
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INTRODUCTION

Since the acceptance of the fact that plant diseases are mainly due to the activities of parasitic micro-organisms much research and effort has gone into the study of the role of the physical and biochemical defence mechanisms operating in plants against pathogens. From such research some commonly accepted concepts have evolved. All plants are not infected by all micro-organisms. Some degree of specificity therefore exists in that, of the many spores or propagules of micro-organisms landing on a plant surface, very few initiate disease. In many cases the dispersal propagule will germinate on the plant surface and attempt entry. In the vast majority of cases the process of infection will either be arrested at the primary step, i.e. at the stage of penetration of the host cuticle and epidermal cell wall, or at some subsequent stage in the attempted colonisation of the host.

The failure of a micro-organism, in this case a fungus, to initiate and successfully complete an infection may be due to characteristics of the host which are either present prior to infection or develop after infection. These characteristics or defence reactions may be either physical or biochemical in nature. For example, disease resistance has been related to the physical resistance of the outer epidermal cell wall to needle puncture in Berberis attacked by Puccinia graminis (Melander and Craigie, 1927), in flax attacked by Melampsora lini (Sharvelle, 1936), and in potato tubers attacked by Pythium debaryanum (Hawkins and Harvey, 1919), and to cuticular differences in strawberry attacked by Sphaerotheca macularia (Peries, 1962) and also in various plants attacked by Botrytis cinerea (Louis, 1963).
Apart from pre-infectional barriers, further defence mechanisms are also initiated in response to infection (Lazarovits and Higgins, 1975a). Histological defence mechanisms which are of the post-infectional type operate mainly by isolating the invading fungus from readily available nutrient sources. These can, in many instances, be very effective means of defence as fungal spores only have a limited supply of endogenous nutrients and require further supplies to continue the processes of penetration and colonisation. Histological or 'mechanical' barriers formed in response to infection are only effective against those pathogens which do not have the necessary enzymes to degrade and thus breach them. The growth of necrotrophic pathogens, that kill the cells of host plants first and then utilize them as a nutrient source, can be arrested or reduced if the cells of the host can rapidly initiate and complete the formation of some sort of physical barrier to colonisation. This may also be thought of as a barrier to nutrient supply.

In the case of biotrophic pathogens i.e. those pathogens that obtain nutrients from living host cells, it is in the interests of the host to initiate responses which will rapidly kill the cells in the immediate area of infection. Such a system will ensure that the nutrient source is removed and the pathogen starved, but would not arrest the rate of colonisation by a necrotrophic pathogen which would, in the long term, benefit from the production of localised areas of dead host tissues.

Largely as a result of the pioneering work of Müller and Börger (1940) on phytoalexins, studies of histological defence mechanisms in plants fell into decline. Phytoalexins are host-produced antifungal...
compounds which constitute an important category of biochemical resistance mechanisms. Studies on such compounds and other biochemical defence mechanisms has stimulated much research on the physiological and biochemical areas of plant-pathogen interactions. In many cases histological and biochemical defence mechanisms probably occur in combination such that a plant subjected to attack by a potential pathogen will possess an overall defence reaction. Following the availability of better light microscopes and improved methods of fixation, dehydration and staining, interest in the histological bases of host defence has been revived. It has been reviewed in detail by Akai (1959).

Two important diseases of wheat (*Triticum aestivum* L.) are 'speckled leaf blotch' and 'glume blotch'. These are due to two septorioid fungi —*Mycosphaerella graminicola* Fckl. (*Septoria tritici* Rob. apud Desm.) and *Leptosphaeria nodorum* Mull. (*Septoria nodorum* Berk.) respectively. Both pathogens are causing ever increasing concern in all areas of the world where wheat is grown (C.M.I., 1966a,b), and recent high yield losses have stimulated increased research into their epidemiology and control (Shipton et al., 1971). Field studies have shown that both pathogens are capable of causing large yield losses in years of heavy infection (Brönnimann, 1968a,b; Jenkins and Morgan, 1969; Brönnimann et al., 1972; Scharen and Krupinsky, 1973; Saari and Wilcoxson, 1974).

The symptoms of both diseases are chlorotic and necrotic lesions on all aerial parts of the host (C.M.I., 1966a,b). Both pathogens are splash dispersed, and recent studies have shown that severe grain losses do not always occur in years in which infection is limited to the early stages of host development (Jones and Odebumni, 1971) — i.e. at growth stages 1 to 3 on the Feekes' Scale (Large, 1954). If infection periods
recurr during the growth of the crop then tillering and subsequent yield is reduced. If the flag leaves or inflorescences of the host are infected (Feekes' Scale 8+) then serious reductions in the 1000 grain weight are likely (Bromann, 1968a,b; Jenkins and Morgan, 1969; Shipton et al., 1971; Bromann et al., 1972; Scharen and Krupinsky, 1973; Saari and Wilcoxson, 1974).

No cultivars of *T. aestivum* immune to either of the pathogens are known. In many regions of the world much effort is being directed towards quantifying the resistance of existing cultivars to one or both of the pathogens (Krupinsky et al., 1977). Only rarely do breeding programmes take into account resistance to *M. graminicola* and *L. nodorum* with a view to increasing the degree of resistance which occurs to some extent in a few of the currently used wheat cultivars. The relative scarcity of resistant cultivars may be due, at least in part, to the fact that for many years wheat has been bred for resistance to powdery mildew (*Erysiphe graminis f.sp. tritici*) and to rust (*Puccinia graminis f.sp. tritici*) and to other biotrophic pathogens. The resistance which rust or mildew resistant cultivars frequently exhibit takes the form of a hypersensitive reaction in which host cells immediately surrounding the site of infection, rapidly die. Such a reaction is very effective against biotrophic pathogens but will not itself arrest the growth of necrotrophic pathogens such as *M. graminicola* or *L. nodorum*. Indeed, such a reaction may be advantageous to the pathogen by providing an area of host tissue which rapidly dies, has no active resistance and is a potential nutrient source.

Various studies have shown that the two septorioid pathogens are epidemiologically very similar. Both are able to survive and sporulate on a range of alternative graminaceous hosts (Harrower, 1977a), but this
aspect of their epidemiology is not thought to be of great significance in Australia. A more important source of inoculum is that derived from soil debris and trash (Harrower, 1974, 1975). In Australia straw after deheading at harvest, is left standing in pastures as animal fodder, a practice that also assists in protecting the soil from wind erosion. However, due to the increasing importance of both pathogens it has been suggested that the ploughing in of infected stubble, burning it, or disposing of it by some other means may benefit the following crop by removing an important source of inoculum. Sexual reproduction is also known to occur on stubble which therefore also acts as a potential source of recombinant pathogen genotypes. It is now realised that effective breeding programmes must be established and some attempts along these lines are now in progress. It is important to understand the mechanisms whereby cultivars are resistant, so that the nature of resistance and its occurrence in breeding material can be identified.

There are several reports of a correlation between the level of resistance of a cultivar and the lengths of the incubation and latent periods of the pathogens on these cultivars (Brokenshire, 1973; Harrower, 1974, 1975, 1976a). Lately, it has also been shown that the rate of mycelial growth of *M. graminicola* in wheat cultivars can be correlated with the level of resistance of the cultivars to produce a ranking order of susceptibility which agrees with that produced by more conventional methods of assessment such as the rate of appearance of foliar symptoms (Harrower, 1977b).

Two explanations are possible for the observed differences in susceptibility of wheat cultivars to either *M. graminicola* or *L. nodorum*. Firstly, biochemical defence reactions in the cultivars may arrest or
retard the growth of the pathogen to different extents. Secondly, structural changes may occur which might hinder the growth of colonizing hyphae of the pathogens to varying degrees. Such histological phenomena may not occur in all cultivars or they may occur at different levels of intensity and, hence effectiveness. Histological differences between the cultivars, whether they be pre- or post-infectional will affect the rate of colonisation of the tissues and will, therefore, be manifest by a different rate of appearance of foliar symptoms.

Preliminary studies of the interaction of wheat with the two septorioid pathogens were carried out to elucidate the details of infection and the life cycles of the pathogens. Initially a very susceptible cultivar, Robin, was used. Further studies used three wheat cultivars which are commonly grown in Eastern Australia, viz. Summit, Pinnacle and Teal. Fleche d'Or, a relatively new cultivar, known to exhibit a fairly high level of resistance to both pathogens (Kuiper, pers. comm.) was also used for comparative purposes. The latter four cultivars were examined for resistance to both pathogens and a ranking order of susceptibility was constructed. Attempts were made to correlate these results with pre-infectional and post-infectional histological changes observed by microscopy.

Studies revealed that both pathogens were extremely destructive. It was then postulated that the observed scarcity of histological changes might have been due to the rapidity with which host cells and tissue functions were disrupted by the pathogens. Consequently, two non-pathogens were also used as inocula, viz. Epicoccum nigrum Link, a common wind dispersed saprophytic fungus and Ascochyta pisi Lib. a pea (Pisum sativum L.) pathogen with a similar life style and epidemiological profile to
the two septorioid pathogens used (C.M.I. 1972).
MATERIALS AND METHODS

(a) **Source of experimental plants.**

Seeds of *T. aestivum* cultivars Robin, Summit, Pinnacle, Teal and Fleche d'Or were obtained from the New South Wales Department of Agriculture branch at Wagga Wagga. Four to eight seeds per pot were sown in normal potting compost and, two to three weeks after emergence of the coleoptile, when plants were at the third to fourth leaf stage, the seedlings were used for experimental purposes.

(b) **Sources and maintenance of fungal isolates.**

*M. graminicola* was isolated from naturally infected wheat plants (cv. Robin) growing at Wagga Wagga NSW. *L. nodorum* was isolated from naturally infected wheat plants growing at Perth WA. Cultures of *A. pisi* were kindly provided by Dr. I.A.M. Cruickshank of the C.S.I.R.O. Division of Plant Industry in Canberra. *E. nigrum* was isolated from the air spora in Canberra during February 1977 and also during January 1978.

*L. nodorum* and *A. pisi* were maintained on Czapek Dox/V8 juice agar (CZV8) at 25°C under constant ultra-violet illumination provided by two Philips 40W/08TL light tubes suspended 60cm above the cultures to promote sporulation. This technique was based on the work of Cooke and Jones (1970). *M. graminicola* was maintained on CZV8 in the dark at temperature of 15°C to promote production of secondary conidia (Lee and Jones, 1974). *E. nigrum* was grown on Potato Dextrose agar (PDA) at 20°C in the dark.
Cultures to be used as inoculum sources were prepared by flooding the entire surface of the culture medium with a spore suspension of the fungus to be grown. Thus, pycnidia, masses of secondary conidia or clusters of conidiospores, depending on the fungus, were generated uniformly over the medium to provide spores of a more uniform age than those which would have been derived from cultures which had been inoculated centrally.

The wheat pathogens, i.e. *L. nodorum* and *M. graminicola*, have been reported (Harrower, pers. comm.) to lose their aggressiveness when they have been grown for several successive asexual generations in culture. For this reason they were re-isolated from artificially infected wheat plants and fresh isolates prepared at intervals throughout the duration of the study. During the study no reduction in aggressiveness of the two pathogens towards wheat was observed.

(c) Production of inocula.

*L. nodorum* and *A. pisi* produced abundant pycnidia in culture and the inocula consisted solely of pycnidiospores. *M. graminicola* did not produce pycnidia in culture and the inoculum consisted solely of secondary conidia which multiplied by budding (Cooke and Jones, 1970). Whenever pycnidiospores of *M. graminicola* were required, artificially infected wheat leaves bearing pycnidia were removed, surface sterilised by a brief immersion in 70% ethanol, allowed to dry and then transferred to a watch glass containing sterile distilled water into which the pycnidiospores were exuded. The sexual or ascigerous stages of the two pathogens was not produced in culture.
(d) **Inoculation techniques.**

Spore suspensions of both pathogens and also of the two non-pathogens were usually prepared from one or two week old cultures prepared as described in (c). Spore suspensions were adjusted to $10^6$ or $10^5$ spores/ml using a haemocytometer. To each 100ml of inoculum a drop of Tween 20 was added to assist in the wetting of leaves and the even dispersal of the inoculum on them. Plants were sprayed with an atomizer until run-off. Control plants were sprayed with sterile distilled water containing Tween 20. After inoculation plants were covered with a clear polythene bag for three to four days to maintain a humid environment which is necessary for spore germination and germ tube growth. The plants were grown in a controlled environment cabinet adjusted to provide a 14 hour light period at 25°C alternating with a 10 hour dark period at 20°C (Schild and Harrower, 1978).

