THE HISTOLOGY OF LOOSE SMUT RESISTANCE IN BARLEY

by

Brad Gabor

A thesis
presented to the University of Manitoba
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ABBREVIATIONS

CW-cell wall
EA-embryo axis
EDL-electron dense layer
EDM-electron dense material
F-foliage leaves
FWL-fungal wall layers
GP-growing point
H-hypha
HC-host cytoplasm
HP-hyphal protoplasm
HVC-heavily vacuolated cell
IH-intracellular hyphae
L-lipid
M-mitochondria
N-nucleus
NA-necrotic area
NC-necrotic cell
NM-nuclear membrane
NU-nucleous
NP-nuclear connection
OE-outer epidermis
P-pericarp
PB-protein body
PL-host plasmalemma
S-sheath
SC-secondary thickening of host cell wall
SM-scutellum
V-vacuole

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ABSTRACT

The barley cultivars Warrior, Conquest, and CI13662 (containing resistance genes Un1, Un3 and Un6, and Un8 respectively), were inoculated with the loose smut (Ustilago nuda) lines 72-66 and 72-146 (containing virulence genes Unv2 and Unv1 respectively), in order to compare the sites and mechanisms of the resistance reactions conferred by the different resistance genes.

A modified version of the Scottish embryo test was used for the preliminary histological observations of the embryos. Glycol methacrylate embedded embryos were stained with Toluidine blue and Calcofluor White M2R for observation under incandescent and fluorescent light microscopy respectively. Finally, Spurr embedded embryos were stained with uranyl acetate and lead citrate for examination in the electron microscope.

Resistance appeared to occur at a different site in each of the three cultivars and probably was the result of different mechanisms. In Warrior a low level of embryo infection (3%) occurred with no subsequent sporulation in the adult plants. This suggested that resistance was conferred before embryo infection. A necrotic reaction observed in the pericarp of Warrior in association with fungal penetration,

has been suggested to be the mechanism of resistance in a cultivar related to Warrior. However, this necrosis was found after inoculation of Warrior with both virulent and avirulent lines, suggesting that it confers little if any resistance in the cultivar.

In Conquest and CI13662 resistance was expressed after embryo infection because 50% or more of the embryos contained hyphae after inoculation, without subsequent sporulation in the adult plants. The host and fungus were apparently in a compatible relationship until the seedling stage in Conquest and until the fungus penetrated the embryo axis in CI13662.

In CI13662 an "incompatible" reaction occurred where the fungus penetrated into the embryo axis and growing point regions, and consisted of necrosis of cells varying from a few to many affected cells. This reaction appeared to confer at least a certain level of, if not complete resistance. It was not known whether the fungus was able to grow beyond the site of the incompatible reaction, or whether resistance was expressed in the developing seedling.

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Chapter I

INTRODUCTION

Loose smut caused by Ustilago nuda (Jens.) Rostr. is a disease of barley (Hordeum vulgare L.) that results in the inflorescence of the plant being largely replaced by sori containing teliospores of the fungus (Fisher and Holton, 1957).

The yield loss caused by U. nuda is approximately equal to the percentage of plants with sporulation in the field (Morton, 1961). On the Canadian prairies this yield loss resulted in approximately an eight million dollar loss to the producer in 1981 (Thomas, 1984).

Control of loose smut can be achieved by using chemical seed treatments, but this raises environmental concerns and increases the producers' costs. An alternative form of control is the use of resistant cultivars (Thomas, 1984), hence the need to understand the basis of resistance that results in prevention of expression of the disease in the field.

Resistance is defined as "the ability of an organism to withstand or oppose the operation of or to lessen or overcome the effects of an injurious or pathogenic factor, or the ability of the host to suppress or retard the activity

of a pathogenic organism" (Federation of British Plant Pathologists, 1973). Resistance in relation to the literature presented here refers to the prevention of the completion of the life cycle of the loose smut organism. As will be seen later, the resistance reaction occurring between the host and pathogen is dependent on the particular host and pathogen involved in the interaction. Given these different interactions, it is of interest to know whether different genes in the host confer resistance at the same or different sites, by the same or different mechanisms, and at what stage of the life cycle. In order to answer these questions a comparison of loose smut resistance conferred by the resistance gene(s) Un1 in Warrior, Un3 and Un6 in Conquest, and Un8 in CI13662 was undertaken. The site and mechanism of the resistance reaction was studied using both light and electron microscopy techniques.

Chapter II

LITERATURE REVIEW

2.1 THE CULTIVAR RESISTANCE GENES (SEE TABLE 1)

2.1.1 Gene Un1

In 1942, Livingston reported that resistance in the cultivar Trebi was controlled by a single gene, which was later shown to be genetically dominant to an allele for susceptibility (Schaller, 1949; Skoropad and Johnson, 1952). This gene was designated Un1 (Robertson et al. 1947). The cultivar Warrior is a single cross derivative of the cultivar Trebi and therefore probably also has resistance gene Un1 (Thomas, 1974b). The Un1 resistance gene effectively controlled loose smut until 1946, when its resistance was overcome by new strains of the fungus (Thomas, 1974b).

2.1.2 Genes Un3 and Un6

The cultivar Jet has one dominant gene conditioning resistance to some races of U. nuda (Metcalfe, 1962; Metcalfe and Johnston, 1963; Schaller, 1949; Skoropad and Johnson, 1952; Tapke, 1955), and the same gene, plus another independently inherited dominant gene conditioning resistance to other races (Konzak, 1953; Larter and Enns, 1962; Metcalfe

and Johnston, 1963). These genes have been designated Un3 and Un6 (Skoropad and Johnson, 1952). Conquest has the cultivar Jet in its background, and therefore probably derives loose smut resistance genes from Jet (Moseman and Metcalfe, 1969). Thomas (1974a) reported that three 1972 field collections of U. nuda had the ability to complete their life cycle in Conquest and other cultivars derived from Jet.

2.1.3 Gene Un8

Metcalfe (1966) reported that resistance in PR28, which was derived from Milton, (CI4966) (Moseman and Metcalfe, 1969), was due to a single and dominant gene Un8, which was inherited independently from other previously described resistance genes. CI13662 is also known to possess the gene Un8 from Milton (Thomas and Metcalfe, 1984). Cultivars with the gene Un8 are currently resistant to all collections of loose smut to which they have been tested (Thomas, 1984).

2.2 THE PATHOGEN VIRULENCE GENES

2.2.1 Genes Unv1 and Unv2

Thomas (1983) reported that the F1 progeny, from the hybridization of culture 72-66 (virulent on cultivars with Un3 and Un6, avirulent on cultivars with Un1 and Un8) and culture Buff No.1 (virulent on cultivars with Un1, avirulent on cultivars with Un3 and Un6, and Un8) were avirulent on cultivars containing the Un1, the Un3 and Un6, and the Un8 re-

sistance genes. This indicated that 72-66 and Buff No. 1 were recessive for virulence on cultivars containing these genes. Virulence in 72-66 was inherited independently from that in Buff No. 1. The genes controlling this virulence were designated Unv2 and Unv1 respectively (Thomas, 1983). The virulence pattern of 72-146 is the same as that of Buff No. 1 (Thomas, 1974a; Thomas, 1983; Thomas and Metcalfe, 1984). This suggests that 72-146 contains the same virulence gene (Unv1) as Buff No. 1 (see Table 1).

TABLE 1

Virulence of two lines of Ustilago nuda on three cultivars of barley

Smut Lines and Virulence Alleles	Cultivars and Resistance Alleles		
	WARRIOR Un1	CONQUEST Un3 and Un6	CI13662 Un8
72-66 (v2)	avirulent*	virulent	avirulent
72-146 (v1)	virulent**	avirulent	avirulent

* avirulent indicates no sporulation in the adult plants.
** virulent indicates sporulation in the adult plants.

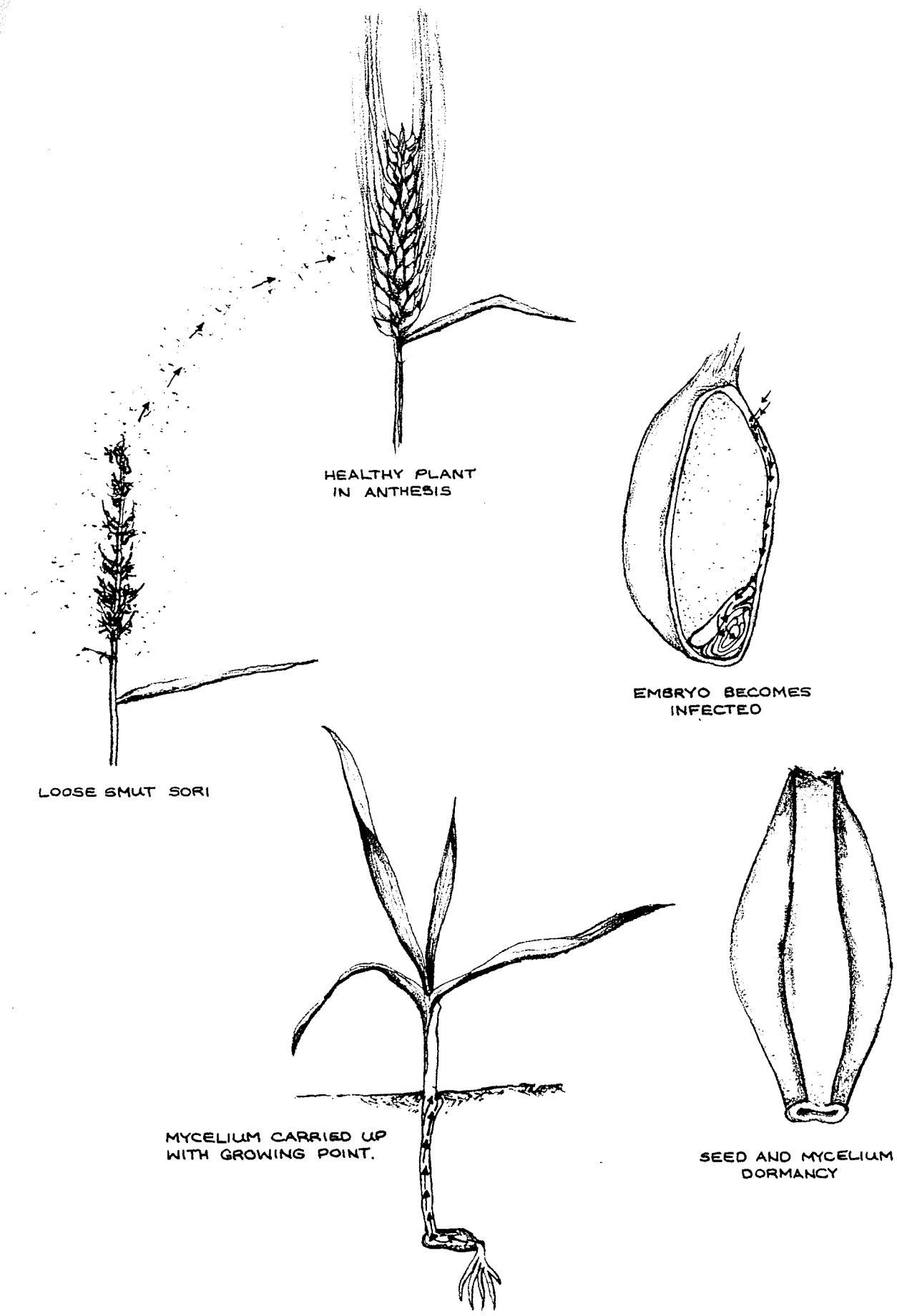
2.3 THE LIFE CYCLE OF LOOSE SMUT (SEE FIGURE 1)

A review of the literature on resistance to loose smut of barley, U. nuda, should include resistance to loose smut of wheat, (U. tritici (Pers.) Rostr.), because the two diseases are similar and have been classified under the same taxon (Ainsworth and Sampson, 1950; Fischer, 1943). Indeed, the

life cycle and infection pathway of both of these loose smuts are identical (Shinohara, 1976). However, recent biochemical and genetic studies by Kim et al. (1984) and Nielsen (1978), supports their classification as distinct species.

Shinohara (1976) has recently reviewed the life cycle of U. nuda. Teliospores are wind disseminated from the infected head of the adult plant, at or near the time of anthesis in the surrounding healthy plants. The spores land in the open florets of the susceptible host, where they germinate producing a promycelium in which meiosis occurs. Four haploid cells are then produced by septation. Cells of compatible mating types, or the hyphae produced by them, then fuse to form the parasitic dikaryotic hyphae. The dikaryotic hyphae then penetrates the pericarp of the developing kernel, completely infecting the embryo at the time of seed maturity . The mycelium in the infected embryo becomes dormant coincident with seed dormancy. When seed germination occurs, the mycelium is stimulated to grow into the growing point, if it is not already there, and is then carried up with the developing shoot to produce the loose smut sori in the floral parts of the plant.

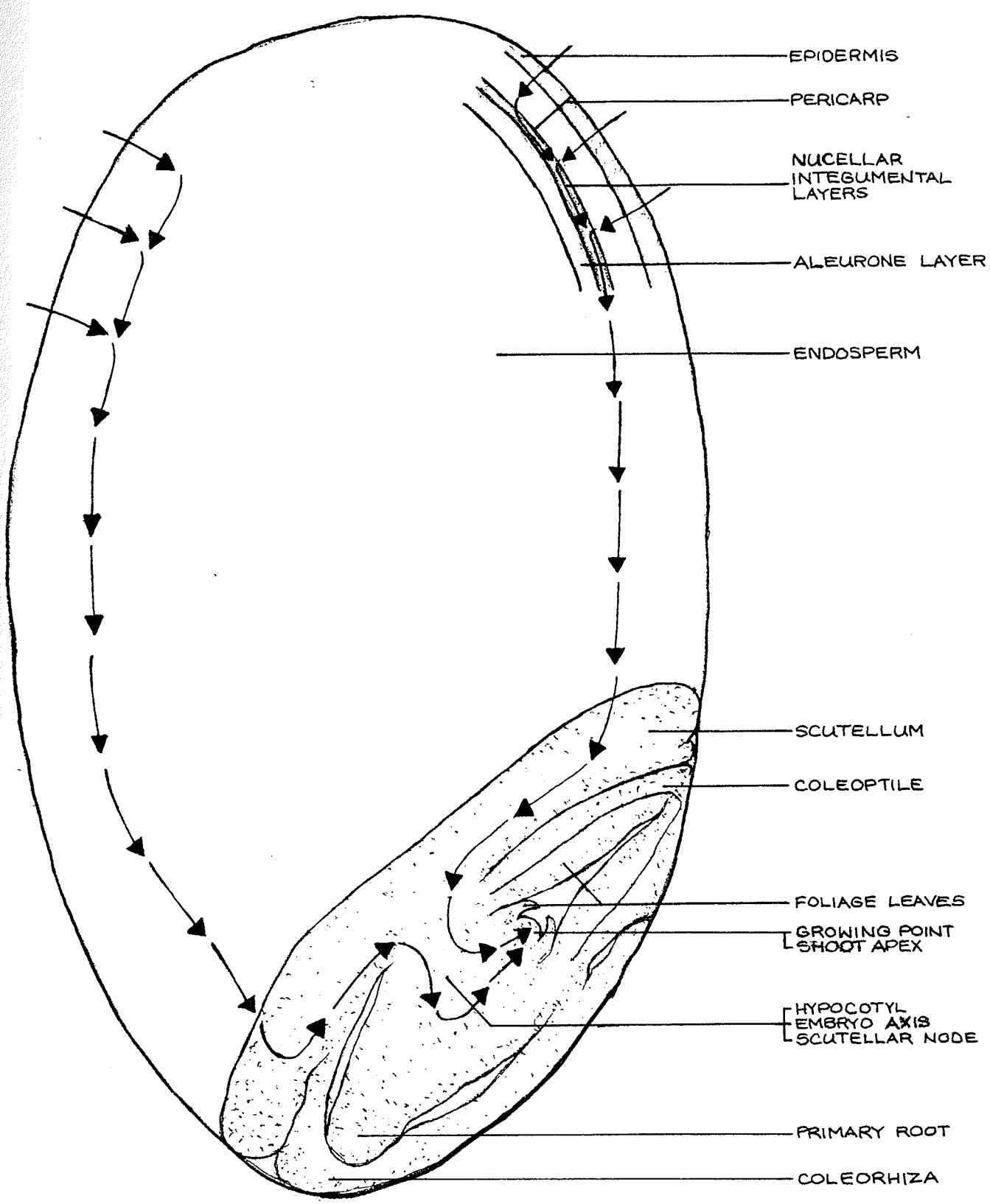
FIG. 1. THE LIFE CYCLE OF USTILAGO NUDA



2.4 THE INFECTION PATHWAY OF LOOSE SMUT (SEE FIGURE 2)

The infection of the susceptible host by loose smut has been studied previously (Batts, 1955; Malik and Batts, 1960; Pedersen, 1956; Shinohara, 1972; Shinohara, 1973). The dikaryotic hyphae penetrate into the pericarp at the distal end of the ovary via appressoria. Once through the outer epidermis the hyphae grow directly into the nucellular and integumental layers, at which point they turn sharply and proceed towards the embryo. The hyphae grow through the scutellum after reaching it, and then into the embryo axis and growing point region of the embryo. Growth in the pericarp has been reported to be mainly intracellular, but intercellular in the integument and embryo. Hyphae seldom enter the endosperm and even then penetration is only slight.