Some variation in the method of inoculation had to be made during the study for specific purposes. Pycnidiospores of *M. graminicola*, due to their relative scarcity, were painted onto leaves with a sable hair brush. Also, in experiments in which excised leaf pieces were used, pycnidiospores of *L. nodorum* and *A. pisi*, the secondary conidia of *M. graminicola* and the conidia of *E. nigrum* were inoculated onto leaf portions by dipping those portions into a spore suspension ($10^5$ spores/ml) of the test fungus to which a drop of Tween 20 per 100ml of inoculum had been added. The control (leaf portions dipped in sterile distilled water with Tween 20) and the inoculated leaf portions were then floated on sterile solution of 40 ppm benzimidazole in 1% glucose contained in petri dishes at room temperature (Samborski et al, 1958). These dishes were illuminated by white fluorescent light tubes.
Epidermal strips or pieces from the leaf sheath were removed from healthy wheat leaves and inoculated by dipping them into spore suspensions of the test fungus adjusted to $10^5$ spores/ml$^{-1}$. The epidermal strips were then placed on cover slips and inverted over van Tieghem rings which had previously been affixed to microscope slides with vaseline. The cavity of each culture chamber so prepared also contained a block of water agar which served to keep the epidermal strip in contact with the cover slip and also helped to maintain a humid atmosphere within the chamber (Skipp and Deverall, 1972).

To study the location of seed borne inoculum of both pathogens, wheat plants were inoculated at different stages of heading. Inoculation was carried out at the following growth stages (Feeskes scale, after Large, 1954) (Plate I 1): 10.1 (head just emerging), 10.5 (head emerged and at anthesis) and at growth stage 11 (early ripening stage - grain still soft). The grains were harvested when ripe, and after being sectioned and stained, compared to healthy, uninoculated, sectioned and stained grain.

(e) **Assessment of foliar symptoms.**

To sort the four cultivars into a ranking order of susceptibility both *M. graminicola* and *L. nodorum* were individually inoculated on to the four wheat cultivars as described earlier. Four plants per pot with five replicates for each cultivar were used.

After various intervals the percentage area of leaves bearing symptoms after inoculation, was visually assessed using a 'Septora Foliar Key' (Plate I 2) which was suitable for both species (James, 1971). Chlorosis and necrosis were assessed together, no attempt being made to
differentiate between the two. Readings were taken for leaf 1, 2 and 3.

Following angular transformation, the percentage leaf area infected was subjected to analysis of variance as for a completely randomised design. This complete analysis was performed only for data collected 9 and 20 days after inoculation.

(f) Measurement of the incubation period.

Inoculated plants were observed daily and compared to uninoculated healthy plants. The incubation period was defined as that period (days) between inoculation and the first visible symptoms. The number of plants and the method used for inoculation was the same as described in (e). The experiment was repeated twice and the mean of the two experiments and the standard deviation was calculated.

(g) Measurement of the latent period.

The same plants as described in (f) were used, and the mean and standard deviation was calculated from two experiments. The time (days) required after inoculation for the production of mature pycnidia was measured by daily inspection of the plants. The maturity of pycnidia was assessed by soaking a portion of an attached leaf, bearing pycnidia, in a drop of water on a microscope slide. The leaf area in contact with the water droplet was lightly tapped. If the pycnidia were mature then pycnidiospores which had exuded from them into the water drop could be seen on subsequent microscopic examination.

(h) Measurement of the sporulation index.
The term 'sporulation index' is used to refer to the extent of pycnidiospore production per unit area of necrotic tissue. Five days after the end of the latent period of the most resistant cultivar i.e. Fleche d'Or, portions of the fourth leaf of each plant were excised. The terminal six centimeters of the fourth leaf of each plant were removed and discarded. The length and breadth of the next two centimeters was measured prior to placing the leaf tissue in 5.0ml of 0.01% thiomersal solution to arrest pycnidiospore germination. The leaf portions were soaked for 30 min., removed and washed off with a further 1.0ml of fresh washing solution. The spore concentration in each sample was then counted with a haemocytometer and the mean of four such readings for each cultivar/pathogen combination was related to the sample with the least pycnidiospores to obtain the sporulation index.

(i) Measurement of pycnidial density.

The leaf portions which were used to assess the sporulation index were also used to determine the number of pycnidia per unit area of necrotic leaf tissue. This was done by decolourizing and mounting the leaf pieces on microscope slides in lactophenol– cotton blue and counting the number of pycnidia on them. Mature pycnidia were identified by the presence of an ostiole and dark pigmented walls.

Statistical analysis for (h) and (i) i.e. pycnidiospores per cm$^2$ leaf portion, mature pycnidia per cm$^2$ leaf portion and pycnidiospores per mature pycnidium was done by pooling the standard deviations obtained for four replicate readings for each pathogen cultivar combination, and calculating an estimate of the standard error of the difference between means, original readings being unavailable.
(j) **Measurement of stomatal density.**

Healthy leaves of the wheat cultivars Summit, Pinnacle, Teal and Fleche d'Or at the first, second and third leaf stage were assessed for the density of stomata on both the upper and lower surfaces. Measurements were made on portions near the leaf tip, near the base of the leaf and also from two points along the leaf lamina. The leaf pieces were mounted in glycerine, and the number of stomata in the field of view under a X40 objective were counted. The area of the field of view was previously calculated. Ten different areas at each of the four points on the leaves were examined.

Statistical examination was by analysis of variance and 'Students' t test, the measurements being considered as an 8 X 3 factorial design.

(k) **Measurement of the thickness of the cuticle and epidermis.**

The combined thickness of the leaf cuticle and the outer wall of epidermal cells was measured using sectioned and stained leaves. The combined thickness was measured using a calibrated eyepiece micrometer for both the upper and lower surfaces of leaves of the four cultivars mentioned in (j) above. Same aged leaves were used and the samples taken were the same as described for stomates. Twenty replicate observations were made on each cultivar. The original results presented as means and individual standard deviations were recomputed to derive a pooled standard deviation, on the assumption that there was constant variance between treatments. From this, an estimate of the standard error for the difference between means and the least significant difference was calculated.
(1) **Sampling.**

Samples of leaves for histological study were confined to those leaves which were present at the time of inoculation. Leaf portions were removed at various times after inoculation and were compared to healthy uninoculated leaves collected at the same time.

(m) **Fixation procedures.**

For surface observation, inoculated and uninoculated leaf portions were either placed into labelled vials containing methylcellosolve (2-methoxyethanol) or heated in 75% ethanol for decolourisation. Leaf portions were left in these solutions for 24 hours.

For the preparation of sections, fixation was carried out as described by Feder and O'Brien (1968). Portions of inoculated and uninoculated leaves and also wheat grains were placed in 3% glutaraldehyde and subsequently trimmed into pieces approximately 0.2cm by 0.5cm in size. They were then transferred to vials containing 3% glutaraldehyde at 0°C. Aqueous glutaraldehyde (24.3% by volume) was diluted with 0.025M Sorensens phosphate buffer (pH 7.0) and leaf portions were left in this solution for 24 hours.

(n) **Dehydration procedures.**

Dehydration of fixed specimens was done as described by Feder and O'Brien (1968). The glutaraldehyde was discarded and methylcellosolve was then added, followed by two changes during the following 24 hours. The methylcellosolve was then discarded and absolute ethanol was added
with two subsequent changes within 24 hours. Leaf portions were further dehydrated in n-propanol and then in n-butanol with two further changes of these solutions. Dehydration and solvent changing was performed at 0°C.

(a) Embedding procedures.

The fixed and dehydrated leaf pieces in n-butanol were infiltrated with purified glycol methacrylate (GMA) at room temperatures for a period of at least one week. Often, longer periods of infiltration were used with regular changes of the GMA solution. The glycol methacrylate was purified as recommended by Tippet and O'Brien (Unpublished data) using the tertiary amine resin Amberlyst A-21. The procedure was as follows:

(i) The moist beads (300g) as supplied by the manufacturers, were rinsed with one litre of distilled water.

(ii) The beads were then transferred to a Buchner funnel and the water filtered off under vacuum.

(iii) The beads were then returned to a beaker and one litre of 5% HCl added. This was done to remove any amines present and also to expand the beads.

(iv) After 30 minutes the HCl was filtered off under vacuum and the beads rinsed twice with distilled water. The beads were then returned to a beaker, covered with fresh distilled water and allowed to stand for 1 hour with occasional stirring to remove any free HCl.

(v) The water was then filtered off under vacuum and steps (iii) and (iv) repeated but using 10% NaOH to convert the resin to the hydroxide form.
(vi) The beads were then rinsed with distilled water until they were neutral to litmus paper. Most of the water was then drawn off under vacuum.

(vii) To dehydrate the beads 1 litre of absolute ethanol was poured over them and allowed to stand overnight. The ethanol was then drawn off and the beads air dried in a beaker for 2 days.

(viii) The dry beads were then divided into four beakers and 700ml of hydroxy ethyl methacrylate (HEMA) was poured over the first lot of beads and stirred. After several hours the mixture was filtered through a Buchner funnel under vacuum and the filtrate poured into the second beaker of beads.

(ix) 250ml of GMA was collected after treatment in the fourth beaker and was found to have a pH of approximately 8.0. It was now ready to be mixed with polyethylene glycol and azo-bis-isobutyronitrile to obtain a low temperature embedding medium.

(x) The regenerated beads, after a rinse in ethanol, could be recycled by repeating steps (i) to (vii).

The method used for the low temperature embedding was that described by Ashford et al. (1972). Purified GMA (95%v/v) was mixed with polyethylene glycol 400 (5%v/v) and also with azo-bis-isobutyronitrile (0.125%v/v).

Two or three pieces of the specimens to be sectioned were then transferred to shallow plastic vial caps which already had a thin layer of polymerized GMA in their bases. The leaf pieces were orientated such
that a block containing the specimen with 0.1 cm of plastic all round could be cut from the circular block which was subsequently formed in each cap. After orientating the specimen an identity tag was placed in the vial cap and filled with GMA and placed in an airtight perspex cabinet (Plate II). Polymerization of the embedding medium was achieved by illuminating the caps by a Philips Actinic Blue 15W fluorescent light tube with a stated maximal energy at 370 nm. This light tube was supported 19 cm above the vial caps containing the specimens.

(p) Sectioning.

Blocks approximately 0.75 cm by 0.4 cm containing the specimens were sectioned on an Om U2 microtome using glass knives. Sections (2.5 µm thick) were placed in a drop of filtered distilled water on glass slides and then dried on a hot plate at a temperature not exceeding 50°C.

Many workers recommend the preparation of fairly thick sections to obtain the best results in histochemical tests, thicker sections (10-15 µm) were cut for this purpose.

(q) Detection of seed borne mycelium.

To study the extent and location of mycelia of both pathogens in infected grain Simmond's (1946) technique of embryo separation was used. Batches of naturally infected grain obtained from the NSW branch of the Department of Agriculture at Wagga Wagga, and also infected grain obtained from heads artificially inoculated at different growth stages were studied.
Approximately 3g of grains were soaked in 9ml of 5% sodium hydroxide solution. Within 24 hours the embryos were observed to have "popped out" from the soft swollen endosperm and a slight pressure or stir with a glass rod easily separated these embryos from the rest of the tissues. Embryos were then picked out with forceps and soaked for 24 hours in distilled water to remove the sodium hydroxide. After soaking, the embryos were dehydrated in 95% ethanol for 2 hours and then in absolute ethanol for a further 2 hours. They were examined either in lactophenol-cotton blue or in glycerine.

Embryos were also embedded in GMA and sectioned as described earlier. These were subsequently stained with 0.5% Toluidine Blue O at pH 4.4 in benzoate buffer (Sidman et al., 1961).

(r) Staining.

(A) Surface structures.

Both inoculated and uninoculated fixed and decolourised leaf pieces as well as fresh leaf pieces were stained to study the behaviour and location of both pathogens and non-pathogens.

(i) Lactophenol cotton blue.

The original method for preparing lactophenol, as described by Amann (1896) was used. Molten phenol (50g) was added to (50ml) of warm water followed by (50ml) of lactic acid and (100ml) of glycerol. For every 100ml of lactophenol 0.05g of cotton blue (Aniline Blue WS) was added and the solution was filtered and stored.

Leaf portions were mounted in the stain, covered with a coverslip
and then warmed gently to expel air from the tissues. Preparations were then ringed with rubber solution.

(ii) Periodic Acid Schiff Reaction.

To study hyphae and spores on both fixed and fresh leaf material Preece's (1959) method was employed. Decolourised basic fuchsin was prepared by dissolving 1g of basic fuchsin in 200ml of boiling distilled water. After cooling, 20ml of 1M HCl was added, mixed well and then 1g of potassium metabisulphite crystals were added and dissolved. The flask was plugged and left overnight. Usually a straw coloured solution was obtained after standing. If not, the solution was cleared with 2g of powdered charcoal and filtered. This solution was prepared monthly.

Sulphurous acid was prepared just prior to use by adding 200ml of distilled water to a 10ml solution of 10% potassium metabisulphite in distilled water. 10ml of 1M HCl was then added to liberate the sulphurous acid.

Leaf portions were dipped in 1% periodic acid for 5 minutes, washed in distilled water for a further 5 minutes and then immersed in decolourised basic fuchsin for 5 minutes. They were then washed in freshly prepared sulphurous acid solution for 5 minutes and rinsed for several minutes. The leaf portions were mounted in either glycerol or Gurr's water mounting medium and sealed with rubber solution.

(B) Histology.

Many of the staining procedures recommended for studying parasitic hyphae in tissue sections (Johansen, 1940; Jensen, 1962; Peacock, 1966) were found to be unsuitable for GMA embedded sections as they made use
of ethanol in a concentration in excess of 40%, or the use of strong acid, alkali or phenol solutions. Staining procedures which made use of any of the above caused the sections to wrinkle and to separate from the slide. Unsatisfactory results were also obtained when GMA sections were stained prior to mounting on slides. This problem was mostly overcome by dry heating as advocated by Ling Lee et al. (1977). Sections were placed on slides and heated to temperatures of between 150°C and 200°C for a few minutes. This technique increased the range of staining techniques which could be used to give the best results with minimal damage to the tissues.

To study the location and effects of parasitic hyphae in tissues of the host the following staining procedures were used:

(i) **Safranin counterstained with picroaniline blue.** (Peacock, 1966)

Safranin (1g) was dissolved in 100ml of distilled water. 25ml of a saturated aqueous solution of Aniline Blue WS was added to 100ml of saturated aqueous picric acid which was then filtered. Slides with sections attached were immersed in safranin for 10 to 15 minutes and then washed with distilled water. They were then stained for a further 5 to 10 minutes in picro-aniline blue solution and again rinsed with distilled water. The slides were transferred to 96% ethanol and washed till no more safranin diffused form the tissue. A further wash in absolute ethanol was then given prior to mounting the sections in Eukitt.

(ii) **Toluidine Blue O.** Sidman et al. 1961)

Toluidine Blue O (0.1g) was dissolved in 200ml of benzoic buffer at pH 4.4 which was prepared by dissolving 0.25g of benzoic acid and 0.029g
of sodium benzoate in distilled water. The solution was filtered and
the pH checked and adjusted if necessary. The stain was applied to the
sections on a slide and allowed to remain for 2 to 15 minutes at room
temperature. It was then rinsed off under running tap water. The
slides were then air dried and mounted in Eukitt.

(C) Histochemical Tests.

Surface studies of non-pathogens on leaves revealed the deposition
of materials at the site of penetration. This material stained a deep
pink with the Periodic acid Schiff and with decolourised basic fuchsin
indicating that some carbohydrates or aldehydes were present. The
sections, when stained with Toluidine Blue 0 or with Safranin picro-
aniline showed a thickening of the epidermal cell walls within which
hyphae were enclosed. Many studies of the histopathology of host:
parasite interactions in which a resistance mechanism is involved have
reported the deposition of a substance generally referred to as callose.
Other studies have shown that these deposited materials may not be callose
but lignin (Ride, 1975). Tests were, therefore, carried out for both
callose and lignin. The methods employed were:

(i) Aniline Blue WS. (Johansen, 1940)

Sections were placed in a 0.005% solution of aniline blue in
50% alcohol for 4-6 hrs, rinsed in water, air dried and mounted in
laevulose syrup. This syrup was prepared by dissolving 10g of laevulose
in 10ml of warm distilled water.

(ii) Rosolic acid (Corallin). (Johansen, 1940)

A 1% solution of rosolic acid was mixed with a 4% solution of
aqueous sodium bicarbonate. Sections were stained for a few minutes, air dried and then mounted in laevulose syrup.

(iii) Resorcin Blue. (Johansen, 1940)

Resorcin blue (0.01g) was dissolved in 25ml of distilled water. The sections were stained for 15 minutes, air dried and then mounted in a drop of laevulose syrup.

(iv) Resorcinol Blue. (Eschrich and Currier, 1964)

3.0g of resorcinol was dissolved in 200ml of distilled water to which 3.0ml of concentrated (over 28%) ammonia solution was added. The solution was warmed for 10 minutes on a steam bath without boiling. The dark reddish brown solution which was obtained was stoppered with cotton wool and kept till a dark bluish colour appeared. This was then warmed for 30 minutes over a steam bath and the warmed solution filtered into an evaporating dish and warmed until no smell of ammonia could be detected.

Three drops of the above stock solution were diluted with 10ml of tap water or neutral buffer solution and sections were stained in the resulting solution for several hours, covered with a coverslip and examined. Sections were then dipped into a dilute solution of HCl (pH 4.0) and again observed.

(v) Chlorine sulphite test. (Siegel, 1953)

Slides were placed in an acidified, aqueous saturated solution of calcium hypochlorite for 5 minutes. They were then transferred to a solution of 1% sodium sulphite, mounted and observed.
(vi) **Maules test.** (Johansen, 1940)

Slides with sections attached were covered with a 1% neutral aqueous solution of potassium permanganate for 15 to 20 minutes. They were then rinsed with distilled water and placed in a 2% solution of hydrochloric acid. The acid was then rinsed off with distilled water and a drop of either ammonium hydroxide or sodium bicarbonate added. Sections were then covered with a coverslip and observed.

(vii) **Methyl Red.** (Johansen, 1940)

Ammonium hydroxide was added to an 0.001% aqueous solution of methyl red until a yellow colour appeared. Sections were then stained with this solution for a few minutes, mounted and examined.

(viii) **Wiesner Test.** (Johansen, 1940)

Drops of a saturated solution of phloroglucinol in 20% HCl were placed on the sections which were then mounted and examined.

(ix) **Phloroglucinol.** (Bradbury, 1973)

Phloroglucinol (5g) was dissolved in 100ml of 75% ethanol and filtered. Sections were then stained for 4 minutes and then a drop of 20% HCl was added. The sections were then mounted and observed.

(x) **Safranin 0 and Fast Green FCF.** (Bradbury, 1973)

Safranin 0 (1.0g) was dissolved in 99ml of 50% ethanol and sections were stained with this solution for at least 5 minutes before being dehydrated for one minute in each of 70%, 90% and 100% ethanol. The sections were then counterstained in a filtered saturated solution of Fast Green FCF in clove oil for 2 minutes. Sections were rinsed in
absolute ethanol and cleared in clove oil for 2 minutes prior to mounting.

(xi) Schiff's Reaction. (McLean and Cook, 1941).

Declourised basic fuchsin was prepared as described earlier and both leaf pieces and GMA embedded material were stained in this solution for a period varying between 15 minutes to 4 hours. The sections were then rinsed in distilled water, mounted and examined.

Schiff's reaction is really a test for aldehydes. During the embedding procedures tissues were fixed in glutaraldehyde and there was a possibility that the fixation material was reacting with the Schiff's reagent. Therefore, an aldehyde blockade was used to block both the naturally occurring aldehydes as well as those introduced during fixation. The procedures used were those recommended by Feder and O'Brien (1968). The leaf pieces and sections embedded in GMA were left for 10 minutes in a saturated solution of 2,4-dinitrophenylhydrazine (DNPH) in 15% acetic acid in water. No staining should occur after this treatment if blockade is complete. Sections and leaf pieces were then treated with periodic acid, stained with decolourised basic fuchsin and sulphurous acid as described earlier.

(xii) Fluorescence microscopy.

Aniline blue was prepared as described by Eschrich and Currier (1964). 0.05% aniline blue was dissolved in 0.06 M $K_2HPO_4$ and then adjusted to pH 9.5 with $K_3PO_4$ solution.

Fresh leaf samples which were either uninoculated or inoculated with both pathogens of non-pathogens individually were decolourised in
several changes of ethanol and the samples stored in 0.06 M \( \text{K}_2\text{HPO}_4 \) buffer (pH 9.0) for one day. Before examination, the samples were soaked for at least 3 hours in aniline blue stain. Best results were obtained with exciter filter UG1 and barrier filter 41.

Leaf portions untreated or inoculated with either pathogens or non-pathogens were also treated with buffer alone and then observed.

Unstained GMA sections of pathogen and non-pathogen-inoculated leaves were stained with aniline blue as described above and mounted in special mounting media (Eschrich and Currier, 1964). Powdered gum arabic (150g) was soaked in 100ml solution of 35% aqueous potassium acetate. A few crystals of thymol were also added and the mixture was allowed to stand for 2 to 3 days at room temperature. The viscous solution was diluted with potassium acetate solution. The pH of the resulting solution was checked to ensure that it was slightly alkaline.

Sections were also mounted in fluorescence free immersion oil and in Buffer alone to use as controls for callose and to check for auto-fluorescence using the filters as described above.

A summary of the colour reactions which would indicate the presence of various substances is shown in Table 1.

(s) Microscopy.

Most of the observations made during the course of this study were made with a Zeiss Ultraphot which had alternative light sources comprising
of either a 12v:100W filament lamp or else an HBO-200W mercury vapour lamp which was used as a source of illumination for fluorescence microscopy.

(t) Photography.

Photomicrography was done using the Zeiss Ultraphot. For certain procedures and for the examination of certain materials, various exciter filters in conjunction with barrier filters, as recommended by Eschrich and Currier (1964), were used. The main films employed were Agfa Ortho Professional for monochrome work and Agfachrome 50L for colour work. For fluorescence photography Kodak high speed Ektachrome daylight film was used.
<table>
<thead>
<tr>
<th>Staining procedure</th>
<th>Colour reaction</th>
<th>Material indicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline Blue (0.005%)</td>
<td>Blue</td>
<td>Callose</td>
</tr>
<tr>
<td>Aniline Blue (Fluorescence) (0.05%:pH9.5)</td>
<td>Greenish yellow, Yellow</td>
<td>Lignin, Callose</td>
</tr>
<tr>
<td>Autofluorescence</td>
<td>Greenish fluorescence</td>
<td>Lignin</td>
</tr>
<tr>
<td>Chlorine sulphite</td>
<td>Bright red fading to brown after 40 minutes</td>
<td>Lignin</td>
</tr>
<tr>
<td>Maules test</td>
<td>Deep red</td>
<td>Lignin</td>
</tr>
<tr>
<td>Methyl Red</td>
<td>Reddish violet or orange-red</td>
<td>Lignin</td>
</tr>
<tr>
<td>PAS basic fuchsin</td>
<td>Red</td>
<td>Fungal tissue, Lignified tissue, Polysaccharides, Pectin, starch and hemicellulose</td>
</tr>
<tr>
<td>Wiesner test (Johansen)</td>
<td>Red-violet</td>
<td>Lignin</td>
</tr>
<tr>
<td>Phloroglucinol (Bradbury)</td>
<td>Orange-red</td>
<td>Lignin</td>
</tr>
<tr>
<td>Rosolic acid</td>
<td>Red</td>
<td>Callose</td>
</tr>
<tr>
<td>Resorcin Blue</td>
<td>Brilliant blue</td>
<td>Callose</td>
</tr>
<tr>
<td>Resorcinol Blue/HCl</td>
<td>Cobalt or sky blue, Red</td>
<td>Callose, Lignin</td>
</tr>
<tr>
<td>Safranin O &amp; Fast Green</td>
<td>Red, Green</td>
<td>Lignin, Cellulose</td>
</tr>
<tr>
<td>Safranin/picroaniline blue</td>
<td>Blue, Red</td>
<td>Hyphae &amp; cellulose, Lignin</td>
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<tr>
<td>Toluidine Blue O (pH4.4)</td>
<td>Pink-purple, Purple blue, Green or greenish blue</td>
<td>Hyphae, Nuclei, Lignin</td>
</tr>
<tr>
<td>(pH1.0)</td>
<td>Green or green-blue</td>
<td>Lignin</td>
</tr>
</tbody>
</table>
RESULTS

A. (a) Gross symptoms produced by *M. graminicola* and *L. nodorum* on a very susceptible wheat cultivar Robin.

Both pathogens attack all foliar regions of the host as well as the inflorescence. Chlorosis and necrosis (Plate III, 1) were typical symptoms produced on the leaves, leaf sheaths, glumes and awns. *L. nodorum* was much more noticeable on the glumes than was *M. graminicola*. The foliar symptoms of both pathogens were exactly similar and it is not possible to distinguish between them, Plate III, 1.

The symptoms were first apparent as isolated, chlorotic lesions. In the case of *L. nodorum* they can appear 48 hours after inoculation while with *M. graminicola* they may take two to three days longer. The chlorotic lesions gradually enlarge and their centres become necrotic. These smaller lesions coalesce to form larger compound lesions. This same process is much slower in the case of *M. graminicola* although the final appearance is the same. Harrower (1976 a) found that both pathogens usually colonize the leaves of inoculated plants by spreading as compound lesions from the leaf tip backwards.

Occasionally, a heavily infected seed with *L. nodorum* may show a lesion on the outside of the seed coat. Pycnidia may sometimes develop on infected glumes but it is very unusual for them to appear on the grain. *M. graminicola* does not produce such severe head symptoms. Frequently, many small chlorotic spots were observed which rarely enlarged to form the extensive necrotic reaction typical of *L. nodorum*. The grain harvested
from infected heads was usually darker in colour and shrivelled compared to grain from healthy heads.