FIG. 1. THE INFECTION PATHWAY OF USTILAGO NUDA



2.5 LOOSE SMUT GROWTH IN BARLEY AND WHEAT

Popp (1951), studying the reactions of several wheat cultivars to different races of loose smut, reported that cultivars with noninfected embryos would invariably give rise to adult plants free from loose smut. Cultivars with infected embryos would give rise to either a corresponding number of sporulating adult plants, give rise to a lower number of sporulating adult plants, or give rise to smut free adult plants, depending upon the cultivar and the particular race of loose smut to which it was inoculated. The stage at which resistance was expressed therefore varied from before the hyphae reached the embryo, to the developing seedling stage, or to various later stages of development. It was concluded that two factors were operative in determining the resistance of wheat cultivars to loose smut. The first resides in the embryo tissue and either prevents entirely the invasion of smut hyphae or inhibits the development of hyphae that gain entrance. The second becomes operative in the developing seedlings and prevents or greatly reduces hyphal development, resulting in little to no sporulation in the adult plants.

In a study by Batts and Jeater (1958), the proportion of wheat embryos infected and the percent adult plants with sporulation were closely correlated. However, other cultivars were reported to be embryo susceptible and field resistant, that is the scutellum was permeated with hyphae but

there was no sporulation in the adult plants. Upon close examination it was noted that the hyphae did not pass from the scutellum to the growing point and hence no spores were produced from infected plants in the adult stage. The cultivars were described as scutellum susceptible because the embryo as a whole was not susceptible. Only one of the embryos examined had hyphae in the region between the scutellum and the hypocotyl, and the host cells in this region appeared swollen and brown in color. The hyphae did not penetrate beyond these affected cells.

Batts and Jeater (1958) were supported by Mantle (1961a), who reported the same type of embryo susceptible/field resistant reaction in some of the wheat cultivars he studied. He noted there was no consistent relationship between the amount of mycelium in the embryo and the number of sporulating adult plants produced. Sporulation of adult plants in the field may result from a slight amount of mycelium in the embryo, and field resistance may be associated with moderate to heavy amounts of mycelium in the embryo.

Popp (1959) correlated the percentage of embryos with infection in the plumular bud with the percentage sporulation in the adult plants. He noted that resistant cultivars frequently had hyphae present in the embryo, however the plumular bud was not infected and therefore adult plants remained smut free.

Kiraly and Lelley (1956) noted that some Hungarian wheat cultivars produced seedlings that were stunted and subsequently failed to produce sporulating adult plants. Histological examination of stunted seedlings revealed that the loose smut hyphae could be found in the culm only as high as the third node. This type of resistance was referred to as a hypersensitive reaction. Hypersensitivity is defined as "the violent reaction of an organism to attack by a pathogenic organism, resulting in prompt death of invaded tissue, thus preventing further spread of infection" (Federation of British Plant Pathologists, 1973). Mantle (1961b) suggested that because there was no mention of necrotic cells in the reaction reported by Kiraly and Lelley (1956), that it should not be referred to as a hypersensitive reaction.

A detailed study of the resistance reaction of the wheat cultivar Kota to three races of loose smut was carried out by Mantle (1961b). In the seedlings, resistance was defined as a reduction in the leaf length and a dark green color accompanied with chlorotic striping, spiral twisting, and curvature of the blade in the flat plane, and sometimes cancerous proliferations of uninfected parenchymatous tissue near the growing point region. Kota seedlings were inoculated with the three races of loose smut, and those with infected scutella were divided into three groups. Group one consisted of normal seedlings with no leaf chlorosis, in which hyphae were considerable and confined to the scutellum. The plants

produced normal uninfected heads. Group two consisted of plants with hyphae in the crown but not in the growing point region. These seedlings tended to be less than half the size of uninfected seedlings due to almost complete inhibition of the third internode extension. Hyphae were often associated with the crown node but did not subsequently penetrate further than the third node. Seedlings in this group tended to recover by the main axis. Group three always had hyphae in the apical growing point and showed the most severe symptoms. This group had slow emergence, accompanied by a reduced third internode extension. Infected apical growing points tended to cease growth in the seedling stage and uninfected tillers would develop to produce healthy spikes in those seedlings that did not die.

The classification of the Kota seedlings was dependent on whether hyphae were confined to the scutellum, partly permeated into the plumular bud, or had reached the growing point. Mantle (1961b) suggested the presence of the fungus may inhibit cell division and reduce cell extension, or possibly inhibit growth by the production of some inhibitory metabolite. Necrosis was not associated with the presence of the fungus in the stunted seedlings. This absence of internal necrosis suggested that subsequent death of the main axis, when the apical growing point was infected, was due to secondary infections by other organisms such as Fusarium spp., etc. (Mantle, 1961b).

Ohms and Bever (1955) investigated the reaction of two winter wheat cultivars to loose smut infection. The cultivar Kawvale had no seedling mortality, hyphae were mostly confined to the scutellum, and elongation of the third internode was reduced. In the cultivar Wabash, infection was accompanied by a severe seedling mortality at the three to five leaf stage. Statistical analysis revealed that the seedlings which died were probably infected. The seedlings which did not die were mostly uninfected. This reaction was termed "over susceptible" and was defined as a false type of resistance. Seedling death was probably due to a reduced resistance to infections by soil organisms.

Seed mortality has also been reported in susceptible barley cultivars (Wells and Platt, 1949). A portion of the embryos were almost completely destroyed by the presence of the loose smut hyphae in the embryos, and the seeds subsequently failed to germinate. Surviving seedlings produced normal sporulating heads.

Gaskin and Schafer (1962) reported differences in the resistance of five winter wheat cultivars to race 6 of U. tritici. Hyphae in the cultivar Hope-Hussar invaded the pericarp and the edge of the endosperm but not the embryo. In the cultivars Kawval, Tremezino and CI191533 hyphae had penetrated the growing points of the embryos. Resistance was expressed between the two and four week old seedling stage. Resistance in Kawval, and Tremezino was absolute by the four

week stage. In CI191533, the proportion of seedlings infected (99%), was dramatically reduced by the four week stage (33%), but a low level of infection persisted to produce spores in the adult plants (16%). In the cultivar Rieti, resistance was mainly expressed between the embryo stage, where 85% of the embryos were infected, and the two week old seedling stage where 25% of the seedlings had hyphae in the growing point. Growth of the hyphae persisted in a proportion of these plants, with 6% developing sori.

Hewett (1979) reported that two types of resistance to loose smut could occur in barley. In one case five cultivars had a high level of embryo infection but sporulation was rarely noted in the adult plants. In the other case, one cultivar had only a few infected embryos and hence the number of infected adult plants was again low. This suggested to him that resistance occurs before embryo infection in some cultivars and after in others.

Rasmusson and Mumford (1961) reported that the cultivar Jet had 36% infected embryos after floral inoculation but produced no sporulation in the adult plants. In contrast, the cultivar Trebi had only 6% of its embryos infected, again with no sporulation occurring in the adult plants. A further study by Mumford and Rasmusson (1963), using three different loose smut races, verified the results for Jet and Trebi. Susceptible cultivars used as controls had high levels of infection in both the embryos and adult plants. A

histological study of the young seedlings of the cultivar Jet revealed that infected shoots and tillers failed to develop, while noninfected tillers developed to produce healthy plants. Mumford and Rasmusson (1963) suggested that the Jet type of resistance involved two mechanisms. Firstly, a more general type of resistance was expressed before embryo infection, which would account for the 50% level of embryo infection in Jet, compared to 90% in the susceptible cultivar Newal, and secondly, resistance was expressed after embryo infection at the young seedling stage, approximately three weeks after germination.

Hewett (1980) demonstrated a good correlation between the percent embryo infection and the percent sporulation occurring in adult plants of winter barley.

In summary, the reactions of barley and wheat to their respective loose smuts can be very complex. In hosts inoculated with the virulent line the number of infected embryos was closely correlated to the number of sporulating adult plants. In hosts inoculated with the avirulent line, growth of hyphae can be stopped before embryo infection. Alternatively, infected embryos can produce adult plants with a low level of , or no sporulation. Fungal growth can end abruptly at a specific site, eg. the pericarp, the embryo, in the seedling stage, or it can end at different stages over the subsequent development of the plants, even going on to produce sporulation in a small percentage of the adult

plants. The presence of the fungus can have little, moderate, or a severe effect on the health of the plant, at any stage from the embryo to the adult plant.

2.6 STRUCTURES FORMED IN INFECTED PLANTS

Batts (1955) reported that loose smut of wheat penetrated the outer epidermis of susceptible cultivars via an appressorium under which a funnel shaped structure developed. Sheathing of the internal hyphae was common. Appressoria were again formed at the point of penetration into cells within the pericarp.

Pedersen (1956) noted that in susceptible barley, hyphae penetrated via appressoria, and grew intercellularly and intracellularly through the pericarp to the integumental layer, and formed no structures upon cell penetration. After a few days the hyphae grew randomly in the pericarp, and produced bulbous structures which appeared to aid in cell penetration. It was suggested that the structures may have been in response to a greater resistance offered by the cells at later stages of development.

Shinohara (1973) noted that appressoria were formed at penetration points, under which funnel shaped, lignituber like structures were formed. Hyphae in the pericarp were enveloped in sheaths which were suspected to be of host composition. Hyphae at the integument, formed pear-shaped struc-

tures, which appeared to augment passage through the integument to the nucellar layer. Shinohara noted two places where incomplete resistance may occur. First, at the outer epidermis as noted by the funnel shaped structures and second at the integumental layer as noted by the pear-shaped structures.

In contrast to the structures reported in the susceptible host by Batts (1955) and Pedersen (1956), there were no funnel shaped structures present in the barley cultivar Trebi inoculated with an avirulent race of loose smut. Host cells were seldom invaded without necrosis of the cells in areas where penetration occurred (Shinohara, 1974).

Malik and Batts (1960) reported that in the susceptible barley cultivar Carlsberg, numerous peg like structures were formed when the hyphae penetrated the epidermis and that the hyphae penetrated the pericarp via appressoria. Sheathing of hyphae was common. Intracellular growth was common in the pericarp and testa, and appressoria were formed on each host cell wall at the point of penetration. Hyphae were mainly intercellular in the scutellum and embryo. In the cultivar Carlsberg the peg like structures were the only structures associated with penetration of host cells by hyphae. In contrast, the cultivars Plumage Archer and Proctor reacted with browning and death of three to four epidermal cells at and around the point of infection. Except for this reaction, the histology of the infection appeared identical to that in

Carlsberg. The cells in the scutellum and embryo of the cultivars Plumage Archer and Proctor appeared to share a compatible relationship with the hyphae, showing no signs of distortion or malformation.

In summary, the hyphae enter the host via appressoria, under which lignitubers (funnel shaped structures) form, which may or may not prevent the entrance of the hyphae into the epidermal cells. Bulbous structures may occur within the pericarp at the point of cell penetration. Hyphal sheathing is common, and host cell necrosis can occur in the pericarp at and around the point of penetration of host cells.

Lignituber formation and mycelial sheathing have been reported in susceptible barley and wheat hosts infected with loose smut. This suggests that in those hosts, resistance offered by these structures is of little if any value (Batts, 1955).

Fullerton (1970) used the electron microscope to study the fine structure of the relationship of 11 species of inflorescence infecting smut fungi (not including U. nuda or U. tritici) on their respective susceptible hosts. He showed that intracellular hyphae were surrounded by an "encapsulated area" extending back from the apical region. Encapsulation refers to any simple or complex structure between the plasmamembranes of the host and parasite, which is neither host nor fungal cell wall. Material similar in appearance to

the host cell wall was deposited into the encapsulation by what appeared to be reverse pinocytosis, eventually developing a sheath around the hyphae. Ensheathed hyphae eventually degenerate. Whether this degeneration was a result of sheath formation or a normal process of senescence is not known. Contrary to the results of Pedersen (1956) and Shinohara (1973), Fullerton (1970) found no special structures, such as appressoria or infection pegs, prior to penetration of host cells. Intracellular hyphae ranged from hyphal branches of limited development to hyphae which branched or passed from cell to cell. It was suggested that hyphae that terminated in host cells may play a similar role to haustoria.

Chapter III

MATERIALS AND METHODS

The barley cultivars Warrior (CI6991), Conquest (CI11638), and CI13662 were used as hosts in this study. The lines of U. nuda were 72-66 and 72-146 (Thomas, 1974a). Table 1 shows the cultivars and the resistance genes that they contain, the loose smut lines and their virulence genes, and the interaction that is normally observed after inoculating the three cultivars with the two lines of smut.

The barley florets were inoculated at anthesis, after the anthers had turned yellow but before the kernel had filled one third of the floret. An aqueous spore suspension (1 g/L) was injected into the florets using a 5 mL syringe with a 22 or 24 gauge needle as described by Thomas (1976). All plants were grown in growth cabinets and/or the greenhouse.