Pycnidia form in the necrotic tissue and they can be seen protuding from the leaf. Plate III 2 shows a pycnidium of \textit{L. nodorum} in host tissue.

(b) \textbf{Surface phenomena}.

Both pathogens, \textit{M. graminicola} and \textit{L. nodorum}, show similar features with regards to spore germination, host leaf penetration and colonisation of host tissues. Generally speaking \textit{M. graminicola} takes longer to germinate and penetrate the host than \textit{L. nodorum}. Wheat leaves were excized at various intervals after inoculation and treated as described in materials and methods. Uncleared leaves were also used to observe surface phenomena.

\textit{M. graminicola} does not produce pycnidia in culture when grown on CDV8 agar, but produces abundant secondary conidia which were initially produced by budding from pycnidiospores (Plate IV 3). These budding colonies of secondary conidia become dark after a week or so.

The pycnidiospores of both pathogens and the secondary conidia of \textit{M. graminicola} behaved similarly in that germination occurred within 24 hours. Germ tubes of \textit{L. nodorum} were usually visible after 6 hours. Germ tubes can arise at one or both ends of the spores (Plate V 2 and Plate VI 2 & 3) or, rarely, one from each spore cell. Frequently, after germination the germ tubes grew along the grooves between adjacent epidermal cells (Plate V 1 & 2, Plate VI 1). The germ tube of both
pathogens often branched (Plate VI 1) before growing over the leaf surface. Frequently, germ tubes grew to a length several times that of the parent spore before penetration occurred (Plate V 1 & 2) and this growth was also frequently restricted to the depressions at the junction of two adjacent epidermal cells.

Terminal swollen appressoria were often observed at the end of germ tubes (Plate VI 1, 2, 3 & 4) and were situated over the junction of adjacent epidermal cells (Plate VI 2, 3 & 4; Plate VII 1 & 2) or near or over the openings of stomatal cavities (Plate V 3). Although the two pathogens can enter through stomatal openings the usual method is by direct penetration through the cuticle and epidermis, which could be near a stoma or frequently through the adjacent cells of the epidermis (Plates VI 1 & 3). A halo was observed around the appressorium at such penetration sites (Plate V 3 & Plate VI 4). At such times, no appressoria were apparent although a penetrating hypha was observed at the end of the germ tubes (Plate V 4). Very often the first germ tube produced lateral branches which grew in the direction of a stoma, but usually only a single branch developed fully to produce an appressorium on or just within the stomatal opening. Germ tubes often branched at the appressorium to produce a secondary germ tube (Plate VI 2) which often, in turn, produced a further appressorium.

Sometimes pycnidiospores of *M. graminicola*, when inoculated on to wheat leaves, produced small masses of secondary conidia by budding rather than producing germ tubes as described earlier. These secondary conidia subsequently germinated producing germ tubes which formed appressoria on the leaf as described earlier. These appressoria, for some
reason, were frequently bi- or tri-lobed, (Plate VI 2). Apart from these no other behavioral differences were found between the two pathogens.

Two to three weeks after inoculation, microscopic observation of chlorotic and necrotic regions of the host revealed pycnidia, arranged linearly, in necrotic tissue. The ostioles of the pycnidia protruded through the stomatal openings on the upper as well as the lower surfaces of the leaf (Plate X 3).

(c) Internal phenomena.

Due to the extreme susceptibility of cv. Robin to both pathogens it was hard to decide whether the fungus grew inter- or intra-cellularly. The development of internal colonising mycelium was similar in both pathogens and although the hyphae of both pathogens were not clearly visible in the sections until pycnidial production was occurring, it was observed that the cells surrounding the area of penetration had collapsed and disintegrated. The breakdown of leaf tissue in advance of the penetrating hyphae suggests that toxic fungal metabolites were produced by these two pathogens. Such areas were visible on the leaf surface as chlorotic lesions which then become necrotic due to cell death and disintegration.

Sectioned material showed that colonising hyphae of the pathogens spread through the leaf tissues and that the epidermal and mesophyll cells rapidly collapsed and disintegrated. The cuticle generally remained intact even in very necrotic tissue (Plate IX 3 & 4). Slides of heavily infected material showed that vascular tissue and cells with secondary thickening, such as the stereomes (Plate VIII 3) remained intact even
after heavy infection (Plate IX 3 & 4). Only during or after pycnidial production did they show signs of distortion which could be due to physical pressure exerted by the fungus.

Usually, when penetration was via direct penetration of the cuticle epidermal cells were the first to show distress by plasmolysis of their cytoplasm, the mesophyll cells only appearing slightly affected. This was very noticeable with the Toluidine Blue 0, pH 4.4 stain. Some parts of the mesophyll cell walls stained pinkish instead of the normal purple staining of healthy cell walls. When penetration was via stomata then the cytoplasm of the mesophyll cells showed immediate signs of plasmolysis and parts of the cell wall stained pink. The chloroplasts and the nuclei degenerated very quickly and eventually whole cells collapsed. It was difficult to decide which cell component was affected first. A comparison of Plate VIII 1, with VIII 2 illustrates the extent of damage caused by _L. nodorum_.

Sections of plant tissue, which had been subjected to high relative humidity conditions, i.e. by covering with a clear polythene bag, two to three weeks after inoculation showed that hyphae of the two pathogens had thickened and tended to aggregate in the sub-stomatal cavities of the host leaves to form pycnidia embedded in this necrotic tissue. The ostioles of these pycnidia protuded slightly through the stomatal apertures (Plate X, 1, 2 & 3). The pycnidial walls of _M. graminicola_ and of _L. nodorum_ are composed of pseudoparenchymatous cells and those in the outermost layers are melanotic when mature. Pycnidiospores which exuded from these pycnidia readily distinguished the pycnidia of the two pathogens from each other. Pycnidiospores of _L. nodorum_ are
shown in Plate IV 1. They have one to two septa and an average length of 25 μm. Pycnidiospores of *M. graminicola* are three to four septate with an average length of 55 μm (Plate IV 2).

B. Behaviour of *M. graminicola* and *L. nodorum* on the four wheat cultivars used in this study.

The cultivars used in this study were Summit and Pinnacle which are both known to be very susceptible to *M. graminicola*. Teal which is moderately resistant (Harrower, 1977) and Fleche d'Or (Kuiper, pers. comm.) which is also moderately resistant were also used.

Both pathogens were shown to be capable of invading the foliar regions and inflorescences of all four cultivars, producing symptoms typical of the diseases, when inoculated either as pycnidiospores or as secondary conidia in the case of *M. graminicola*.

(a) Assessment of foliar symptoms.

The results obtained by assessment of the percentage leaf area affected using the Septoria Foliar Key (Plate I 2) are represented in Figs. I, II and III. These trends indicate that for each host-pathogen combination percentage leaf area infected increases approximately linearly with time. There is a suggestion of some cultivar differences and the three leaves studied can be placed in a ranking order of susceptibility of leaf 1 > leaf 2 > leaf 3.

The data for day 9 and 20 were subjected to more extensive analysis (Tables 2 and 3). The results reveal that the cultivars fall approximately into two groups, Summit and Pinnacle being consistently
Fig. I. Mean percentage leaf area affected of the first leaf of four wheat cultivars at various times after inoculation with (a) *L. nodorum* or (b) *M. graminicola*.

- Summit,
- Pinnacle,
- Teal,
- Fleche d'Or.
% Leaf area affected.

Time after inoculation (days).

FIG. I
Fig. II. Mean percentage leaf area affected of the second leaf of four wheat cultivars at various times after inoculation with (a) *L. nodorum* or (b) *M. graminicola*.

○● Summit, ■□ Pinnacle,
□□ Teal, □□ Fleche d'Or.
% Leaf area affected.

Time after inoculation (days).

FIG. II
Fig. III. Mean percentage leaf area affected of the third leaf of four wheat cultivars at various times after inoculation with (a) *L. nodorum* or (b) *M. graminicola*.

- Summit,
- Pinnacle,
- Teal,
- Fleche d'Or.
% Leaf area affected.

Time after inoculation (days).

FIG. III
TABLE 2. Mean percentage leaf area affected 9 and 20 days after inoculating four wheat cultivars with *M. oraminicola*.

9 DAYS AFTER INOCULATION:

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Leaf 1</th>
<th>Leaf 2</th>
<th>Leaf 3</th>
<th>Cultivar mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>33.3</td>
<td>5.8</td>
<td>1.3</td>
<td>13.5</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>21.8</td>
<td>6.3</td>
<td>3.2</td>
<td>10.4</td>
</tr>
<tr>
<td>Teal</td>
<td>3.9</td>
<td>2.7</td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>6.1</td>
<td>0.2</td>
<td>4.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Leaf mean 16.3 3.8 2.7

LSD (P = 0.01) = 5.5

20 DAYS AFTER INOCULATION:

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Leaf 1</th>
<th>Leaf 2</th>
<th>Leaf 3</th>
<th>Cultivar mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>84.0</td>
<td>53.0</td>
<td>20.3</td>
<td>52.4</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>63.5</td>
<td>56.8</td>
<td>40.0</td>
<td>53.4</td>
</tr>
<tr>
<td>Teal</td>
<td>58.5</td>
<td>28.3</td>
<td>19.3</td>
<td>35.4</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>44.9</td>
<td>19.1</td>
<td>14.8</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Leaf mean 62.7 39.3 23.6

LSD (P = 0.01) = 7.5
### TABLE 3.
Mean percentage leaf area affected 9 and 20 days after inoculating four wheat cultivars with *L. nodorum*.

#### 9 DAYS AFTER INOCULATION:

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Leaf 1</th>
<th>Leaf 2</th>
<th>Leaf 3</th>
<th>Cultivar mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>23.3</td>
<td>20.6</td>
<td>7.1</td>
<td>17.0</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>42.3</td>
<td>24.1</td>
<td>6.1</td>
<td>24.1</td>
</tr>
<tr>
<td>Teal</td>
<td>6.2</td>
<td>15.4</td>
<td>4.7</td>
<td>8.7</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>5.0</td>
<td>9.7</td>
<td>3.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Leaf mean</td>
<td>19.2</td>
<td>17.4</td>
<td>5.4</td>
<td></td>
</tr>
</tbody>
</table>

LSD (P = 0.01) = 4.5

#### 20 DAYS AFTER INOCULATION:

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Leaf 1</th>
<th>Leaf 2</th>
<th>Leaf 3</th>
<th>Cultivar mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>68.3</td>
<td>63.3</td>
<td>40.9</td>
<td>57.5</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>93.0</td>
<td>59.0</td>
<td>22.6</td>
<td>58.2</td>
</tr>
<tr>
<td>Teal</td>
<td>42.5</td>
<td>46.9</td>
<td>16.9</td>
<td>35.4</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>45.3</td>
<td>37.6</td>
<td>13.2</td>
<td>32.1</td>
</tr>
<tr>
<td>Leaf mean</td>
<td>62.3</td>
<td>51.7</td>
<td>23.4</td>
<td></td>
</tr>
</tbody>
</table>

LSD (P = 0.01) = 13.5
more susceptible to both pathogens than Teal and Fleche d'Or. The differences between leaves mentioned above is confirmed as statistically significant at least for infection by *M. graminicola*. The experimental design did not permit direct comparisons between the two pathogens.

(b) **Incubation Period.**

Table 4 records the incubation periods, that is the period from inoculation until the appearance of foliar symptoms visible to the unaided eye, on the four wheat cultivars when inoculated individually with the two pathogens. The data suggest a gradation of susceptibility from Summit with the shortest incubation period to Fleche d'Or with the longest, but the wide standard deviations calculated from a small sample size demand extreme caution in interpretation.

(c) **Latent Period.**

The results obtained for the latent periods, i.e. the time from inoculation until the first appearance of mature pycnidia, are shown in Table 5. The trend of susceptibility is again Summit > Pinnacle > Teal > Fleche d'Or. As this sample was also very small it was not subjected to statistical analysis.

(d) **Sporulation Index.**

The results obtained for the sporulation indices of the two pathogens are presented in Table 6. As with other parameters there is a tendency for Summit to behave as the most susceptible cultivar and
### TABLE 4. Incubation periods of *M. graminicola* and *L. nodorum* on four wheat cultivars (days) ± SD.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>M. graminicola</th>
<th>L. nodorum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>4.0 ± 1.4</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>8.0 ± 0.0</td>
<td>4.5 ± 2.1</td>
</tr>
<tr>
<td>Teal</td>
<td>8.0 ± 4.2</td>
<td>6.0 ± 4.2</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>10.5 ± 3.5</td>
<td>8.0 ± 4.2</td>
</tr>
</tbody>
</table>

### TABLE 5. Latent periods of *M. graminicola* and *L. nodorum* on four wheat cultivars (days) ± SD.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>M. graminicola</th>
<th>L. nodorum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>16.0 ± 1.4</td>
<td>11.0 ± 1.4</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>18.0 ± 1.4</td>
<td>13.5 ± 2.1</td>
</tr>
<tr>
<td>Teal</td>
<td>21.0 ± 1.4</td>
<td>18.0 ± 1.4</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>27.5 ± 3.5</td>
<td>21.5 ± 2.1</td>
</tr>
</tbody>
</table>
### TABLE 6. Sporulation of *M. graminicola* and *L. nodorum* on four wheat cultivars.