3.1 CONTROLS

The virulence patterns shown in Table 1 were verified by growing seeds from inoculated plants to maturity in the greenhouse, and the proportion of adult plants with sporulation was recorded. All spikes with sori were collected in paper envelopes, and a representative sample of teliospores from each envelope was smeared on complete medium agar (Vo-

gel, 1956) in petri dishes for a germination test. This test was made to rule out any contamination by false loose smut, U. nigra (Tapke). U. nigra has a sporidial type of growth on complete medium agar, compared to U. nuda, which has the hyphal type of growth (Tapke, 1941). The germination test is the only reliable means of distinguishing between these two species (Thomas, 1981).

The teliospores collected from this control study were used in all subsequent experiments.

3.2 EMBRYO TEST

Embryos from inoculated plants were extracted and examined using the "Scottish Method" described by Khanzada et al. (1980). The following describes the method and any modifications made to it. Seeds were soaked for approximately 22 h at room temperature in a 5% sodium hydroxide solution containing 15 mg/100mL Trypan blue (cat. no. 508, Allied Chemical). The sodium hydroxide solution causes the endosperm to swell and therefore helps separate it from the embryo. Complete separation of the embryo from the rest of the seed was facilitated by agitation in a 1 L beaker in water at approximately 50 C. The separated embryos were then flushed through a 10 mesh sieve with hot water (50 C) and caught on a 40 mesh sieve. The extracted embryos were then dehydrated in 95% ethanol for 2 min. and transferred to a funnel containing a 3:1 solution of lactophenol:water. The

embryos remained afloat while most remaining chaff sank to be run off through the bottom of the funnel with the aid of a rubber tube and a clamp. The lactophenol:water solution was filtered through a screen and used to flush the embryos several more times to remove all of the chaff. The embryos were then just covered with pure lactophenol in a beaker and boiled for 2 min. to clear the tissues. After clearing, the embryos were quickly rinsed in distilled water to remove the lactophenol, transferred into glycerin for 2 h, and mounted in glycerin for viewing. The Trypan blue stained mycelium was readily visible in the embryos at 6 to 40X magnification, allowing unambiguous classification of embryos into infected and non-infected classes.

3.3 PERICARP STUDY (FLUORESCENCE MICROSCOPY)

The outer epidermis of developing barley kernels was examined at approximately 48 h intervals for 12 days after inoculation. The developing kernels, less the lemma and palea, were stained in a 0.01% solution of Calcofluor White M2R (cat. no. 4359, Polysciences, Inc.) in distilled water for 3 min., followed by two rinses, 1 min. each, in distilled water. The kernels were mounted in distilled water on glass slides, and examined with a Zeiss incident light fluorescence microscope equipped with the HBO 50 light source, exciter filter BP 405/6, dichromatic beam spliter FT 425, and barrier filter LP 435.

Examination of the necrotic areas in the pericarp required an alternate method of preparation because non-specific staining occurred in sections prepared by the above method. The method of Rohringer et al. (1977) was used and modified as follows. Sections approximately 0.5 mm thick were cut by hand with a razor blade through the necrotic areas of the kernels. These sections were fixed 16 h in a lactophenol:ethanol solution (1:2, v/v) at room temperature. Sections were then washed twice for 15 min. in 50% ethanol, twice for 15 min. in 0.05 M sodium hydroxide, three times for 10 min. in distilled water, and placed in 0.1 M Tris/HCl buffer, pH 8.5, for 30 min. The sections were then stained for 1 min. in 0.1% Calcofluor White M2R in the same buffer, washed four times for 10 min. in distilled water, washed for 30 min. in 25% aqueous glycerin and mounted on glass slides in pure glycerin containing a trace of lactophenol. Sections mounted in glycerin can be stored for at least two years without degradation. Sections were examined using the Zeiss incident light fluorescence microscope as described above.

3.4 EMBRYO STUDY (INCANDESCENT AND FLUORESCENCE LIGHT MICROSCOPY OF GMA EMBEDDED EMBRYOS)

Mature seeds of Conquest and CI13662 inoculated with 72-66 and 72-146, and the uninoculated controls, were imbibed with distilled water for approximately 15 h at room temperature. A 1 mm thick longitudinal section was made through the growing point of the embryos using a razor blade. Non-embryo material was trimmed away and the embryo

sections were fixed in 3% glutaraldehyde (JBS-Chem, J.B.E.M. Services Inc.) in 0.025 M phosphate buffer, pH. 6.8, for 16 h at 3 C, after vacuum infiltration for approximately 15 min., to sink the sections to the bottom of the fixative vials. After fixing, the sections were washed in the phosphate buffer six times over the next 24 h, then transferred through an ethanol dehydration series (20, 40, 60, 80, 90, 95, and 3X 100%) with 30 min. changes, at ice water temperature, brought to room temperature and washed twice in molecular sieved ethanol. Glycol methacrylate (GMA) was prepared by mixing 93 g of 2 hydroxyethal methacrylate, (cat. no. 3699, Polysciences Inc.) with 9 g Carbowax 400 (Polyethylene Glycol 400, J.T. Baker Chemical Co.), and 0.2024 g benzoyl peroxide (J.T. Baker Chemical Co.). The sections were transferred through a graded ethanol:GMA series (3:1, 1:1, 1:3) using 4 h intervals. The sections were held in GMA for 3 weeks, with one change at 24 h. The GMA was polymerized at 60 C for 24 h in an oxygen free, nitrogen flushed vacuum oven. The polymerized blocks were sectioned on an LKB 11800 pyramitome. Sections were mounted on glass slides by placement on a drop on 10% acetone which was then evaporated on a warming plate. Sections were stained with 1% Toluidine blue (The Coleman and Bell Co.) in 1% borax for 5 s, or 0.1% Calcofluor White M2R for 3 min. and mounted in glycerin for observation with the Zeiss incident light fluorescence microscope equipped with the HBO 50 light source, exciter filter BP 436/8, dichromatic beam splitter FT 460, and LP 470 barrier filter.

3.5 EMBRYO STUDY (ELECTRON MICROSCOPY)

Material for electron microscopy was fixed in glutaraldehyde and washed in phosphate buffer as previously described. Sections were then post-fixed for 5 h in 2% osmium tetroxide (JBS-Chem, J.B.E.M. Services Inc.), given four washes in distilled-deionized water over 2 h, and passed through the previously described ethanol dehydration series. Sections were then passed through a propylene oxide:ethanol series (1:3, 1:1, 3:1 and 2X 1:0) in 30 min. intervals, before embedding with Spurr resin mixture (Spurr, 1969). The Spurr formula used was, 20 g vinylcyclohexene dioxide (VCD, cat. no. 0280, Can-EM Chemicals), 16 g diglycidyl ether of polypropylene glycol (DER 736, cat. no. 0249, Can-EM Chemicals), 52 g nonenylsuccinic anhydride (NSA, cat. no. 0270, Can-EM Chemicals), and 0.8 g dimethylaminoethanol (DMAE, cat. no. 0242, Can-EM Chemicals). The Spurr resin mixture was polymerized for 16 h at 60 C. Ultrathin sections were cut with a glass knife using a Reichert OM-U2 ultramicrotome. All sections were taken at the growing point region, mounted on formvar and carbon coated 100 mesh copper grids, and stained with 5% uranyl acetate in 50% ethanol aqueous solution for 10 min., then rinsed by 20 dips into each of four beakers of distilled water, and stained in lead citrate for 6 min. followed by a repeat of the same distilled water rinse. All electron microscope examinations were carried out with a Phillips 420 transmission electron microscope at an

accelerating voltage of 80 kV. Electron images were taken on Kodak 3 1/4 X 4 in. sheet film.

Chapter IV

RESULTS

4.1 EMBRYO TEST

Florets of Warrior inoculated with 72-66 (Table 2) had only 3% of the embryos with detectable hyphae. All other cultivar/strain combinations gave at least 50% of the embryos with detectable hyphae. Sporulation only occurred in adult plants of Conquest from seed from 72-66 inoculated florets and adults of Warrior from seed from 72-146 inoculated florets.

TABLE 2

Percentage of infection observed in embryos and the % sporulation observed in adult barley plants after inoculation with Ustilago nuda

U. nuda lines	% embryos/adult plants observed Warrior	Conquest	CI13662
72-66	3/0	51/49	81/0
72-146	84/90	50/0	58/0

Examination of infected Conquest embryos with a stereomicroscope showed the mycelium along a pathway through the scutellum, down into the embryo axis and then to the growing

point. (Fig. 4). There was no visible difference between 72-66 and 72-146 in the amount or the position of the hyphae in infected embryos of Conquest. No hyphae were detected in healthy, non-inoculated embryos (Fig. 3).

There appeared to be a compatible relationship between the host tissue and the fungal hyphae in Conquest and Warrior inoculated with both 72-66 and 72-146. Embryos of Warrior infected with 72-146 had the same amount and positioning of hyphae as those of Conquest infected with this line (Fig. 5). However, only 3% of the embryos of Warrior inoculated with 72-66 contained hyphae and these embryos were usually only partially infected (Fig. 6).

The reaction of embryos of CI13662 infected with both smut lines appeared to be compatible as in Conquest and Warrior, until after the hyphae penetrated through the scutellum. However, hyphae in the vicinity of the scutellum attachment to the embryo axis, the growing point, and in a few cases in the foliage leaves, were associated with heavy staining of a few to many cells, indicating that internal changes had occurred as a reaction to hyphal penetration (Figs. 7 and 8).

4.2 PERICARP STUDY

The majority of inoculated loose smut spores that were observed tended to be clumped on the stigma and style. However, spores were present from the top of the floret to the brush end of the young developing kernel, examined when 3-4 mm long. Spore germination and hyphal growth was observed in the upper part of the floret away from the stigma, style, ovary, and anthers, as well as on these organs and on pollen grains. Hyphae and appressoria were found over the entire kernel surface, from the brush end to the embryo region.

A yellow autofluorescence (AF) was observed under appressoria. However, approximately one third of the appressoria formed were not associated with AF. The AF regions varied in size, from the intercellular spaces around a few cells, to the entire cellular contents of many cells. Necrotic cells, varying in number, were often associated with the AF regions. The AF and necrotic regions were detected as early as five days after inoculation. Cross sections through the necrotic regions revealed that AF and necrosis extended from the outer epidermis deep into the pericarp (Fig. 9). A comparison of the amount of AF and necrosis occurring in Conquest, Warrior, and CI13662, inoculated with both smut lines, revealed no quantifiable differences amoung any of the combinations of the host and the fungus. The uninoculated controls of all three cultivars showed no AF or necrosis.

4.3 EMBRYO STUDY (INCANDESCENT AND FLUORESCENCE LIGHT MICROSCOPY OF GMA EMBEDDED EMBRYOS)

The observations from the transmitted incandescent light and incident fluorescence light studies complement each other and therefore will be described together, with specific reference to incandescent or fluorescence light microscopy where appropriate.

The pathway of infection in Conquest infected with 72-66 appeared to be identical to that for the same host infected with 72-146. Hyphae were apparently able to penetrate from anywhere on the face of the scutellum. Growth was then through the embryo axis and into the growing point region. It was difficult to make a distinction between intercellular and intracellular hyphal growth. Hyphae were always found in the embryo axis region but not always in the growing point. There was no observed difference between embryos containing hyphae of 72-66 and those containing 72-146 (Figs. 10 and 11). The relationship could be described as compatible at this stage of infection because no necrosis or other adverse reaction was detected.

Embryos of CI13662 infected with 72-66 appeared to be identical to those infected with 72-146. Hyphae were observed to penetrate from anywhere on the face of the scutellum as in Conquest. Again it was not obvious whether hyphal growth was intercellular or intracellular. In some regions of the infected scutellum the host and fungus appeared the same as previously noted in Conquest, however, near the scu-

tellum point of attachment to the embryo axis, the host cells appeared to react adversely (Fig. 13). This "incompatible" reaction to the presence of the hyphae was also observed in the growing point, and in the foliage leaves (Figs. 12 and 14). Hyphae could not be detected in or between the cells in the regions in which the incompatible reaction occurred, but hyphae were located in apparently healthy cells near the sites of the reaction (Fig. 13). The number of necrotic cells occurring in the incompatible reaction varied from a few to 50 or more. Contents were not easily discernible in affected cells. The affected cells also appeared distorted and collapsed (Figs. 12, 13, 14, and 15).

Fluorescence microscopy clearly defined the regions in which the reaction occurred (Fig. 14), while Toluidine blue staining gave more detailed information on the hyphae and host cells within these regions (Fig. 15).

4.4 EMBRYO STUDY (ELECTRON MICROSCOPY)

Figs. 16 and 17 are of embryos free from loose smut infection and are representative of the fine structure in the growing point region of healthy embryos.

In embryos of Conquest hyphal growth was mainly intracellular (Fig. 18). Hyphae were always surrounded by a "sheath" of material similar in appearence to the host cell wall (Fig. 19). An electron dense layer (EDL), was between the sheath and the fungal cell wall (Fig. 19). Hyphae were con-

sidered viable because of high lipid content, and the presence of nuclei and organelles (Figs. 19, 20, 21, 22, and 23). Fungal nuclei were easily distinguishable in hyphae in Conquest (Fig. 22 and 23). There was no apparent degradation in host cells containing the fungus (Figs. 20 and 21).

In some hyphae, at least eight distinctive layers were visible in the region between the host and hyphal plasmalemmas, but distinguishing between host and hyphal cell wall material in these layers was difficult (Figs. 24 and 25).

In embryos of CI13662 infected with 72-146, hyphal growth was mainly intracellular, and hyphae were surrounded with the characteristic sheath mentioned earlier (Fig. 26). A major difference between the hyphae in CI13662 and those in Conquest was the greater thickness of the EDL in CI13662. Hyphal condition in the embryo region of CI13662 varied from hyphae that appeared to have a certain degree of degeneration (Fig. 26), when compared to the compatible reaction occurring in Conquest (Fig 20 and 21), to apparently necrotic hyphae, as indicated by organelles which were difficult to identify, electron dense protoplasm, and a very thick EDL (Figs. 27 and 28). Large vacuoles containing electron dense material were observed in cells of CI13662 with intracellular hyphae (Fig. 27), and in cells adjacent to cells with intracellular hyphae (Fig. 28).

Increased secondary thickening of walls occurred in those cells neighbouring necrotic cells (Figs. 29 and 30), as well as in and beside heavily vacuolated cells (Figs. 26 and 29). Increased vacuolization of host cells and extensive secondary thickening of cell walls was not apparent in noninfected embryos of CI13662 (Fig. 17), or infected embryos of Conquest (Fig. 18). Hyphae were not always detected in the large regions of necrotic cells in CI13662 (Fig. 31).

Chapter V

DISCUSSION

The results herein described suggest that resistance in each of the three cultivars is the result of different mechanisms. First, in Warrior inoculated with the avirulent line (72-66), there is a low level of embryo infection (3%) compared to the high level (84%) produced after inoculation with the virulent line (72-146). This indicates that resistance is expressed before embryo infection, probably in the pericarp. The presence of hyphae in 3% of the embryos indicates that the resistance in the pericarp is not complete, and therefore resistance also occurs after embryo infection, because no sporulation occurred in the adult plants.

It was previously suspected that the resistance of Trebi, which carries the same resistance gene as Warrior, was related to a necrotic reaction of the cells of the epidermis and pericarp in response to hyphal penetration (Shinohara, 1974). However, the presence of the necrotic reaction in Warrior inoculated with the avirulent line 72-66, and also with the virulent line 72-146, as well as in Conquest and CI13662 inoculated with both smut lines, suggests that necrosis at this stage, may not play a role in conferring resistance to loose smut in the cultivars Warrior, Conquest, or CI13662.

It should be noted that loose smut spores were present and had germinated on every embryo examined for pericarp necrosis. With viable germinating spores in every developing kernel, it would not be unreasonable to expect 100% infection or close to it in the embryo susceptible cultivars. The lack of 100% infection suggests that a certain level of resistance is expressed in even highly susceptible cultivars. It may be possible that the necrotic reaction observed in the pericarp is at least partially responsible for the levels of infection below 100%.

The fact that the necrosis of pericarp cells does not appear to prevent embryo infection, suggests the fungus can survive this necrotic reaction, or that the inoculum concentration used was so high that the chance of some successful penetrations was inevitable.

Due to the similar amounts of autofluorescence and necrosis occurring in the pericarp of the three cultivars inoculated with both 72-66 and 72-146, when compared to the uninoculated controls, which showed no signs of autofluorescence or necrosis, further studies on the resistance occurring in the pericarp were not carried out.

A second mechanism of resistance apparently occurs after embryo infection in Conquest inoculated with 72-146. This combination produced a high level of infected embryos but no subsequent sporulation in the adult plants. Apparently infection develops the same in Conquest as in the parent from

which it derives its resistance. In this parent (Jet), the infected main shoots fail to develop while uninfected tillers developed into healthy plants (Mumford and Rasmusson, 1963).

Comparative examination of the extracted embryos of the three cultivars (Figs. 3-8), revealed an interesting difference in the reaction of the embryos to the presence of the fungus and a possible third mechanism of resistance. The relationship of the hyphae and the embryos appeared compatible in the Conquest and Warrior embryos which contained hyphae. This compatible relationship has been described in previous studies on susceptible cultivars inoculated with virulent races of loose smut (Batts, 1955; Pedersen, 1956). In contrast, the reaction of CI13662 to the presence of the fungus in the embryos appears to be one of incompatibility (Figs. 7 and 8).

Previous reports of this type of incompatible or necrotic reaction in embryos, in the presence of loose smut hyphae, are vague. Batts and Jeater (1958) noted that in scutellum susceptible , field resistant wheat cultivars, hyphae failed to enter the growing point region. In one embryo, in the region between the scutellum and embryo axis, host cells appeared swollen and brown in color. Wells and Platt (1949) reported that susceptible barley cultivars had embryos which were partially or completely destroyed by the fungus. However, details of the type of destruction were not given.

Therefore, this study provides the first detailed report of a third type of reaction in a resistant barley cultivar.

Infected embryos of CI13662 had hyphae in the scutellum in what appeared to be a compatible relationship, similar to that described in Conquest. However, the regions around the embryo axis, as well as those near the growing point and/or in the foliage leaves contained a few, to many necrotic host cells.

The extensive necrotic cell reaction in the embryo axis and growing point in some embryos appeared to stunt development (Fig. 12). As well, from the inoculated CI13662 seeds that were planted as controls, the number of plants produced was less than that expected to be produced by the noninfected embryos alone. This may be indicative of death of infected embryos, prior to or soon after germination. However, from these limited data it is impossible to determine if all infected embryos die, such a determination requires statistical analysis of larger populations and further histological studies. The death of embryos from loose smut infection has been referred to as a false type of resistance (Ohms and Bever, 1955). This type of resistance could be very desirable in a cultivar because the fungus can not complete its life cycle, thus the crop remains free from smut in subsequent generations.

Electron micrographs of infected embryos of CI13662 (Figs. 29-31) verify the reaction apparent in light micrographs (Figs. 12, 14, and 15). Death of host cells upon invasion by a pathogen is often referred to as a hypersensitive reaction. The hypothesis that the hypersensitive reaction stops parasite development by cutting off the supply of nutrients (Stakman, 1915) has given way to the idea that phytoalexins and lignification barriers play an important role in the prevention of penetration (Keen, 1982; Vance et al. 1980).

Phytoalexins are often phenolic compounds and are defined as "substances which inhibit the growth of certain microorganisms and which are produced in higher plants in response to a number of chemical, physical and biological stimuli" (Federation of British Plant Pathologists, 1973). These substances accumulate in the dead cells associated with the hypersensitive reaction, perhaps due to the presence of phytoalexin degrading enzymes in living cells (Keen, 1982).

The bright yellow fluorescence of necrotic regions, as well as the light blue staining of necrotic regions with toluidine blue suggests that phenolic compounds may be present because these reactions are typical for phenolic compounds. However, verification of the presence of phytoalexins, and their role in resistance against loose smut is beyond the scope of this study and will not be discussed further.

The reaction occurring in CI13662 inoculated with 72-146 appears to be a hypersensitive reaction. The presence of the fungus in the tissue is associated with necrosis of the host cells in and around the growing point region of the host. Hyphae in this region are surrounded by a thickened EDL and appear to be necrotic, but whether this is due to normal senescence or a consequence of the thick EDL is not known. Hyphae that appear viable can also be found in the growing point region near the incompatible reaction (Fig. 26).

Whether the fungus is prevented from further invasion at this stage, or whether it is able to grow beyond the cell necrosis only to encounter resistance in the seedling stage was not examined in this study. If hyphae can grow past the necrotic areas, the reaction would not conform to the definition of hypersensitivity. Therefore, classification of this reaction as hypersensitive should be refrained from until a thorough examination of developing seedlings verifies the exact site of restriction of hyphal growth.

Aside from the obvious necrotic reaction in infected embryos of CI13662, there was extensive vacuolization of host cells (Figs. 26-31). This vacuolization may be a primary indication of cell resistance or indicative of early cell necrosis.

Extensive secondary thickening of host cell walls occurred adjacent to necrotic and heavily vacuolated cells (Figs. 26, and 29-31). Secondary thickening of host cell

walls can occur as a defense mechanism in response to fungal penetration (Vance et al. 1980). Presumably penetration through the secondary thickening of these walls would be difficult if not impossible for hyphae. All cells containing hyphae were not necrotic, and necrotic cells without hyphae were observed (Figs. 29 and 31)(although the hyphae may have been missed in the sectioning), suggesting that the factor(s) causing necrosis may not involve direct contact with hyphae.

The previous studies that have suggested mainly intercellular growth in the embryo (Batts, 1955; Malik and Batts, 1960; and Pedersen, 1956), were done using the light microscope. Figs. 10 and 13 illustrate the difficulty of determining the location of the hyphae with light microscopy. Our light microscope results however, suggested that hyphal growth was mainly intracellular in the scutellum of Conquest (Fig. 11). This was verified by the electron microscope results, which showed that hyphal growth was mainly intracellular in the growing point region of the embryos of Conquest and CI13662 (Figs. 18, 20, and 26).

Fullerton (1970) reported that intracellular hyphae of other smut species were surrounded by a sheath. All hyphae examined in Conquest and CI13662 embryos were surrounded by this characteristic sheath. A distinct EDL, not reported by Fullerton (1970), was present adjacent to the sheath. The thickness of the EDL varied from approximately 60 nm in Conquest inoculated with 72-66 (Figs. 19, 20, and 22), to an

extreme thickness of approximately 500 nm in CI13662 inoculated with 72-146 (Figs. 27 and 28). Fullerton (1970) suggested that sheath formation may cause hyphal degeneration. This study indicates that the thickness of the EDL may be a better indicator of hyphal degeneration (Figs. 27 and 28).

Cytochemical studies of the stem rust fungus (Puccinia graminis f.sp. tritici) in a compatible wheat host, revealed up to six possible layers in the haustorium mother cell walls and four layers in the hyphal walls (Chong et al., 1985). At least eight distinct layers were visible between the host and hyphal plasmalemmas in both Conquest and CI13662 infected with 72-146 (Figs. 24-26). The layer constituting the sheath appears to be a continuation of the host cell wall, however the distinction between host and fungus composition will have to be determined cytochemically.

Figures 3-8. Extracted barley embryos from the embryo test.

Fig. 3. Uninoculated Conquest control. No hyphae present in the scutellum (S_M) or embryo axis (EA) (X 30).

Fig. 4. Conquest infected with 72-66. Hyphal penetration (arrows) occurs from all sides of the scutellum, growing into the embryo axis (X 30).

Fig. 5. Warrior infected with 72-146. Hyphal penetration (arrow$) occurs from all sides of the scutellum, growing into the embryo axis (X 30).

Fig. 6. Warrior infected with 72-66. Hyphal penetration (arrow) into the embryo is slight and hyphae are limited to a portion of the scutellum (X 30).

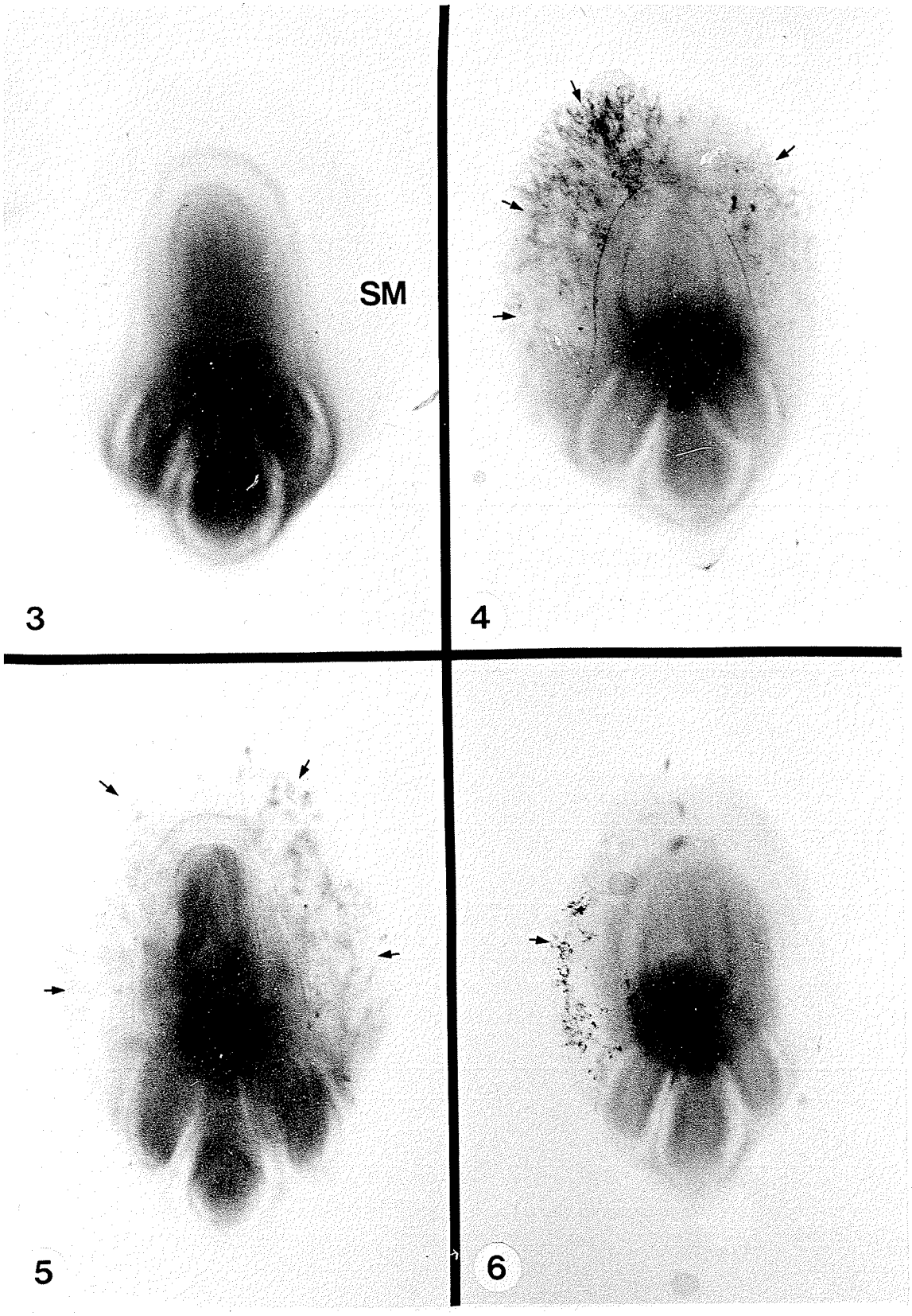
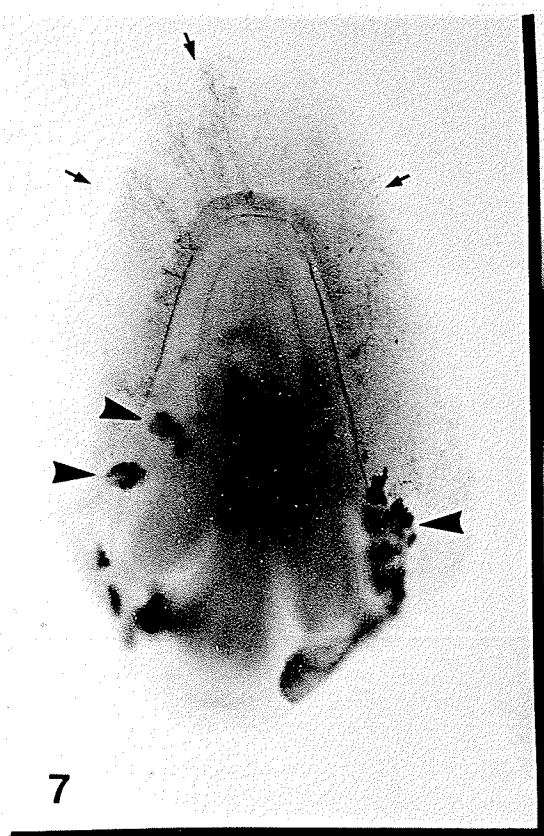

SM
3
4
5
6

Fig. 7. CI13662 infected with 72-66. Hyphal penetration originates on all sides of the scutellum (arrows) incompatibility occurs around the embryo axis and into the growing point (arrowheads) (X 30).

Fig. 8. CI13662 infected with 72-146. The incompatible reaction extends into the foliage leaves (arrowheads) (X 30).

Fig. 9. Conquest inoculated with 72-66. Autofluorescence and necrotic cells (NC) occur in the outer epidermis (OE), and pericarp (P) (X 150).