<table>
<thead>
<tr>
<th>Pathogen-cultivar combination</th>
<th>Pycnidiospores per cm² leaf portion ((x10^3))</th>
<th>Sporulation index</th>
<th>Mature pycnidia per cm² leaf portion</th>
<th>Pycnidiospores per mature pycnidium ((10^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. graminicola</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summit</td>
<td>213.6</td>
<td>15.3</td>
<td>14.0</td>
<td>15.4</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>101.7</td>
<td>7.2</td>
<td>9.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Teal</td>
<td>41.9</td>
<td>3.0</td>
<td>5.3</td>
<td>8.2</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>14.0</td>
<td>1.0</td>
<td>2.8</td>
<td>5.0</td>
</tr>
<tr>
<td>LSD (P = 0.05)</td>
<td>45.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **L. nodorum**                |                                               |                   |                                     |                                 |
| Summit                        | 266.4                                         | 4.8               | 25.2                                | 10.6                            |
| Pinnacle                      | 121.4                                         | 2.2               | 12.8                                | 9.8                             |
| Teal                          | 55.7                                          | 1.0               | 5.4                                 | 10.5                            |
| Fleche d'Or                   | 57.7                                          | 1.0               | 6.2                                 | 8.9                             |
| LSD (P = 0.05)                | 55.6                                          |                   |                                     |                                 |
Fleche d'Or as the least. The effect of cultivar upon sporulation index was much more marked with *M. graminicola* than with *L. nodorum*. This can probably be attributed to the difference between pathogens in the number of spores produced per pycnidium. There is a threefold (and statistically significant) difference between the most and least susceptible cultivars in the case of *M. graminicola*, but no significant differences with *L. nodorum*.

The number of mature pycnidia produced per unit leaf area closely paralleled spore production on the same leaves. Significant differences were noted between Summit, Pinnacle and Teal, but between Teal and Fleche d'Or differences were barely (*M. graminicola*) or not (*L. nodorum*) significant.

(e) **Stomatal density.**

The mean number of stomata per mm$^2$ leaf area for the four wheat cultivars are presented in Table 7. No significant differences ($P = 0.01$) were found between the upper and lower leaf surfaces. Leaf 1 was significantly different from leaf 2 and 3, the difference between the latter two leaves being non significant.

(f) **Epidermal and cuticle thickness.**

The results of the combined thickness of the epidermis and cuticle of healthy cells are presented in Table 8. No significant differences in thickness were found with the marginal exception of Pinnacle and Teal, in that the epidermal wall and cuticle of Pinnacle was slightly thicker.
TABLE 7. The number of stomata per mm\(^2\) leaf area on both the upper and lower surfaces of the first three leaves of the four wheat cultivars (U = upper leaf surface, L = lower leaf surface).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Leaf 1</th>
<th>Leaf 2</th>
<th>Leaf 3</th>
<th>Cultivar mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>38.4</td>
<td>30.8</td>
<td>41.9</td>
<td>31.9</td>
</tr>
<tr>
<td>L</td>
<td>21.4</td>
<td>29.7</td>
<td>34.7</td>
<td></td>
</tr>
<tr>
<td>Pinnacle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>26.2</td>
<td>35.6</td>
<td>39.9</td>
<td>29.3</td>
</tr>
<tr>
<td>L</td>
<td>15.9</td>
<td>32.3</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td>Teal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>37.7</td>
<td>39.6</td>
<td>36.9</td>
<td>34.1</td>
</tr>
<tr>
<td>L</td>
<td>28.7</td>
<td>32.6</td>
<td>29.2</td>
<td></td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>34.3</td>
<td>38.9</td>
<td>39.2</td>
<td>30.4</td>
</tr>
<tr>
<td>L</td>
<td>20.1</td>
<td>28.0</td>
<td>21.6</td>
<td></td>
</tr>
<tr>
<td>Leaf mean</td>
<td>27.8</td>
<td>33.4</td>
<td>33.7</td>
<td></td>
</tr>
</tbody>
</table>

Mean for upper leaf surface = 36.6 stomata/mm\(^2\)
Mean for lower leaf surface = 26.7 stomata/mm\(^2\)
LSD (P = 0.01) = 5.9 stomata/mm\(^2\)
TABLE 8. A comparison of the combined thickness of the cuticle and outer epidermal cell walls on both the upper and lower leaf surfaces of four wheat cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>upper leaf surface (μm)</th>
<th>lower leaf surface (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summit</td>
<td>5.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Pinnacle</td>
<td>6.1</td>
<td>5.9</td>
</tr>
<tr>
<td>Teal</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Fleche d'Or</td>
<td>5.7</td>
<td>5.5</td>
</tr>
</tbody>
</table>

LSD \( (P = 0.01) \) = 1.4
for the upper leaf surface only.

(g) **Comparisons of gross symptoms produced on the four wheat cultivars.**

On the four wheat cultivars, isolated necrotic lesions developed after inoculation with both pathogens. These subsequently coalesced to form irregularly shaped dead blotches on the leaf lamina. All foliar regions as described for Robin were attacked. Pycnidia of both pathogens were seen to form in the sub-stomatal cavities of all four wheat cultivars and were clearly visible in the dead tissues. The leaf sheaths also became discoloured and caused the leaves to fall away from the plants.

On Summit and Pinnacle, symptoms appeared on artificially inoculated plants within three to four days. About 5 or 6 days later, the initial chlorotic lesions turned yellowish-brown, coalesced and killed the leaves rapidly.

On Teal, the pathogens caused smaller lesions, which took longer to appear and were at first chlorotic, later becoming necrotic and more sharply defined. Pycnidial production was slower.

On Fleche d'Or, symptoms varied from small chlorotic flecks to small straw coloured necrotic lesions which developed very few pycnidia. The lesions took much longer to coalesce.

(h) **Surface phenomena of *M. graminicola* and *L. nodorum* on the four wheat cultivars.**

No differences were apparent when both pathogens were inoculated
singly onto the wheat cultivars used in this study either in their mode of spore germination, appressorium formation or in the cuticular and stomatal penetration. These events have already been described for the cv. Robin.

Within two to four weeks after inoculation, depending on the cultivar, the ostioles of pycnidia of both pathogens could clearly be seen protruding from the stomata in necrotic leaves.

(i) Internal phenomena.

Stained sections of the four wheat cultivars, examined at various intervals after inoculation, showed that the basic pattern of tissue colonisation was the same as described for cv. Robin. In Pinnacle and Summit, the most susceptible cultivars, cells collapsed well in advance of the colonising hyphae (Plate IX 1). The disease progressed slightly slower compared to cv. Robin. If entry was via or near stomata the mesophyll cells were the first to show disruption. The stomatal guard cells did not show signs of distress until much later, which might be due to the slight lignification of these cells. When entry was by direct penetration of the cuticle and epidermal cells, these latter cells collapsed first, (this mode of entry is illustrated by Plate VIII 4 in which hyphae of _L. nodorum_ are entering the epidermal cells of Teal). Occasionally, some areas of affected cell walls stained greenish blue with Toluidine Blue O, or red with safranin picro-aniline blue. This could be due to lignification since healthy cell walls usually stain a deep purple (Plate XV 2 & XVI 1) with Toluidine Blue O, and blue with the safranin picro-aniline blue stain (Plate XVI 2).
On Teal and Fleche d'Or, the disruption of cells and their contents was even slower. This can clearly be seen in Plate IX 2 where \textit{L. nodorum} can actually be seen inside the tissue of Teal which does not appear badly disrupted, even 6½ days after inoculation, although signs of plasmolysis of the mesophyll cells were present. Some lignification of parts of the cell walls was present in these two cultivars also.

Studying the more resistant cultivars, it also became clear that intercellular hyphae without haustoria could incite chlorosis of the mesophyll cells. The hyphae could also grow intracellularly (Plate VIII 4). Apart from that, no other histological factors were found which might be responsible for the four wheat cultivars showing differential levels of susceptibility. One thing was certain from the slides, the more resistant a cultivar was the more mycelium was needed to produce the same area of necrosis as was produced on the susceptible cultivars.

Attempts to isolate phytotoxins form both pathogens grown in liquid cultures by the methods of Kent and Strobel (1976) were unsuccessful and no disease symptoms appeared on any cultivar after the application of culture filtrates.

C. Extent of mycelium in seed.

Seeds harvested after inoculations at different stages of heading were fixed, embedded, sectioned and stained with Toluidine Blue O pH 4.4 as described earlier. Examination revealed that the mycelium had not entered the wheat embryo, endosperm or the aleurone layer. It was only found in abundance in the subpericarpic region, mostly aggregating towards
the ventral groove (crease) (Plate XI 1, 2, 3 & 4). Results obtained by Simmond's (1946) embryo testing technique showed that no mycelium was present in the embryos of naturally or artificially infected wheat seeds.

D. Study of non-pathogens on wheat leaves.

(a) Gross Symptoms.

*A. pisi*, on peas, is reported to behave in a similar manner to *L. nodorum* and *M. graminicola* on wheat. It attacks the leaf, stem and pods of pea and the lesions produced are light brown with a darker, frequently prominent margin and pale centre. Stem lesions are sunken and pycnidia are produced in both leaf and stem lesions in the sub-stomatal cavities (CMI, 1972).

On wheat leaves after inoculation with *A. pisi*, minute chlorotic flecks were produced four to five days after inoculation. These flecks did not coalesce and no pycnidia were produced in them.

*E. nigrum* is a common saprophyte on plant debris. After inoculation onto wheat leaves it did not produce any chlorosis but tiny reddish flecks were visible on excized leaf portions seven to eight days after inoculation. It did not sporulate on the wheat surface.

The above behaviour of *A. pisi* and *E. nigrum* was similar on all the four wheat cultivars.

(b) Surface phenomena.
When inoculated onto wheat leaves, produced germ tubes within 24 hours. These germ tubes branched frequently and tended to grow along the depressions between adjacent epidermal cells before producing appressoria (Plate XIII 1 & 2). After appressorium production, cuticle penetration was attempted resulting in the appearance of material which stained strongly with PAS and Shiff's reagent. After this the germ tube usually continued to grow and produced other appressoria.

On all four wheat cultivars *F. nigrum* produced profuse surface mycelium due to branching of germ tubes. Appressoria were observed and hyphae were closely adpressed to the host cell walls and in many instances, appeared to be causing slight indentations (Plate XIV 1). Where this indentations occurred i.e. at the point of contact of fungus and host (or attempted penetration) at the junction of epidermal cell walls, a pink deposit was observed after staining with Periodic acid and Schiff's reagent (Plate XIV, 1 & 2). The nucleus of the adjacent epidermal cell also migrated to this site.

When it occurred penetration by *A. pisi* and *F. nigrum* took two to three days, followed by sluggishness of protoplasmic streaming, the nucleus at the site of penetration enlarged, became distorted and finally disintegrated, indicating that cell death had occurred. In contrast with the two pathogens, within minutes of penetration of the cell, protoplasmic streaming stopped. The sluggishness and the stopping of protoplasmic streaming observed was very marked when compared to a healthy epidermal cell, (Plate XII ) in which the healthy nucleus moves around due to protoplasmic streaming.

(c) Internal phenomena.
Host reaction to a non-pathogen resulted in a thickening of the cell walls in infected tissue. This response appeared to be harmful to both host cells and the fungus.

Studies with non-pathogens also showed that resistance to *A. pisi* and *E. nigrum* was exhibited after penetration and the reaction was of three types. In some cells the cytoplasm was still intact but partially pulled away from the outer epidermal walls (Plate XVII 1). In this periplasmic space were lodged the non-pathogenic hyphae and the deposits formed in response to attempted penetration which could be viewed in surface phenomena (Plate XIV 2). Cell walls near such penetration sites showed signs of lignification as shown in Plate XVII 1 by Toluidine blue O stain, and Plate XVII 2 by the Safranin Picro-aniline blue stain. The third form of reaction shown in response to *A. pisi* and *E. nigrum* infection was deposition of a granular, dark purple (Toluidine blue O; Plate XVIII 2) and blue staining material (Safranin Picro-aniline; Plate XVIII 1) between the intercellular spaces of the mesophyll cells. Some infected cells also had a very dense granular cytoplasm in which the chloroplasts and the nucleus were no longer distinguishable. Hyphae of *A. pisi* were found intracellularly in the epidermal cells. The epidermal, mesophyll and vascular tissue did not appear disorganised even though heavily infected.