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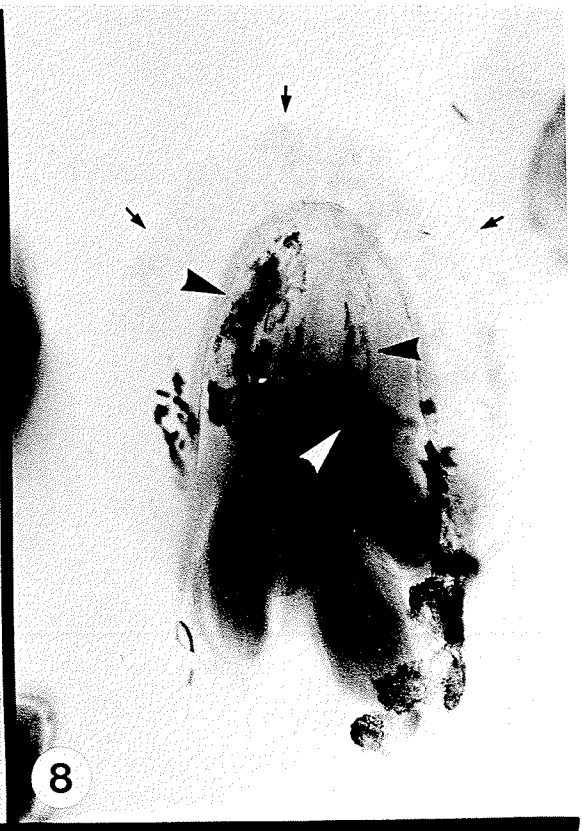

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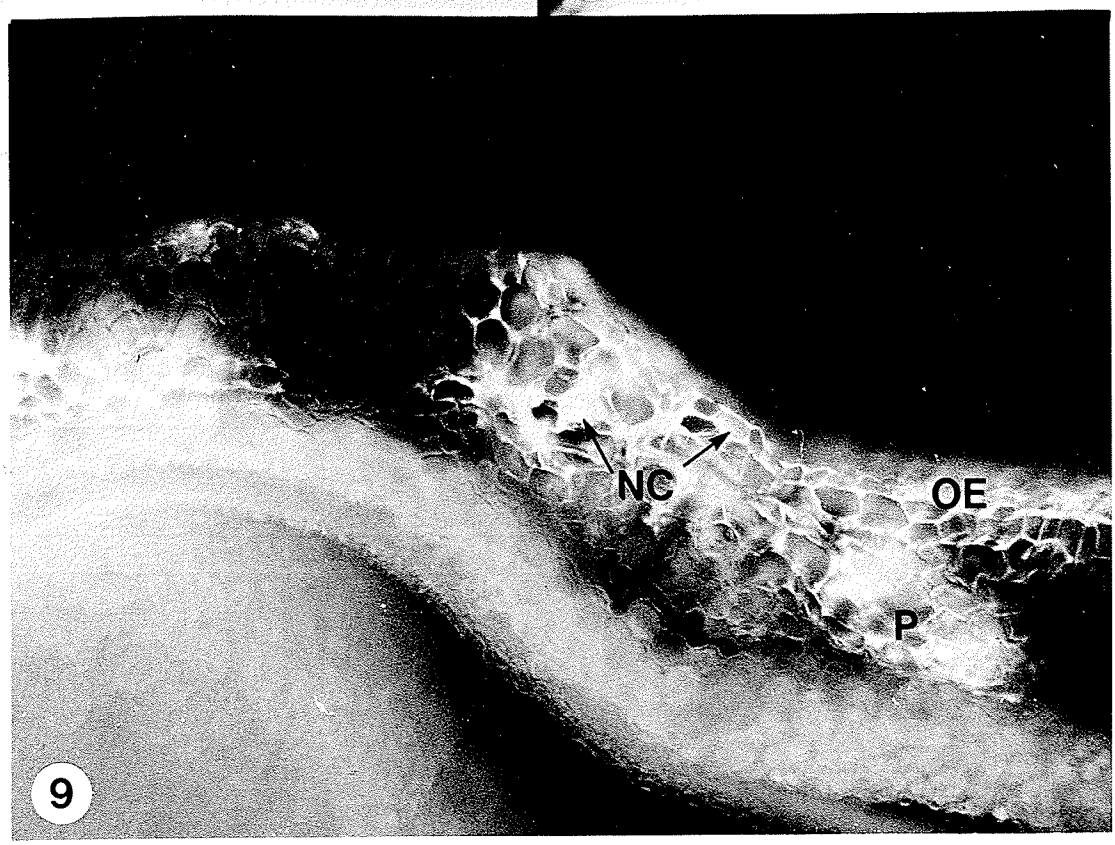

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Figs. 10-15. Embryos embedded with GMA, examined by light and fluorescence microscopy.

Fig. 10. Conquest infected with 72-66. Hyphal growth in the embryo axis (arrowheads), no apparent reaction in cells of the host (X 500).

Fig.11. Conquest infected with 72-146. Hyphal growth in the scutellum appears to be mainly intracellular (arrowheads), with no apparent reaction in cells of the host (X 1000).

Fig. 12. CI13662 infected with 72-66. The incompatible reaction (arrowheads) occurring in the growing point and foliage leaves appears to have stunted the embryo (X 100).

Fig. 13. Scutellum of CI13662 infected with 72-146. The incompatible reaction is present (arrowhead), however not all hyphae (H) are directly in contact with a zone of incompatible reaction (X 250).

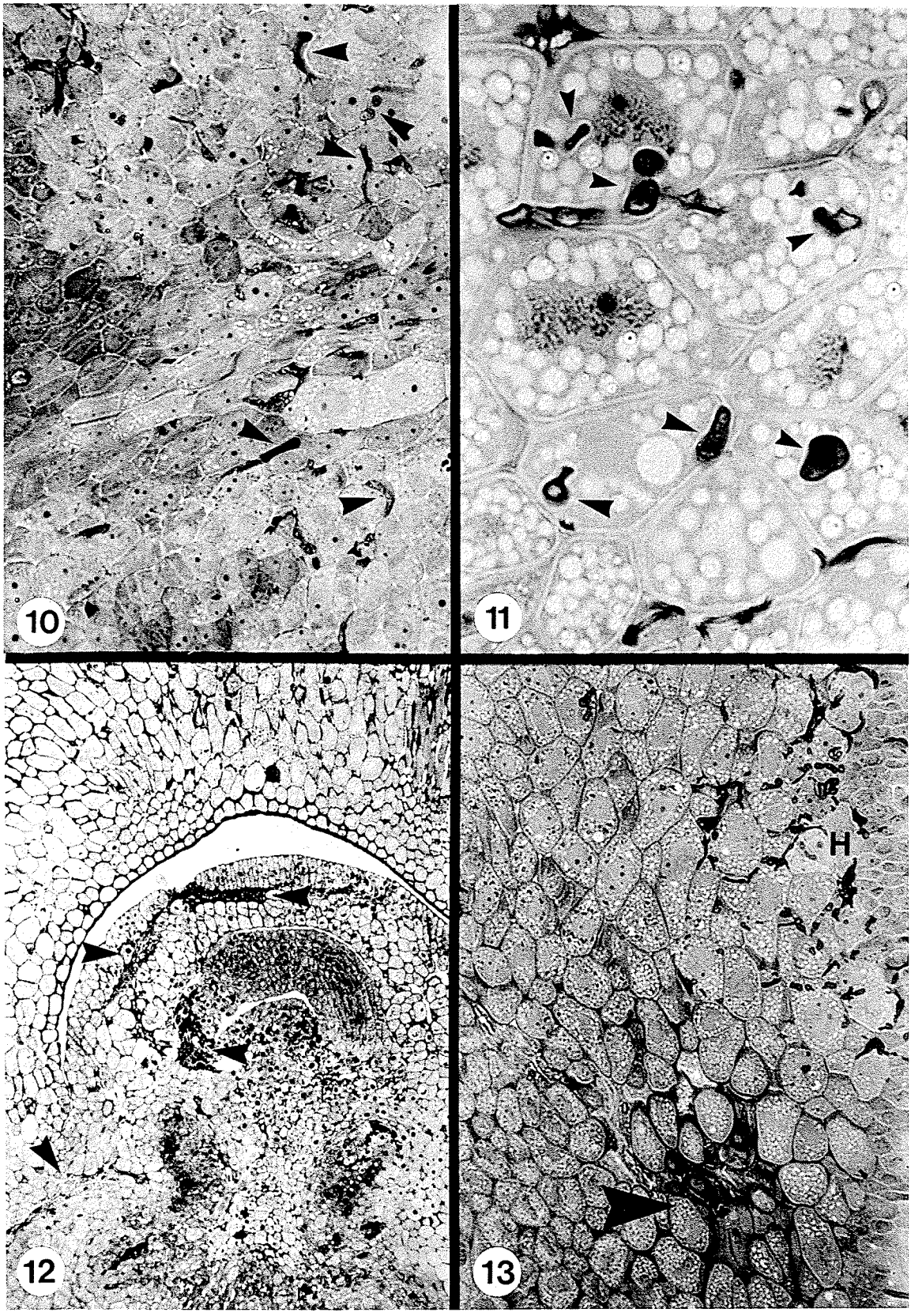
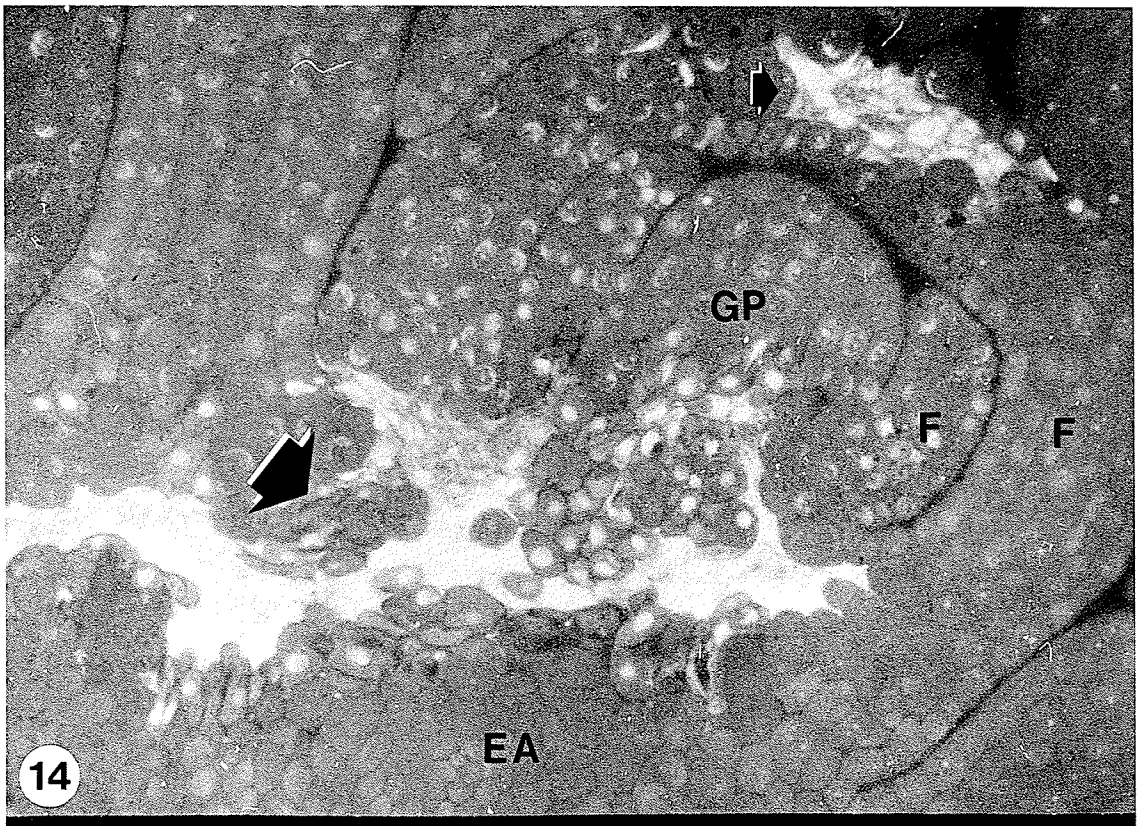
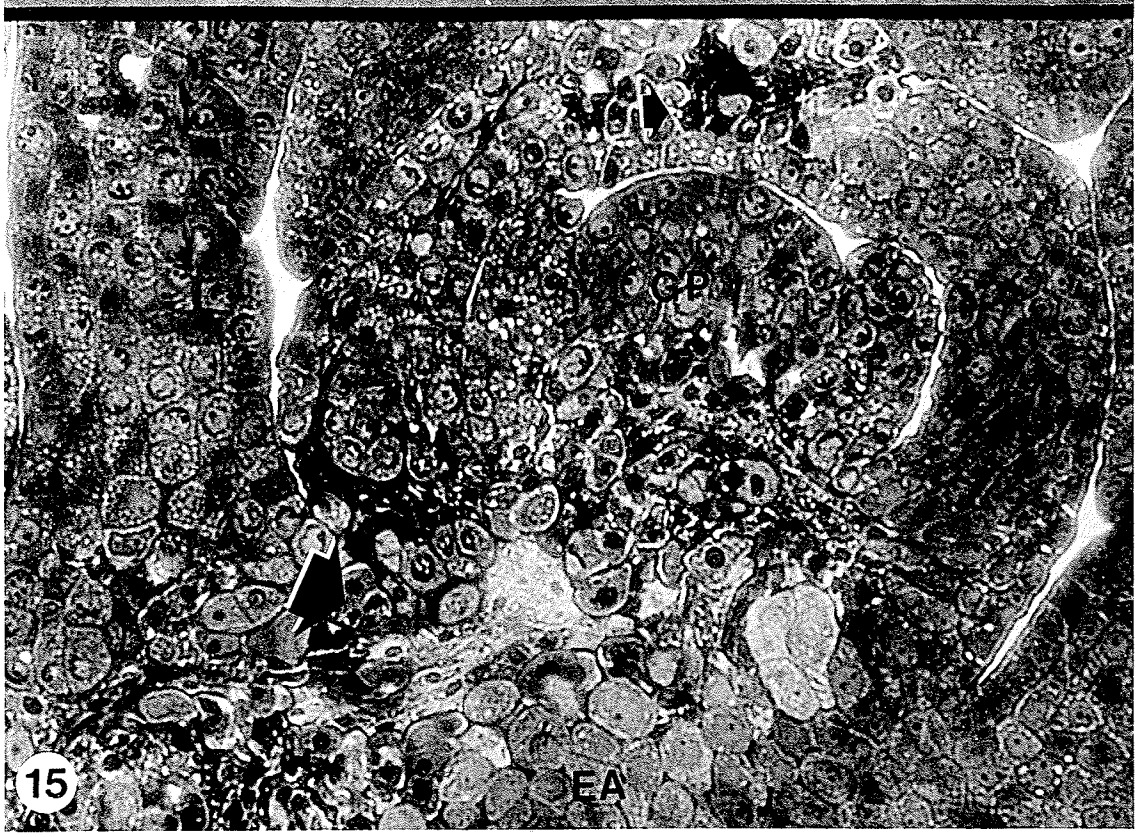

10
11
12
13
H

Fig. 14. CI13662 infected with 72-66. The incompatible reaction (arrows) in the embryo growing point, (GP) as seen by fluorescence microscopy with the Calcofluor White M2R New stain (X 350).

Fig. 15. CI13662 infected with 72-66. Toluidine blue stain. The reaction (arrows) is not as clearly defined as in Fig. 16, however, cellular detail is greater (X 350).


GP
F
F
EA
14


GP
EA
15

Figs. 16-31. Barley embryos embedded in Spurr for electron microscopy.

Figs. 16-17. Representative illustrations of the fine structure present in healthy, noninfected embryos of CI13662. All cells are intact with characteristic membrane bound organelles (nucleus (N) with the nucleous (NU), vacuoles (V), lipids (L), and protein bodies (PB) within the plasmalemma (PL) and cell wall (CW). Fig. 16. X 3062; Fig. 17 . X 6650.