The substance deposited by the host cells in response to attempted penetration by non-pathogens was assumed to be callose because, in many studies of resistant host-pathogen interactions, such deposits have been reported (Akai, 1959; McKeen et al. 1969; Heath, 1971; Sargent et al. 1973; Weinhold and Motta, 1973; Lazarovits and Higgins, 1975 a). These
deposits have also been described as lignitubers, there are suggestions that the deposits formed in response to non-pathogens are lignified (Ride, 1975).

Attempts to identify the material formed in response to non-pathogens was done by different staining techniques. Staining with Toluidine blue 0 and Safranin Picro-aniline blue indicated lignin deposition on the cell walls although the lignitubers (papillae or callose deposits) themselves did not stain indicating that they were not lignified. These lignitubers stained a deep pink with Periodic Acid and Shiff’s reagent but did not stain with Shiff’s reagent on its own. In sections after aldehyde blockade and treated with Periodic Acid Schiff and de-colourized basic fuchsin, these deposits still did not stain indicating that lignin was not the component of such deposits. A summary of the negative and positive staining reactions obtained after different staining techniques are presented in Table 9.

Positive reactions for callose were given by the Aniline blue Aq (0.005%) stain, Resorcinol blue, and Resorcinol + HCl stains. The latter stain indicated that, to some extent, cell walls of the epidermis and mesophyll cells also became lignified.

Autoflourescence, aniline blue (0.05%, pH 9.5) and fluorescence microscopy with different exciter and barrier filters also showed a positive reaction for callose in such deposits and a positive reaction for lignin in cell walls. A comparison with healthy cell walls showed no signs of lignification, no staining with Toluidine blue 0 stain and no autofluorescence. The aniline blue fluorescence and the Resorcinol
blue (HCl) reactions are considered to be specific for callose and lignin (Eschrich and Currier, 1964). Another method of confirming callose is by comparing the yellow fluorescence given by deposits formed in response to non-pathogens with the reaction given by the sieve plates in the phloem (Plate XXII 1). Autofluorescence shown by the epidermal and mesophyll cell walls can be compared to the autofluorescence produced by the xylem (Plate XXII 2).
**TABLE 9.** Reactions obtained when healthy, pathogen and non-pathogen inoculated cultivars of wheat were stained for callose and lignin with different staining techniques. (+) positive result; (-) negative result.

<table>
<thead>
<tr>
<th>STAIN</th>
<th>COLOUR</th>
<th>SUBSTANCE</th>
<th>REACTION (NON-PATHOGEN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline Blue .005%</td>
<td>Blue</td>
<td>callose</td>
<td>+</td>
</tr>
<tr>
<td>Aniline Blue 0.05% in buffer</td>
<td>yellow</td>
<td>callose</td>
<td>+</td>
</tr>
<tr>
<td>pH9.5, Exciter and Barrier filter combinations</td>
<td>greenishyellow</td>
<td>lignin</td>
<td>-</td>
</tr>
<tr>
<td>Autofluorescence, immersion oil (Zeiss) and buffer pH9.5 (Filter comb.)</td>
<td>green</td>
<td>lignified cell walls</td>
<td>+</td>
</tr>
<tr>
<td>Chlorine Sulphite</td>
<td>bright red to brown</td>
<td>lignified cell walls</td>
<td>-</td>
</tr>
<tr>
<td>Maules test</td>
<td>deep red</td>
<td>lignified cell walls</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red</td>
<td>red violet to orange red</td>
<td>lignified cell walls</td>
<td>-</td>
</tr>
<tr>
<td>Basic fuchsin</td>
<td>red</td>
<td>lignified cell walls</td>
<td>-</td>
</tr>
<tr>
<td>Wiesner Test Johansen</td>
<td>red violet</td>
<td>lignified cell walls</td>
<td>-</td>
</tr>
<tr>
<td>Phloroglucinol (Bradbury)</td>
<td>orange red</td>
<td>lignified cell walls</td>
<td>+</td>
</tr>
<tr>
<td>Rosolic Acid</td>
<td>red</td>
<td>callose</td>
<td>-</td>
</tr>
<tr>
<td>Resorcin blue</td>
<td>brilliant blue</td>
<td>callose</td>
<td>-</td>
</tr>
<tr>
<td>Resorcinol + HCl</td>
<td>cobalt or sky blue red</td>
<td>callose</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lignified cell walls</td>
<td>+</td>
</tr>
<tr>
<td>Safranin 0/fast green FCF</td>
<td>red</td>
<td>lignified cell walls</td>
<td>+</td>
</tr>
</tbody>
</table>

54
<table>
<thead>
<tr>
<th>STAIN</th>
<th>COLOUR</th>
<th>SUBSTANCE</th>
<th>REACTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toluidine blue 0</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH4.4</td>
<td>green or</td>
<td>lignified</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>blue green</td>
<td>cell walls</td>
<td></td>
</tr>
<tr>
<td>pH1.0</td>
<td>green or</td>
<td>lignified</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>blue green</td>
<td>cell walls</td>
<td></td>
</tr>
<tr>
<td>**Safranin/picro-</td>
<td>red</td>
<td>lignified</td>
<td>+</td>
</tr>
<tr>
<td>aniline blue</td>
<td></td>
<td>cell walls</td>
<td></td>
</tr>
</tbody>
</table>

**Note:**

1. In healthy tissue epidermal and mesophyll cell walls always gave a negative reaction for callose and lignin.

2. In pathogen infected tissue some positive results were obtained for lignification of epidermal cell walls for the four wheat cultivars with Toluidine blue 0 or Safranin picro-aniline blue stain but these were not always consistent. Staining for callose always gave negative results.
PLATE I


2. Assessment Key for foliar symptoms due to Septoria spp.
on cereals, after James 1971.
GROWTH STAGES IN CEREALS

STEM EXTENSION → HEADING → RIPENING

TILLERING

STAGE 1
STAGE 2
STAGE 3
STAGE 4
STAGE 5
STAGE 6
STAGE 7
STAGE 8
STAGE 9
STAGE 10
STAGE 10.1
STAGE 11

SEPTORIA KEY [FOLIAR]

% LEAF AREA AFFECTED

1 5 5 10 10 25 25 50 75

PLATE I
PLATE II.

Embedding apparatus.

b; Philips actinic blue 15 W light tube, c; plastic caps, f; aluminium foil, n; nitrogen cylinder, p; air tight perspex cabinet, r; rubber tubing, w; beaker of water.
PLATE III.

1. Typical chlorotic and necrotic symptoms caused by (1) 
   *L. nodorum* and (3) *M. graminicola*, compared to a healthy
   leaf (2) on cultivar Robin.

2. A mature pycnidium of *L. nodorum* in a leaf stained with
   Lactophenol cotton blue, on cultivar Robin.

   c; chlorotic lesions, l; leaf, m; melanotic pycnidium
   wall, n; necrotic lesions, o; ostiole, p; pycnidium.
PLATE IV.

1. Pycnidiospores of 

2. Pycnidiospores of

3. Secondary conidia of

\( d; \) pycnidiospores, \( s; \) septa, \( sc; \) secondary conidia.
PLATE V.

1, 2, 3 & 4. Pycnidiospores of L. nodorum on leaves of cv. Robin.

a; appressoria, e; junction of epidermal cell walls,
g; germ tube, gc; guard cells, h; halo where enzymatic degradation has taken place, hy; subcuticular hyphae,
p; pycnidiospore, sa; stomatal aperture.
PLATE VI.

1, 2, 3 & 4. *M. graminicola* on leaves of cv. Robin.

a; appressorium, e; junction of epidermal cell wall,
gt; germ tube, h; halo formed due to enzymatic degradation,
s; stomata, sc; secondary conidia, p; pycnidiospore.
PLATE VII.


a; appressorium, e; junction of epidermal cell walls, g; germ tube, hy; subcuticular hyphae, l; leaf hair, n; host nucleus, p; pycnidiospore, pp; penetration point.
PLATE VIII.


2. T.S. of leaf of cv. Robin, 4 days after inoculation with

   \textit{L. nodorum}.


4. T.S. of leaf of cv. Teal, 4 days after inoculation with

   \textit{L. nodorum}.

\textit{ch; chloroplasts, ep; epidermal cells, gc; guard cells,}
\textit{hy; hyphae, m; mesophyll cells, n; healthy host nucleus,}
\textit{pc; plasmolysed cytoplasm, sc; sub-stomatal cavity, st;}
\textit{stereomes, x; conductive tissue, n; dead nucleus.}
PLATE IX.


2. Penetration through closed guard cells of cv. Teal by hyphae of *L. nodorum*, 6½ days after inoculation.

3. T.S. of necrotic leaf of cv. Robin, 42 days after inoculation with *M. graminicola*.

4. T.S. of necrotic leaf of cv. Robin, 33 days after inoculation with *L. nodorum*.

PLATE X.


3. T.S. of necrotic leaf of cv. Robin showing pycnidia of *L. nodorum* in both the upper and lower leaf surface.

ct; conductive tissue, gc; guard cells, hy; internal hyphae, l; remains of leaf, le; lower epidermis, o; ostiole, p; pycnidium, pc; pycnidiospores, ue; upper epidermis, w; wall of pycnidium.
PLATE XI.

1. Hyphae of *L. nodorum* on the inside surface of the seed coat, of cultivar Robin.

2. Aggregated hyphae of *L. nodorum* in the ventral groove of the seed, of cultivar Robin.

3. Hyphae of *M. graminicola* in the pericarpic region of the seed, near the embryo, of cultivar Robin.

4. An enlargement of Plate XI(3).

   a; aleurone layer, e; endosperm, em; embryo, hy; hyphae, nuc; nucellar layer, p; pericarp, vc; ventral groove.
PLATE XII.

1, 2 & 3. Healthy epidermal cells of cv. Summit, showing the movement of a healthy nucleus.

e; junction of epidermal cells, h; leaf hair, n; nucleus.
PLATE XIII.

1. Pycnidiospores of *A. pisi* on the leaf surface of cv. Teal, 4 days after inoculation.

2. Hyphae of *A. pisi* on the leaf of cv. Teal, 6½ days after inoculation.

*a; appressorium, e; junction of epidermal cells, g; germ-tube, p; pycnidiospores, pl; initiation of papillae, s; stomata.*
PLATE XIV.

1. Papillae of the epidermal cell walls of cv. Teal, 13 days after inoculation with *E. nigrum*.

2. The same area as shown in Plate XIV (1) at a different focus and at x700.

e; junction of epidermal cells, et; epidermal cell wall thickening, hy; hyphae on the surface of the leaf, n; nucleus.
PLATE XV.

T.S. of healthy leaves of cv. Teal after staining with:

1. Safranin and Picro-aniline blue.

2. Toluidine blue O at pH 4.4.

cl; chloroplasts, ct; conductive tissue, ep; epidermal cells, gc; guard cells, m; mesophyll cells, n; nucleus, ph; phloem, st; stereomes, x; xylem.
PLATE XVI.

T.S. of healthy leaves of cv. Summit after staining with Toluidine blue O at pH 4.4.

1. Epidermal and mesophyll area.

2. Conductive tissue and mesophyll area.

cl; chloroplasts, ct; conductive tissue, sp; epidermal cells, gc; guard cells, m; mesophyll cells, n; nucleus, ph; phloem, st; stereomes, x; xylem.
Thickening of epidermal cell walls of cv. Teal in response to infection by *A. pisi*, 13 days after inoculation (T.S.).

1. Stained with Toluidine blue 0 at pH 4.4.

2. Stained with Safranin and picric-aniline blue.

c; callose deposits, cl; chloroplasts, ep; epidermis, epw; epidermal cell wall, h; leaf hair, hy; hyphae, lep; lignification of epidermal cell walls, m; mesophyll, n; nucleus, ph; phloem, st; stereome, x; xylem.
PLATE XVIII.

A. pisi in leaves of cv. Teal, 13 days after inoculation (T.S.).

1. Stained with Safranin and picro-aniline blue.

2. Stained with Toluidine blue 0 at pH 4.4.

ch; chloroplasts, e; epidermal cell wall, dg; dark staining granular material, hy; intracellular hyphae.
PLATE XIX.

Hyphae of *E. nigrum* in leaves of cv. Teal, 13 days after inoculation (T.S.).

1. Stained with Toluidine blue 0 at pH 1.0.

2. Stained with Safranin and picro-aniline blue.

c; cell wall, hy; hyphae, lw; lignified cell walls, m; mesophyll cells, n; nucleus, st; stereome, x; xylem.
Leaf of cv. Teal stained and mounted in Aniline blue (pH 9.5), 6½ days after inoculation with A. pisi.

1. Surface of leaf epidermis.

2. The same area as in Plate XX (1) but focused on the cell wall thickenings at the site of attempted penetration.

3. T.S. of leaf stained and mounted in Aniline blue at pH 9.5.

ch; chloroplast, d; deposit, e; junction of epidermal cells, ep; epidermal cell, g; germ-tube, m; mesophyll cell, pp; penetration
PLATE XXI.

1. Yellow fluorescence at the junction of epidermal cell walls near a stoma. Cultivar Teal inoculated with A. pisi.

2. The same area as in Plate XX (1) & (2), under ultraviolet.

3. The same area as in Plate XX (3), under ultraviolet.

d; deposit, e; junction of epidermal cells, ep; epidermal cells, f; fluorescence, lh; leaf hair, m; mesophyll cells.
1. Fluorescence of callose deposits in the epidermis in response to infection by *A. pisi* and also due to naturally occurring callose in the sieve plates of the phloem (cv. Teal).

2. Autofluorescence of the xylem vessel walls, guard cells and some epidermal and mesophyll cell walls in leaf tissues infected by *A. pisi* (cv. Teal).

3. Autofluorescence of the same areas as in Plate XXII (2). The colour print does not show the greenish fluorescence which was actually visible (cv. Teal).

ct; conductive tissue, ep; epidermal cell, f; fluorescence, m; mesophyll cell, p; papillae, st; stereome, x; xylem.
DISCUSSION

Two of the most important foliar diseases of wheat in Australia today are 'glume blotch' caused by *Leptosphaeria nodorum* and 'speckled leaf blotch' caused by *Mycosphaerella graminicola*. Both diseases are found in all wheat growing areas of Australia but, in any one season their relative distributions need not overlap. Reports from other wheat growing areas are that over successive seasons these two pathogens can cause a grain loss of between ten and sixty percent (Brönnimann, 1968 a & b, Saari and Wilcoxson, 1974). Various control strategies are currently available to reduce the often disastrous effects of these pathogens on the crop. Many sanitary control procedures have been proposed and successfully tested under field conditions. Also, the use of fungicides has been shown to be effective in arresting the development of severe epiphytotics (Kuiper, pers. comm.). However, many of the forms of control advocated are impracticable. For example straw removal is often incompatible with current agricultural practice and so the beneficial effect of reducing intercrop inoculum carried on surface litter cannot be realised. Secondly, the cost of repeated fungicide treatments through the growing season can prove to be far more costly than the actual yield loss which would have occurred had there been no protective measures taken.

For sometime there has been an increasing interest in the selection of wheat lines and cultivars which show moderate resistance to either or both pathogens. An active breeding programme has recently been initiated on a large scale over the whole of Australia in the form of the Australian Septoria Nursery (R. Wilson, pers. comm.). The degree of importance of these two diseases may be illustrated by the fact that this is a joint venture financed by several state Departments of Agriculture.
Various cultivars have been identified as having some degree of resistance to either _L. nodorum_ (Bockman et al. 1975) or to _M. graminicola_ (Rosielle, 1972) or to both. However, many cultivars resistant to biotrophic pathogens which have been considered serious for many years are nevertheless, highly susceptible to _M. graminicola_ and _L. nodorum_.

The aim of this study was to determine if a range of wheat (_Triticum aestivum_) cultivars, shown to differ in their level of susceptibility to both pathogens, possessed any histological features, either prior to or after infection, which might account for the observed differences in susceptibility.

At the commencement of the study relative susceptibility data were obtained by artificially inoculating plants of the four wheat cultivars, Summit, Pinnacle, Teal and Fleche d'Or. The main assessment parameter used was the quantity of foliar symptoms, i.e. chlorosis and necrosis, at different times after inoculation. The results obtained showed a trend of susceptibility for the three leaves to be Leaf 1 > 2 > 3. Percentage leaf area affected increased approximately linearly with time and there was some suggestion of cultivar differences. Analysis for days 9 and 20 showed that Summit and Pinnacle were more susceptible to both pathogens than Teal and Fleche d'Or.

Subsequently other parameters were employed. The relative lengths of incubation and latent periods of both pathogens on the four wheat cultivars showed that the trend of susceptibility was Summit > Pinnacle > Teal > Fleche d'Or. The results for the sporulation indices i.e. the quantity of pycnidiospores produced per unit area of necrotic host tissue showed that Summit was the most
susceptible cultivar and Fleche d'Or the least. The intensity of pycnidial production per unit area also showed significant differences between Summit, Pinnacle and Teal but hardly any between Teal and Fleche d'Or with either pathogen. The experimental design did not allow direct comparison between the two pathogens.

All results roughly place the four wheat cultivars into the same ranking order of susceptibility to both pathogens, i.e. Summit > Pinnacle > Teal > Fleche d'Or, the difference between Teal and Fleche d'Or being marginal. This subjective order of ranking agrees with the results of Harrower (1977 b) which were obtained by chemical analysis of M. graminicola infected tissue.

Pre-penetration studies showed that pycnidiospores of both pathogens germinated within a few hours under conditions of high humidity. After a period of germ-tube elongation, appressorium formation and subsequent penetration usually occurred within 6 to 24 hours. It has not been established whether there is a difference between the pathogens in the time required to initiate an infection. The results obtained by inoculating leaves of all four cultivars with either of the two fungi and then assessing the time required for the first appearance of foliar symptoms suggests that the leaves possess post-penetration defence mechanisms of either a biochemical or histological nature.

Both L. nodorum and M. graminicola caused a progressive chlorosis and necrosis of all aerial parts of the host plant. The first symptoms were pale chlorotic spots which gradually enlarged and coalesced. Both pathogens caused similar symptoms. The centres of coalesced lesions were found to become rapidly necrotic and it was in those areas within the
sub-stomatal cavities on both surfaces of infected leaves that the pycnidia of the pathogens were formed. Stems were also affected and exhibited chlorotic lesions which rapidly turned necrotic. Such lesions often circled the stem thereby weakening it and arresting the translocation of water and solutes.

On inflorescences _L. nodorum_ was the more destructive of the two pathogens. Lesions occurred on all floral parts and those on the glumes turned necrotic and had a distinctive purplish-brown colouration. On heads which were infected shortly after emerging from the 'boot' the glumes were severely damaged and the grain harvested from such heads was often shrivelled and of a darker colour than normal grain. By contrast, _M. graminicola_ did not produce such severe head symptoms. Frequently, many small chlorotic spots were observed and these rarely enlarged and coalesced to form the extensive necrotic reaction typical of _L. nodorum_.

The life histories of both pathogens in their asexual phase only was studied and documented as the ascigerous or sexual phase could not be obtained. This was done by microscopic examination of the surface phenomena at various intervals after inoculation and making comparisons with healthy leaves and by sectioning and staining both healthy and inoculated leaf material at various times after inoculation. The location and general histological effects of both pathogens could be clearly seen in prepared slides.

The life histories of both fungi are very similar. It has long been known that the pycnidiospores of both pathogens are splash dispersed from cirrhi or spore tendrils which are exuded from pycnidia under conditions of leaf surface wetness or high relative humidity (Harrower,
Pycnidiospores are splashed on to adjacent leaves and shortly after, if conditions remain moist, they germinate to produce germ-tubes which elongate, branch and produce appressoria on the leaf surface. This process was observed within 24 hours after inoculation. From the appressorium an infection peg was produced which penetrated the cuticle and occasionally, the outer wall of the epidermal cells.

Mycelial proliferation occurred in the sub-stomatal cavities of necrotic tissues, resulting in the production of pycnidal initials which eventually enlarged to form pycnidia. Mature pycnidia of both pathogens were evident in necrotic areas after the end of the latent period and were characterised by their dark pigmented hyphae in the outer wall and by the ostiole. The hyphae of both pathogens are structurally indistinguishable and the pycnidia (in section) and pycnidiospores are the only means by which the two can be differentiated.

*M. graminicola* differs from *L. nodorum* in culture in that pycnidia of the former are never produced on CDV8 agar. The former produced abundant secondary conidia which were initially produced from pycnidiospores and then by a budding process. Yeast-like colonies of secondary conidia were formed which became dark in colour after approximately seven days. Also, on inoculated wheat leaves, pycnidiospores of *M. graminicola* occasionally produced secondary conidia by budding. Although no dense colonies were ever found on such leaves small groups of secondary conidia could be found. It is possible that these may act as a source of inoculum under natural conditions and would have a very much shorter 'latent period' than inoculum comprised solely of pycnidiospores which are only formed after completion of the infection cycle. This hypothesis is supported by the successful use of these secondary conidia as inoculum in
many experiments in which they produced germ-tubes and appressoria in a similar fashion to pycnidiospores. It would be of interest to determine what factors are responsible for the production of germ-tubes by the secondary conidia and also for the budding process which can also occur on host leaves. However, such an investigation was considered to be outside the scope of the current study.

Surface studies of wheat leaves of all four cultivars clearly showed that neither _L. nodorum_ nor _M. graminicola_ are dependent on stomatal openings for entry into the host tissues. Germ-tubes of both pathogens grew into stomata within the aperture of which appressoria were produced with subsequent penetration of adjacent tissues. More commonly, these appressoria were produced at the junction of two epidermal cells. This area forms a shallow and narrow groove on the leaf surface and appressorium production might occur due to an accumulation of leaf exudates in this region. In latter case direct penetration of the cuticle and epidermis occurred resulting in the formation of a halo.

Within the host tissues it has been reported that hyphae of both pathogens ramify intercellularly to colonise the host tissue (CMI, 1966 a,b). These observations are supported by the present study with the exception that some intracellular hyphae were noted in the more resistant cultivars in dead host cells. Sections of infected host tissue clearly showed that cellular disruption occurred in advance of the colonising hyphae of both pathogens. Such a reaction indicated that toxins or enzymes are involved in pathogenesis and this facet of the study will be discussed later.

The sequence of events in infected tissue was difficult to study
in sectioned material. Histological observations revealed that the cells were killed very rapidly in the zone of pathogen colonisation. Also, cellular disruption was evident in advance of the colonising hyphae. Such tissues proved to be difficult to section due to loss of tissue integrity and loss of adhesion to adjacent tissue. Observations suggested that the cuticle and outer epidermal cell walls were enzymatically degraded during penetration. Similar observations were made from sections of tissues at more advanced stages of colonisation. The cell walls in those tissues, excepting those of conducting tissues, appeared distorted and uneven in outline. In more resistant cultivars some variations existed between the thickness of different areas of the same cell wall in which penetration had occurred.

Lonergan (1976) showed that both pathogens are capable of producing cellulose degrading enzymes in culture. _L. nodorum_ was found to be the more prolific producer of such enzymes. It is possible that enzymes of the cellulase complex are involved in penetration of host tissue, by both pathogens. The role of such enzymes may be to predispose the host cell walls to penetration by softening them. Such enzymes could also play an important role in providing nutrients for the pathogens as they ramify through tissues of the host by degrading cellulose to readily absorbable low molecular weight carbohydrates. Both pathogens were found intercellularly in the epidermal and mesophyll cell zones, and in the early stages of infection, before drastic cellular disruption, they were found intracellularly. However, neither pathogen was ever observed in xylem vessels. Some distortion of the conductive tissue did occur, but at much later stages of infection, and this is probably due to the pressure exerted by the developing pycnidia.
In all four cultivars a rapid reaction of mesophyll cells was noted which made it difficult to assess which cellular components were first affected. It has been suggested (Wheeler, 1975) that when chlorosis is the first observable disease symptom the chloroplasts of the host cells are first affected soon after penetration of the host cell. This was supported by this study in that sections of tissues which were fixed when they first became chlorotic revealed that the chloroplasts, in the mesophyll cells had shrunk and become distorted. Infected tissue which was fixed at a slightly earlier stage i.e. before the appearance of chlorosis also showed some distortion of the chloroplasts. At later stages of disease development the mesophyll cells were distorted, shrivelled and had no discernable organelles in their cytoplasm. In necrotic tissues all that could be made out clearly was the still intact xylem vessels and other conductive tissue. All other tissue was distorted and stained darkly with all stains used.

Histological examinations of inoculated leaves of all four cultivars supported the view that both pathogens act as necrotrophs, and as cells were killed well in advance of the invading hyphae it seemed reasonable to suppose that toxins were involved. Attempts were made to extract a toxic principle from culture filtrates of both pathogens by repeating the techniques of Kent and Strobel (1976). The extract, so obtained failed to produce the typical symptoms of L. nodorum and M. graminicola, on leaves of any of the four wheat cultivars. Further attempts at extraction of the toxic principle were abandoned when Scharen (pers. comm.) cautioned that it now appears possible that the toxic effects reported by Kent and Strobel (1976) may have been due to chemicals which had been used in the extraction process.
Baker (1969) and Brockenshire (1975) have both reported that *L. nodorum* and *M. graminicola* can be found in the sub-pericarpic region of the wheat caryopsis. Hyphae of both pathogens, when found in this region were of a larger diameter than those which were seen colonising leaf and stem tissues. Harrower (1976 b) postulated that the mycelium of *L. nodorum* might be located deeper in the caryopsis than merely in the subcarpic zone. The results obtained in this study by sectioning grains which had been inoculated at different stages of heading revealed that the mycelium of both pathogens was located in the region just below the pericarp and in the pericarp itself. Thus supporting the results of Ponchet (1966), Baker (1969) and Brockenshire (1975). No hyphae were apparent in sectioned material in any other part of the grain. By using the embryo separation technique of Simmonds (1946) it was further demonstrated that hyphae of the pathogens were not present either in the embryo or in tissues closely adjacent to it. In many cases an aggregation of pathogen hyphae was found within and below the pericarp in the region of the ventral groove. On no occasion were pycnidia of either pathogen found on the grain and occasionally with *L. nodorum*, seed infection resulted in one or two small brown isolated lesions on various parts of the grain.