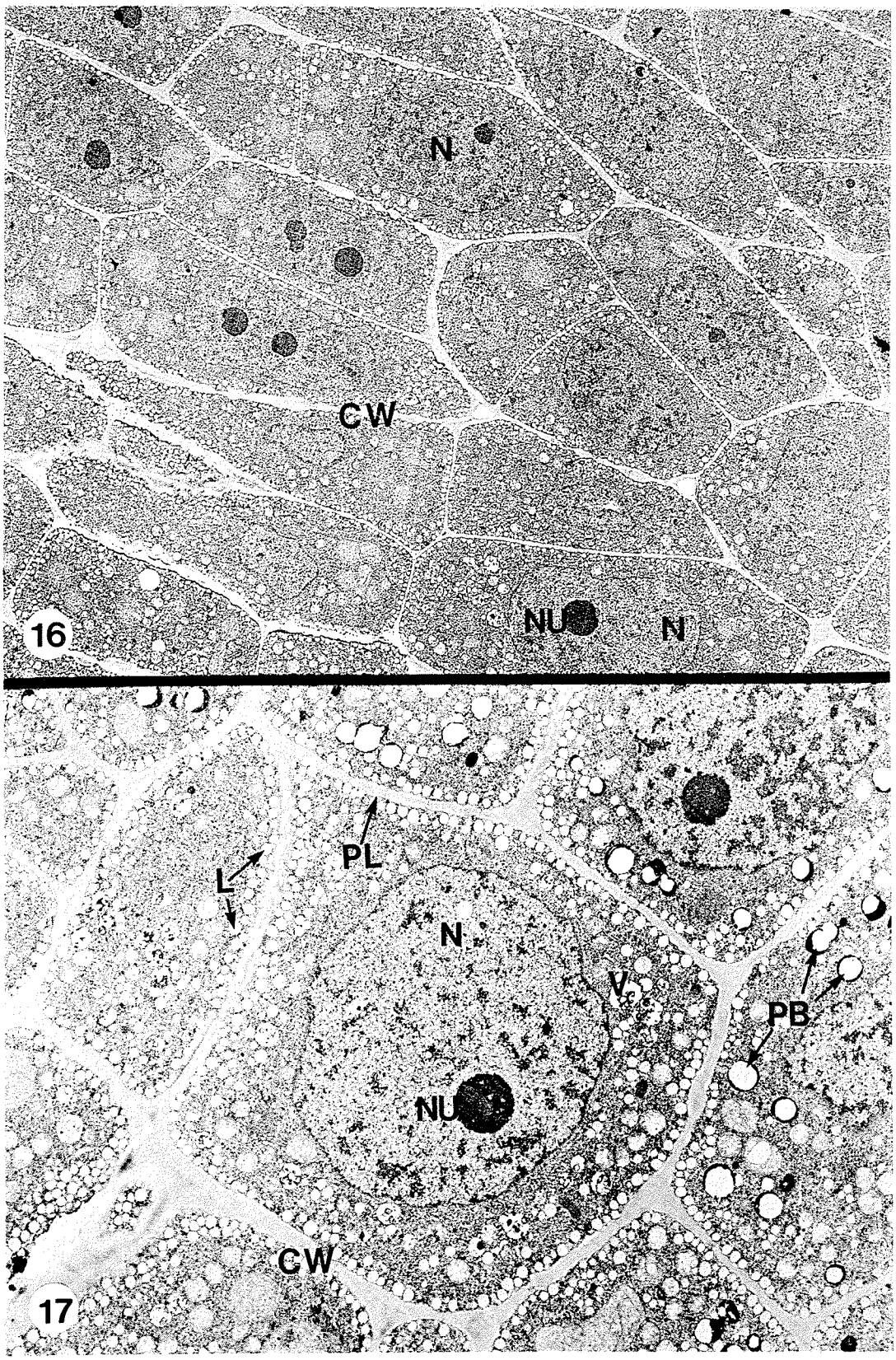
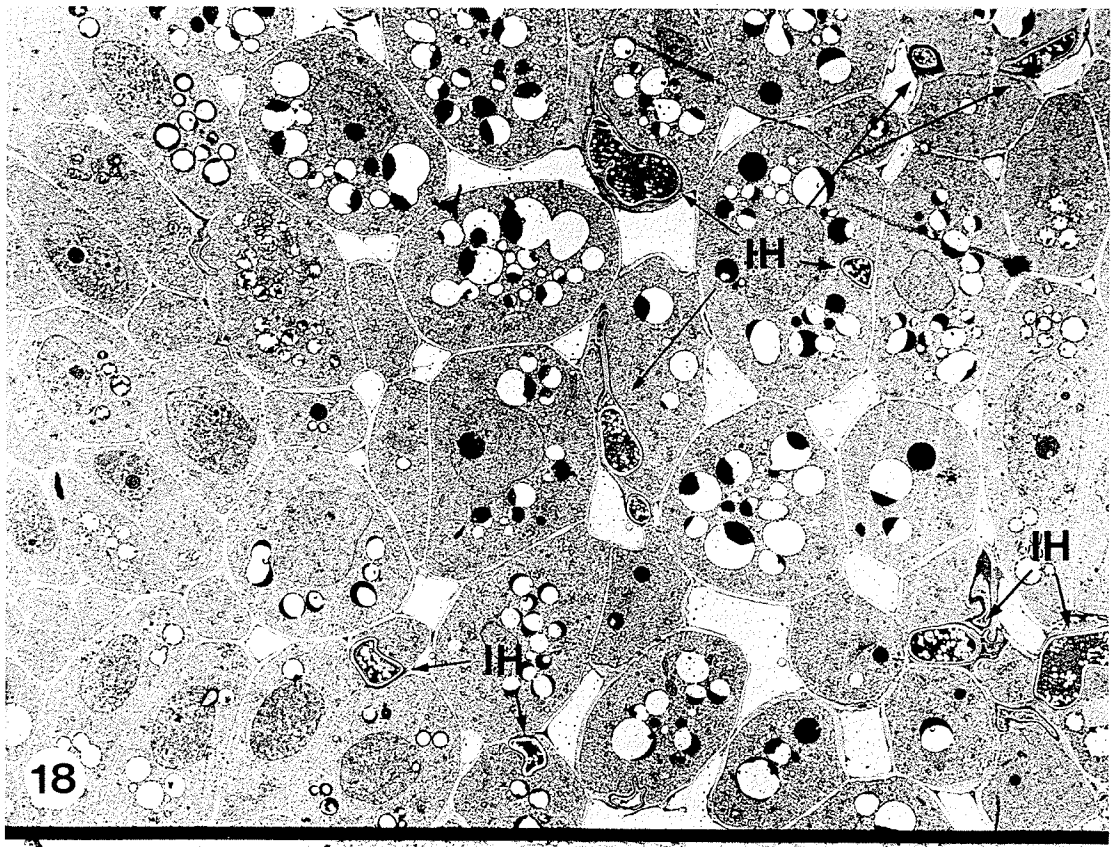

N
CW
NU
N
16
PL
L
N
V
PB
NU
CW
17

Fig. 18. Conquest infected with 72-66. Hyphal growth is mainly intracellular, and the host and hyphae are in a compatible relationship (X 1435).

Fig. 19. Conquest infected with 72-66. Sheath (S), and electron dense layer (EDL), surrounding a viable hypha which contains mitochondria (M) and lipids (L) (X 17850).


IH
IH
IH
18

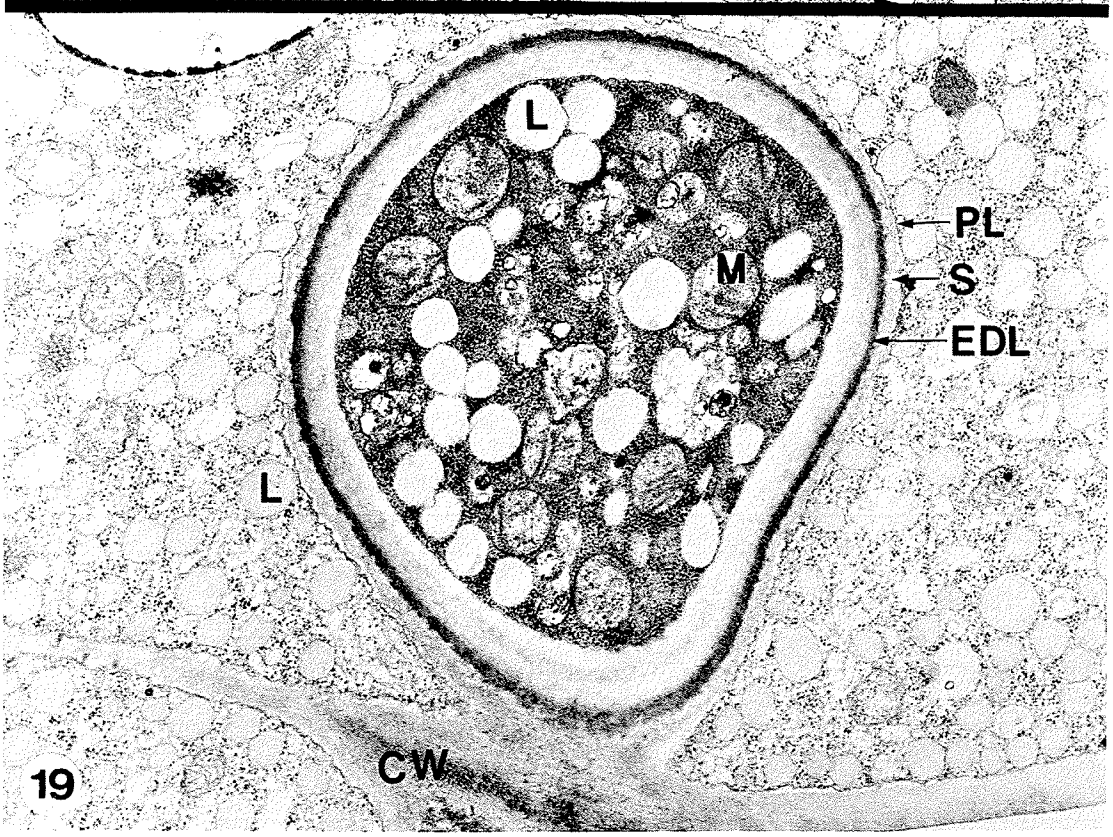
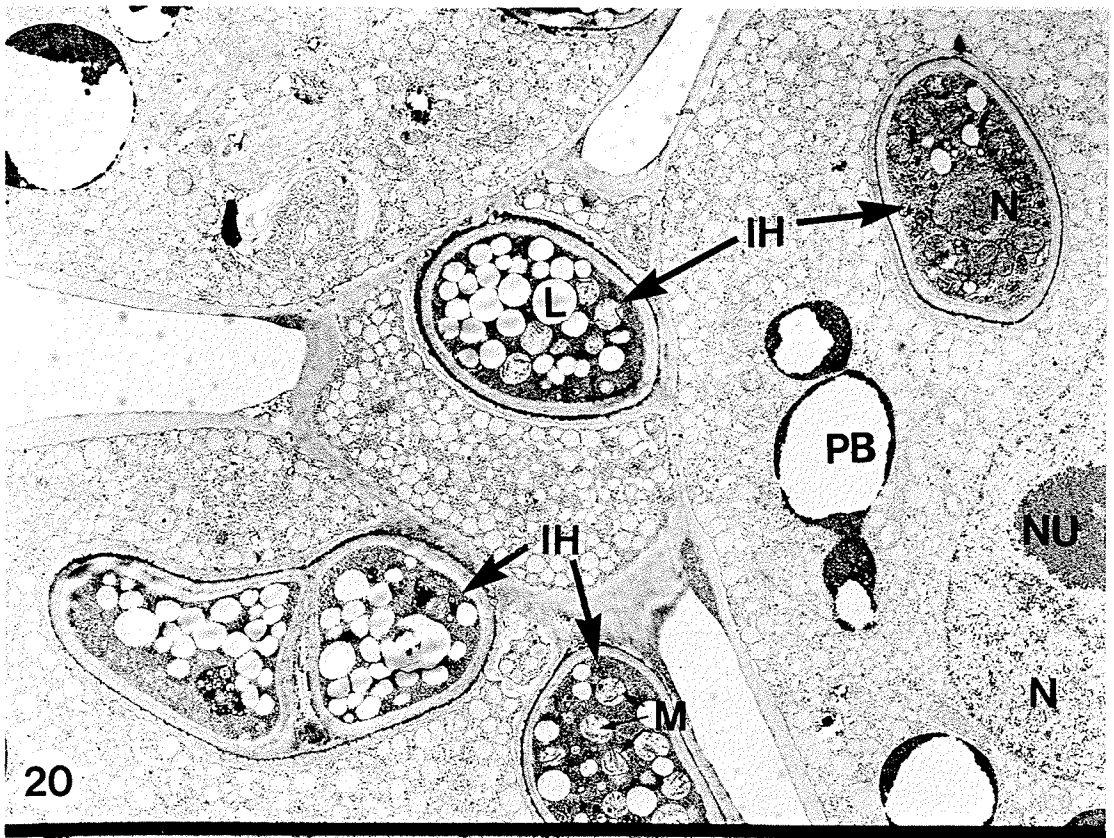

L
PL
M
S
EDL
L
CW
19

Fig. 20. Conquest infected with 72-66. Viable intracellular hyphae (IH) containing nucleus(N), mitochondria (M), and lipids (L) (X 6650).

Fig. 21. Conquest infected with 72-146. Viable intracellular hyphae (IH) containing nuclei (N), mitochondria (M), and lipids (L) (X 5775).



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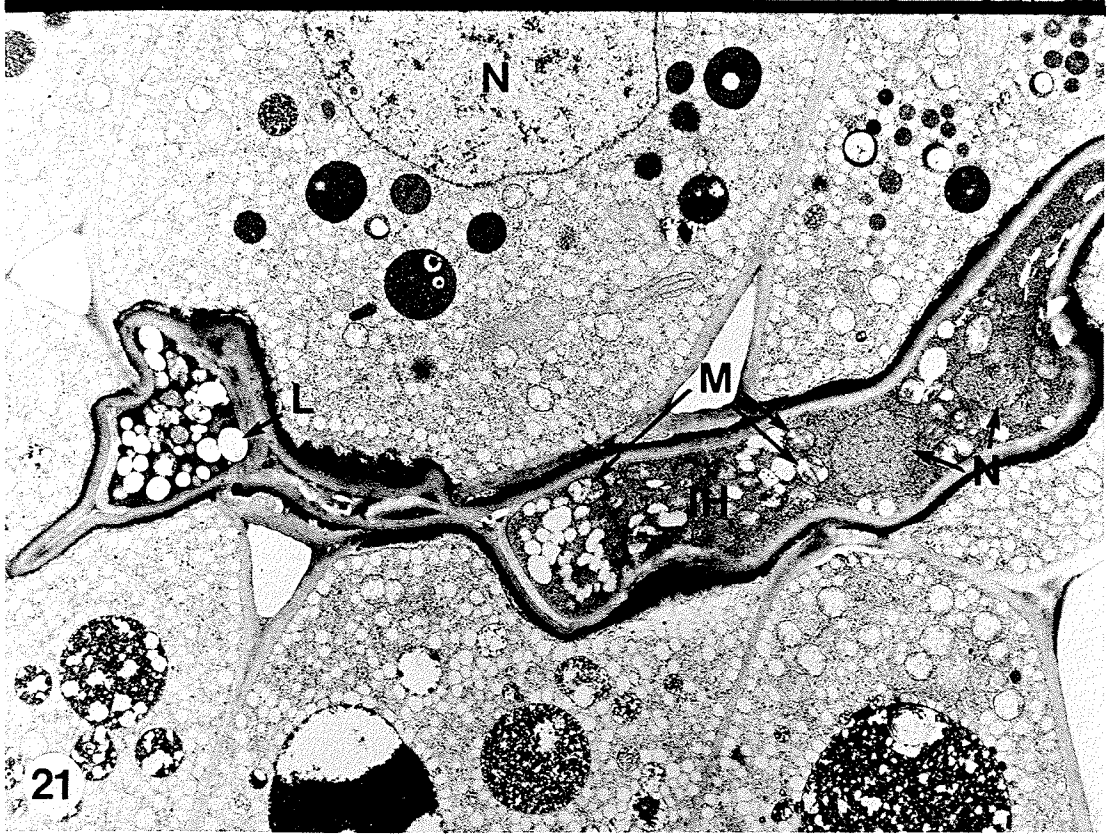

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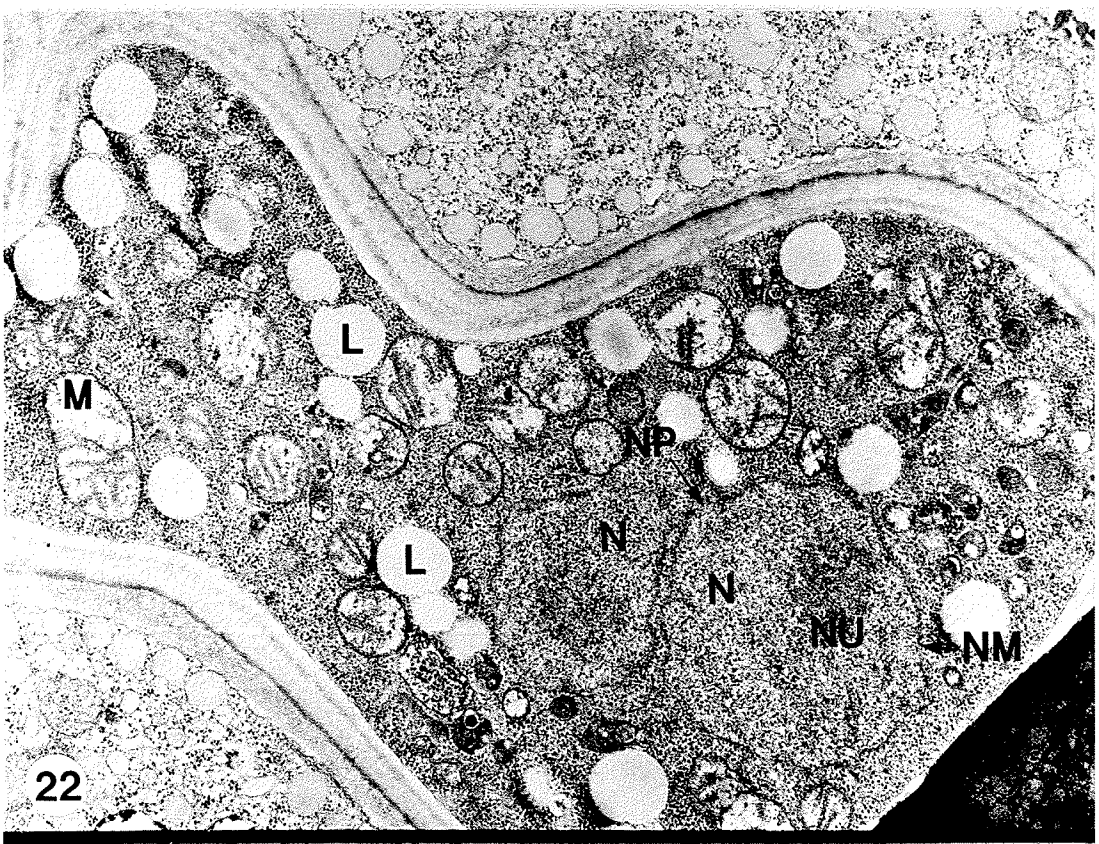
Fig. 22 Conquest infected with 72-66. Dikaryotic hyphal nuclei (N), surrounded by the nuclear membrane (NM), mitochondria (M), and lipids (L) are present. (NP) indicates a connection between the two nuclei. (X 18275).

Fig. 23 Conquest infected with 72-146. Similar to Fig. 22 except only one nucleus is visible (X 18275).



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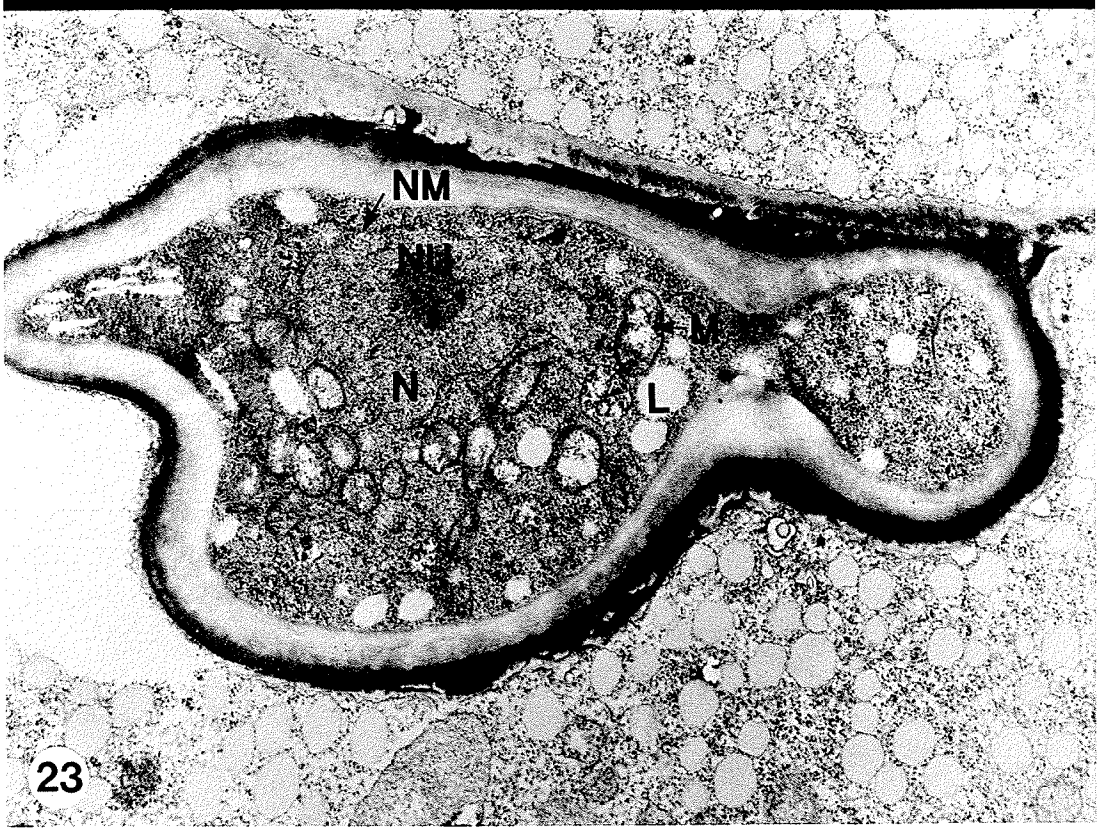
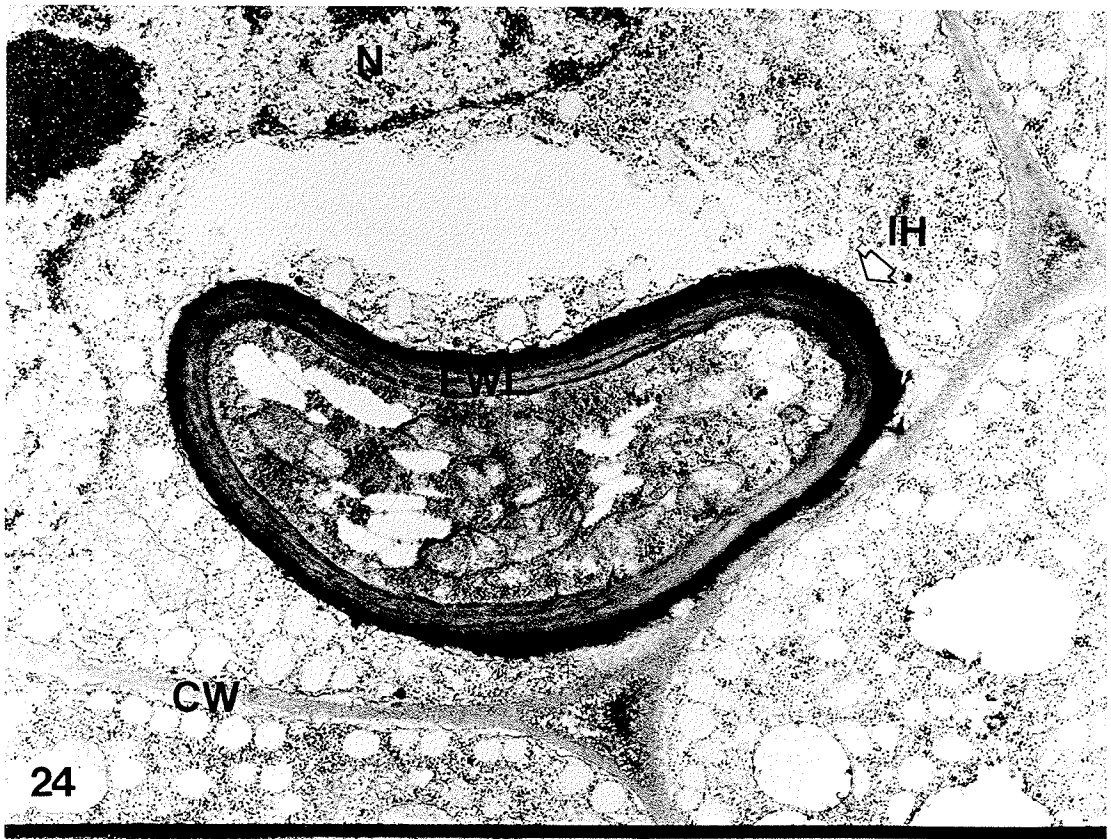


23

Fig. 24. Conquest infected with 72-146. Viable hypha (IH) in a viable host cell. At least eight distinctive layers are present between the host and fungal plasmalemmas (X 18375).

Fig. 25. Increased magnification of Fig. 24, showing at least eight layers between the plasmalemmas (X 183750).


N
IH
EWL
CW
24

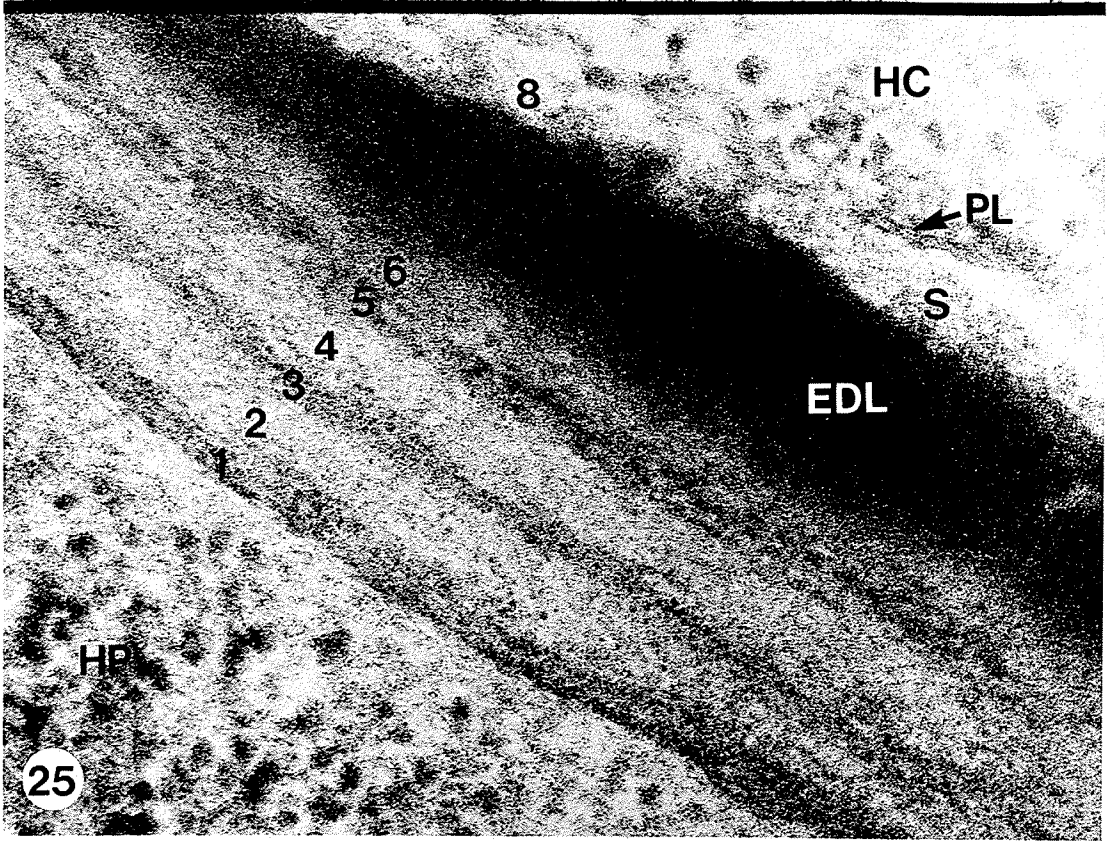

8
HC
PL
S
6
5
4
3
2
1
EDL
HPL
25

Fig. 26. CI13662 infected with 72-146. Intracellular hyphae (IH), secondary thickening of host cell walls (SC), and large vacuoles (V). The fungal wall layers (FWL) are visible between the plasmalemmas of host and fungus (X 8575).

Fig. 27-28. CI13662 infected with 72-146. Relatively thick EDL. Hyphal protoplasm (HP) is electron dense, indicating necrosis. Extensive vacuolization (V) has occurred in host cells, with electron dense material (EDM) present within the vacuoles (Fig. 27. X 23625; Fig. 28. X 11112).