Since the pycnidia of both pathogens are produced in the sub-stomatal cavities of necrotic host tissue, it was thought that the number of stomata may play an important role in determining the inoculum potential of such tissue, and also in the event that stomatal penetration could be of importance in determining susceptibility of the four wheat cultivars, the number of stomata per unit area of leaf of all four cultivars was assessed. No significant differences in stomatal frequency between the cultivars was revealed. Significant differences (*P = 0.01*) were found for the upper and lower leaf surfaces and between leaf 1 and 2.
Microscopic observations revealed that pycnidia were arranged linearly in lesions and that, due to the considerable enlargement of pycnidia at maturity, it would generally be impossible for all stomata to bear pycnidia. Pycnidia of both pathogens obtain their nutrients via colonising hyphae which are attached to them and which ramify into adjacent host tissues. It seems reasonable to suggest that pycnidia will not be tightly grouped and will not be closely correlated to stomatal density due to competition between adjacent pycnidia for nutrients.

Due to the fact that direct penetration was observed, the relative thickness of both cuticle and outer wall of epidermal cells was measured for all four cultivars. Often, thick cuticles and epidermal cell walls may render a host resistant either by preventing physical penetration or by greatly increasing the time required for penetration and so causing a prolonged exposure of germ-tubes and appressoria to unfavourable environmental conditions on the leaf surface (Akai, 1959). This may be especially important with L. nodorum as Harrower (1975) has shown that pycnidiospores of this pathogen are extremely susceptible to dessication. However, in this study, no significant differences were found between the combined thicknesses of cuticle and epidermal cell walls in the four cultivars with the marginal exception of Pinnacle and Teal. It is concluded that no physical basis exists in these four wheat cultivars prior to infection which is responsible for the observed differences in susceptibility to the pathogens.

In leaves of all four wheat cultivars there was slight evidence of lignification. Callose production or the deposition of extracellular material in response to infection was not observed. This could be due to the disruptive capabilities of both pathogens being so great that
host cells are unable to initiate or maintain the deposition of materials which would constitute a defence reaction. Clearly, all histological barriers must have a biochemical basis and, once the metabolism of host cells is disrupted, perhaps due to the production of a toxin by the pathogen, then host cells in the affected area will have a decreased vigour and a decreased capability to produce those compounds required for the production of histological barriers.

To test this hypothesis it was decided to use a non-pathogen of wheat which on its normal host, produces similar symptoms as those produced by _L. nodorum_ and _M. graminicola_ on wheat. The species chosen was _Ascochyta pisi_ which is a pathogen of pea (_Pisum sativum_) on which it causes the production of chlorotic and necrotic lesions and in the tissues of which it also grows intercellularly (CMI, 1972). It is a necrotroph with a similar life cycle to both pathogens used in this study. _Epicoccum nigrum_ was also used as a non-pathogen of wheat, it is a common air borne saprophyte and is occasionally present on healthy plants.

As described earlier, wheat leaves of all four cultivars were artificially inoculated with pycnidiospores of _A. pisi_ or _L. nodorum_ or with secondary conidia of _M. graminicola_ or conidia of _E. nigrum_; uninoculated leaves were used as controls. With _A. pisi_ and _E. nigrum_ no symptoms were apparent on infected leaves even one week after inoculation. Occasionally minute chlorotic flecks were seen in response to _A. pisi_ and small reddish brown spots in response to _E. nigrum_. The latter may be some form of resistance mechanism initiated in response to the presence of the fungus and will be discussed later.

Observations made on living epidermal strips clearly showed that
the nuclei of epidermal cells were in constant motion and they slowly
migrated throughout the cytoplasm. Following penetration by the two
non-pathogens, the host cell nucleus moved to a position adjacent to
the tip of the penetrating hypha. These nuclei then gradually enlarged
and became distorted, subsequently disintegrating. On occasions they
shrank prior to disintegrating. Following infection by L. nodorum and
M. graminicola however, the nucleus did not migrate but rapidly enlarged
or shrank and then disintegrated. This behaviour of the host nuclei
may be typical of a susceptible interaction in contrast to the migration
of nuclei to a site adjacent to germ-tubes which has been associated by
several workers (Mathewson, 1977) with a hypersensitive reaction.

A reaction was also noted in the wheat:E. nigrum interaction in
which deposits were observed in sectioned and unsectioned material where
hyphae of E. nigrum came in contact with wheat epidermal cells. This
also occurred in the wheat:A. pisi interaction. It was clearly seen
in sectioned material that hyphae or penetration pegs of the non-pathogens
were embedded in a deposit laid down over them on the inner surface of
epidermal cell wall. Granulation of host cell cytoplasm was also noted
in the wheat:non-pathogen interactions. This may be a similar response
to the hypersensitive reaction which was described earlier. Also, a
dark staining granular material was found in the intercellular spaces of
the mesophyll cells which was produced in response to an attempted
invasion by either of the non-pathogens. This material was thought to
be produced by the adjacent host cells in response to the presence of a
potential pathogen. Although it was not possible to identify this material
it is probable that it acts as an efficient barrier to the diffusion of
fungal metabolites which would otherwise diffuse through tissues causing
metabolic disruption. The forms of histological defence reactions observed
would tend to isolate healthy host tissues from the disruptive activities of the non-pathogens. A similar situation has been described by Mathewson (1977) in his studies of tomato (*Lycopersicon esculentum*) inoculated with a non-pathogenic and pathogenic races of *Phytophthora infestans*.

It has been demonstrated by Rattan (1971) that *A. pisi* can produce enzymes which would cause the same symptoms in wheat as are caused by the two pathogens. It is possible that the metabolites produced by these two pathogens are host specific.

Several studies (Aist, 1976; Bushnell and Bergquist, 1975; Hyde and Colhoun, 1975; Lin and Edwards, 1974; Heath, 1971; Stanbridge *et al.*, 1971; Friend and Knee, 1969; Knoch and Akai, 1969; Leath and Rowel, 1969;) have shown that deposits which in general appearance are similar to those found in this study in response to the non-pathogens may have an important role in resistance. Such structures are termed papillae or lignitubers but their role in resistance is not universally accepted. Their formation may be initiated in response to both pathogen and non-pathogen alike. If formed rapidly they might reduce the amount of diffusible material from the invader which could influence the host cell, perhaps causing a hypersensitive reaction. Several studies have identified callose as the main component of such papillae (Lazarovitz and Higgins, 1975a; Heath, 1971). Other workers such as Ride (1975) have suggested that the main component of the papillae is lignin.

The specific role of callose in the host defence reaction is not clear. It has been found in cells and tissues which had been subjected to mechanical or chemical injury (Currier, 1975; Politis and Wheeler, 1973). Other workers report that it forms in response to infection by
various micro-organisms, (Moericke, 1955; Wu and Dimitman, 1970; Aist and Williams, 1971; Heath, 1971; Hiruke and Shukla, 1973; Petru and Ulrychova, 1974). Muller and Lerch (1957) have proposed that callose is a bi-product of host metabolism due to pathogen invasion. Callose has been identified as β (1-3)-glucan by Aspinal and Kessler (1957). Heath (1971) showed that callose was less permeable to small molecules than were other cell wall components and therefore concluded that it can restrict or prohibit the passage of nutrients to the fungus and also the passage of enzymes and toxins into adjacent host tissue.

The deposits formed on the epidermal cell walls in response to A. pisi and F. nigrum infection reacted with different stains to give a colour reaction indicative of callose production. Some of the colour reactions obtained with the different stains also indicated that the epidermal and mesophyll cell walls were being lignified. At later stages of infection the new cell wall, just below the callose deposit, also showed lignification. Results obtained with Aniline blue and fluorescence microscopy revealed yellow fluorescence that is characteristic of callose. A comparison with the colour reaction obtained from naturally occurring callose in the sieve tubes further confirmed the deposited material as callose. Positive results were also obtained with resorcinol blue, for callose and lignin. Furthermore, autofluorescence revealed lignification of epidermal and mesophyll cell walls in non-pathogen invaded tissue. The colour obtained was the same as for the xylem tissue.

It is possible that lignin and callose are both formed in response to invasion. Callose is deposited around the penetrating hyphae while adjacent cell walls become lignified to prevent the further
spread of metabolites. Ride (1975) has shown that lignification occurs in wheat plants in response to invasion by both pathogens and non-pathogens.

The main conclusions of the study are that the significant differences found between cultivars with respect to the extent of leaf area affected, the sporulation index, the number of pycnidiospores liberated from leaves, and the number of pycnidia produced per unit leaf area suggests that the extent of colonisation of M. graminicola and L. nodorum and the quantity of vegetative mycelium so produced is dependent on some resistance mechanism, of a non-histological type active within the range of cultivars tested. It has recently been shown by Harrower (1978) that, when L. nodorum and M. graminicola are applied in different proportions at the same time, L. nodorum is the better coloniser while in some situations M. graminicola is the more efficient sporulator. Mycelium produced by the pathogens on the death of the host tissue, acts as a nutrient gathering organ for the energy dependent pycnidia. A certain threshold of mycelial proliferation may be necessary for sporulation. Pycnidia are energy sinks dependent on the provision of nutrients from adjacent vegetative hyphae. Energy is lost from the system through the liberation of spores. In L. nodorum (Scharen, 1966; Harrower, 1974) and in M. graminicola (Harrower, unpublished data) cyclic sporogenesis occurs in the pycnidia. This reproductive strategy is energy demanding and may in part, explain the different latent periods observed in pathogen cultivar combinations which in themselves have been used as an estimation of the degree of susceptibility.

No evidence of histological barriers to infection were initiated
in response to invasion by either _M. oramnicola_ and _L. nodorum_. This is probably due to the rapid and disastrous metabolic disruption of host cells both at the site of infection and adjacent to that site which effectively stops them from initiating or completing the formation of mechanical barriers. Both species cause a gross disturbance of cell function, a loss of tissue integrity and rapid cell death. Such tissue would be incapable of completing the formation of histological barriers, which could arrest or retard the colonisation of those pathogens. In contrast, in the wheat non-pathogen situation the level of disturbance is not severe. Although the cells at the point of infection may be killed soon after, few cells in the areas adjacent to infection are metabolically disturbed so rapidly that they cannot initiate a reaction either of the biochemical type or of the histological type which itself would require some biochemical activity.

Although the role of callose and other histological phenomena as observed in response to non-pathogens is not clear, the cell wall thickenings, the granular deposits and the callose deposits in response to invasion by _A. pisi_ and _F. nigrum_ must have some function. The results support the conclusions of Heath (1971), Bushnell and Bergquist (1975) and Aist (1976) in that they arrest the growth of the invading fungus by preventing it from utilizing nutrients from the immediate vicinity and also prevent the diffusion of fungal metabolites from entering the host tissue.

No histological features were found which could explain the differential levels of susceptibility of the four wheat cultivars used in this study. It is likely that phytoalexins or other biochemical
resistance mechanisms may act against the two *Septoria* species. It is possible that the two least susceptible cultivars, i.e. Teal and Fleche d'Or, may be able to partly inhibit or neutralize the effects of toxins or enzymes produced by *L. nodorum* and *M. graminicola*. Morgan (1974) and Baker and Smith (1977) have reported antifungal compounds active against *L. nodorum* in some cultivars of winter wheat.

Future work should involve further studies of the activities of non-pathogens on wheat to ascertain what limits their pathogenicity. Also further studies on the biochemical defences which occur in infected tissues might define what factors limit lesion expansion in resistant cultivars. Studies by Farkas and Kiraly (1962), Lazorovits and Higgins (1975, a&b) showed that phenolic compounds which inhibited sporulation of the pathogen accumulated in infected tissues as a result of resistant reactions. Histochemical tests of infected areas would show whether Teal, Fleche d'Or and other similar cultivars accumulate more phenolics than the susceptible cultivars. Also, because of the ease with which a hypersensitive type of reaction occurs in the wheat cultivars studied, the interactions between infection by biotrophs and *Septoria* fungi might be worth studying. For example, attempted infection by a rust, resulting in many hypersensitive sites could predispose the host to a higher level of infection by *M. graminicola* and *L. nodorum* then would otherwise occur.

Although attempts were made to isolate a toxic principle from both pathogens using the techniques of Kent and Strobel (1976) no conclusive results were obtained concerning the role of toxins in pathogenesis. It seems worthwhile to undertake further studies on the role of toxins in disturbing the metabolic processes which themselves are important in the determination of effective resistant mechanisms.
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