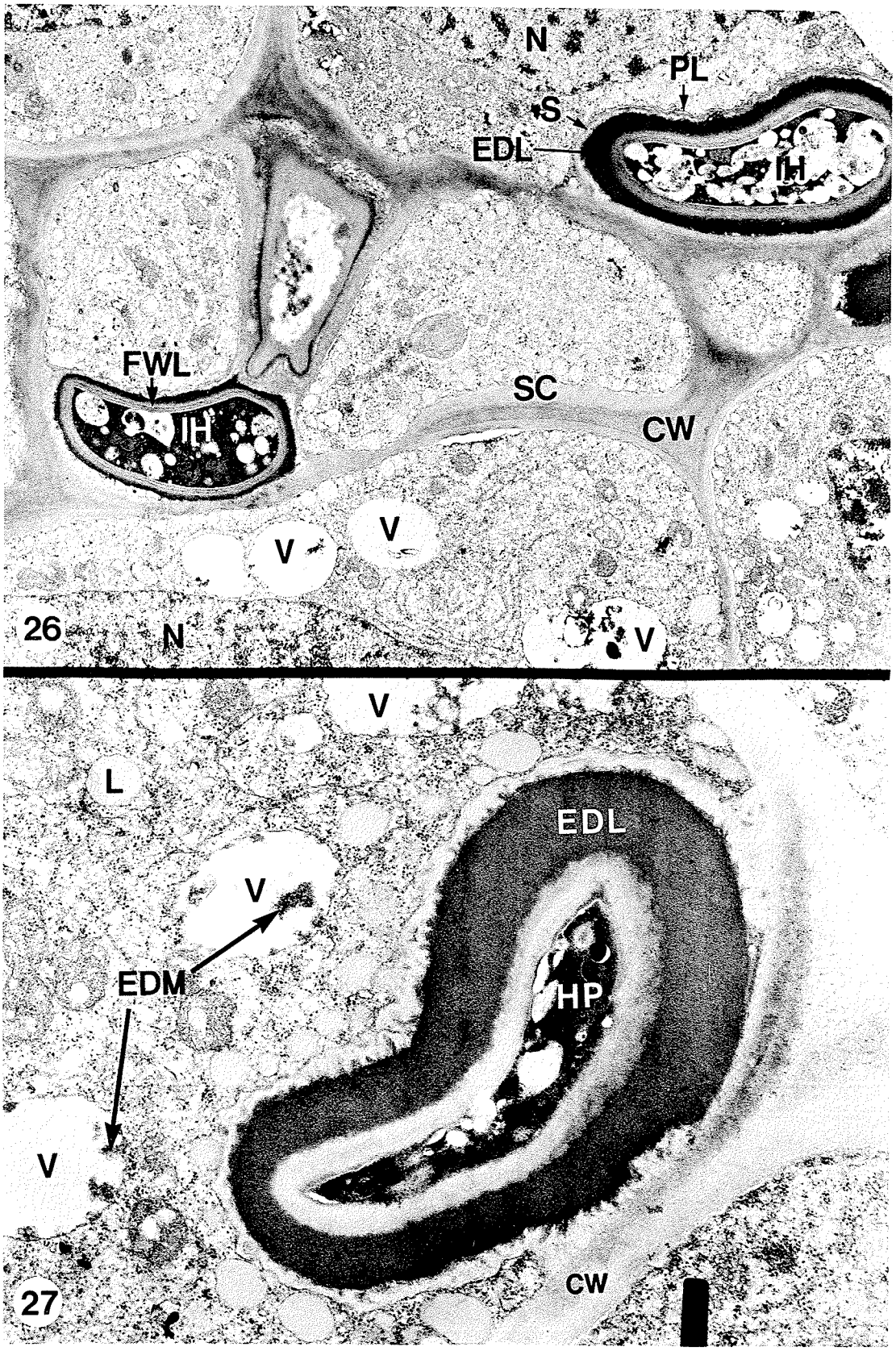

N
PL
S
EDL
IH
FWL
IH
SC
CW
V
V
26
N
V
V
L
EDL
V
EDM
HP
V
27
CW

Fig. 28. See figure 27.

Fig. 29. CI13662 infected with 72-146. Hyphae (H) are present near necrotic cells (NC). Shows irregular shape of cell walls as well as secondary thickening (SC) of walls and extensive vacuolization (HVC) (X 2012).

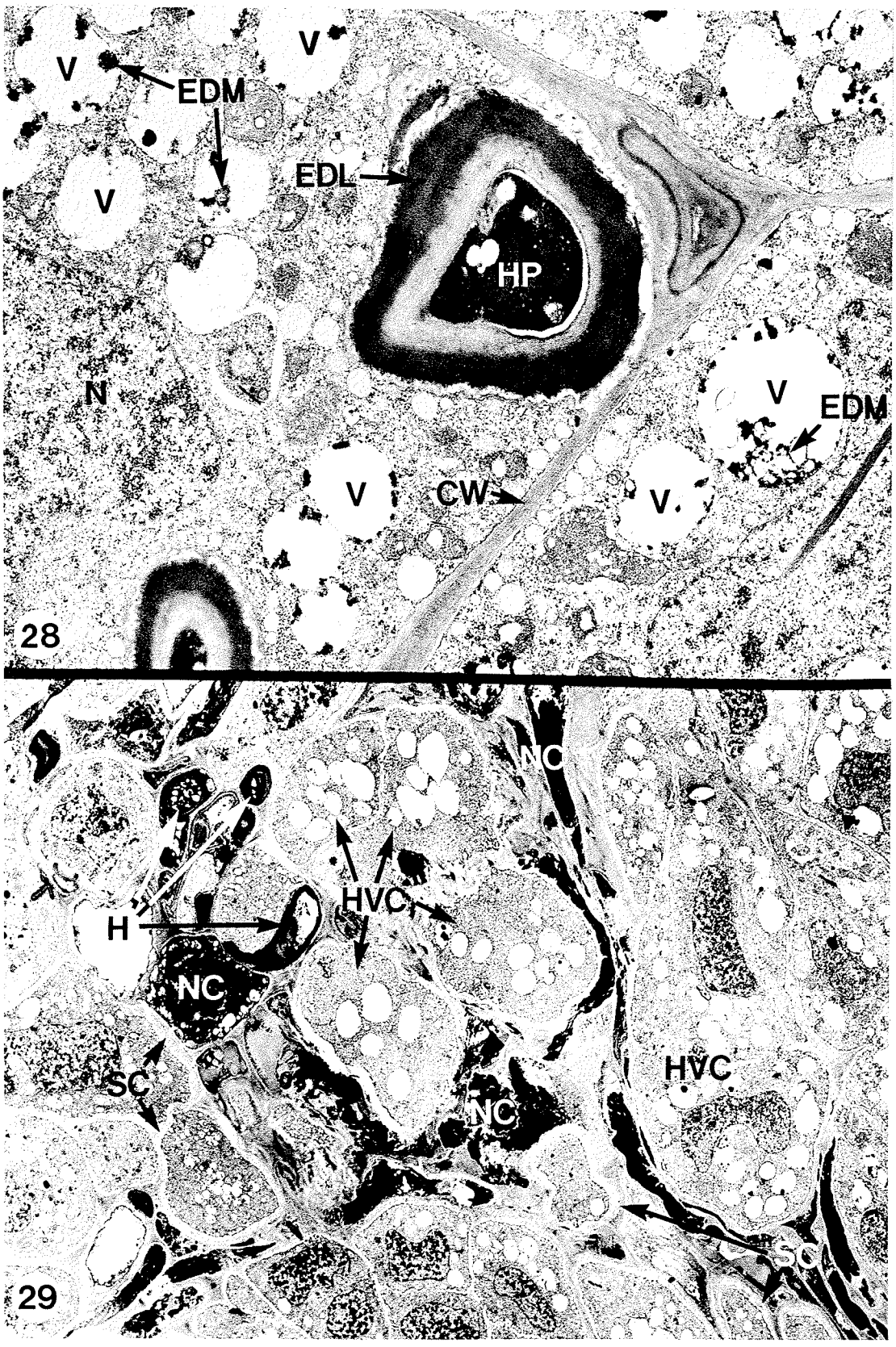
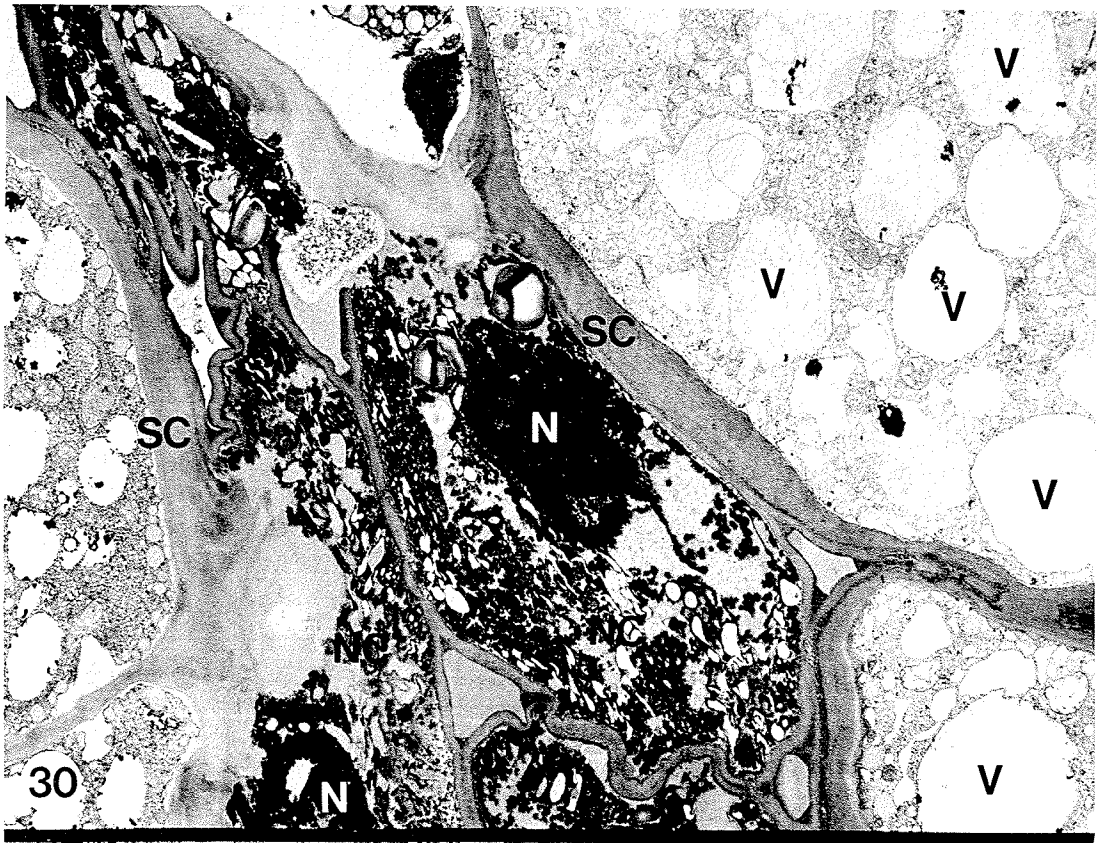
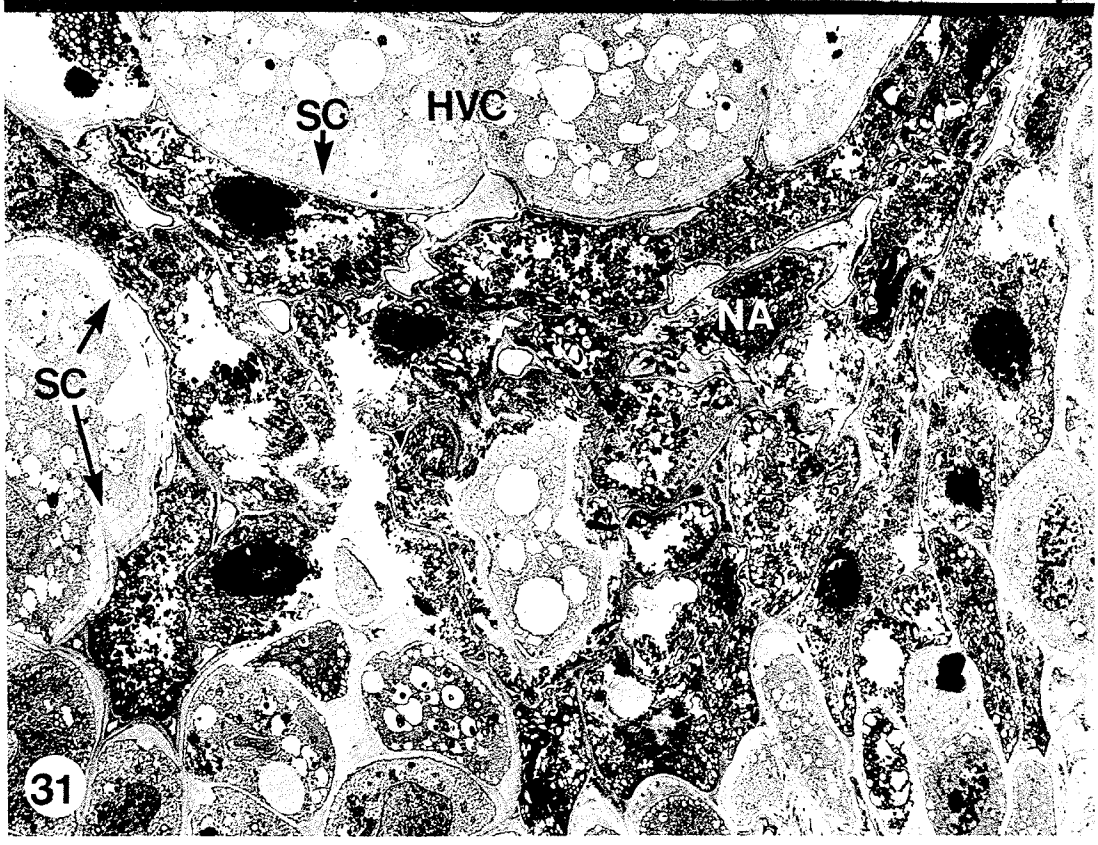

V
V
EDM
EDL
V
HP
N
V
EDM
V
CW
V.
28
WC
HVC
H
NC
SC
NC
HVC
SC
29

Fig. 30. CI13662 infected with 72-146. Necrotic host cells (NC) lacking membrane bound organelles. The host nucleus (N) is very electron dense. Extensive secondary thickening (SC) has occurred in the walls of apparently viable, extensively vacuolated, adjacent cells (X 5775).

Fig. 31. CI13662 infected with 72-146. Large necrotic areas are present in the growing point. Secondary thickening (SC) of cell walls is obvious in heavily vacuolated cells (X 2012).


V
V
V
V
SC
SC
N
N
N
V
V
30


SC
HVC
NA
SC
31

Chapter VI

CONCLUSION

It appears that the resistance genes Un1, Un3 and Un6, and Un8, in Warrior, Conquest, and CI13662 respectively, confer resistance at different stages of plant growth and possibly by different metabolic mechanisms.

The Un1 gene in Warrior appears to confer resistance before embryo infection, which is evident from the low level (3%) of embryo infection after inoculation with 72-66. Resistance is also expressed after embryo infection because there is no sporulation in the adult plants produced from seed lots with 72-66 infected embryos.

The genes Un3 and Un6 in Conquest appear to confer resistance to line 72-146, after embryo infection. This is apparent from the compatible relationship occurring at the embryo stage, with no sporulation occurring in the adult plants. Young seedlings from inoculated seed were not examined. However, resistance is probably expressed when seedlings are approximately three weeks old, as was found in Jet (Mumford and Rasmusson, 1963), because Conquest derives resistance genes from Jet.

The gene Un8 in CI13662 appears to confer resistance at the embryo stage. Hyphae can penetrate through the scutellum, sharing what appears to be a compatible relationship with the cells in this tissue. Resistance is probably associated with the necrotic host cell reaction observed in the presence of the fungus in the region around the attachment of the scutellum to the embryo axis, as well as in the embryo axis, the growing point, and the foliage leaves. Hyphae in the growing point region of CI13662 were necrotic or appeared to have a certain amount of degeneration of cellular contents. Whether this necrotic reaction is indicative of death of the whole of the fungal mycelium, or whether resistance is conferred at a later stage as in Jet, is not known.

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