

I dedicate this work to my mother, Bronislawa, and
my late father, Jan Nowak, who also generously
financed the production of this thesis.

INFECTION OF CEREALS BY Claviceps purpurea
AND THE ROLE OF SOME ENZYMES IN PARASITISM

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by

BARBARA IRENA SHAW (née NOWAK) B.Sc.

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Department of Biochemistry,
Imperial College of Science and Technology,
London, S.W.7.

INFECTION OF CEREALS BY *Claviceps purpurea* AND THE ROLE OF SOME ENZYMES
IN PARASITISM

BARBARA I. SHAW

ABSTRACT

Infection of wheat and rye by *Claviceps purpurea* and its subsequent development to a sclerotium was investigated by light and electron microscopy. These studies revealed that initial penetration occurred via the stigma and ovary wall. Further colonization was intercellular and, initially, fungal growth was directed towards the ovule and vascular traces. The ovary was destroyed and replaced by fungal tissue (the sphacelium) within about a week and honeydew exudation commenced. Fungal penetration did not extend into the rachilla, but remained distal to a core of thick-walled cells in the rachilla throughout fungal development. Sclerotial differentiation was detected near the base of the sphacelium within two weeks of inoculation and further sclerotial proliferation resulted in a hard compact ergot sclerotium.

Activity of some of the enzymes produced by the fungus during saprophytic and parasitic growth was assessed. These included a range of pectolytic enzymes, β -glucanase and β -glucosidase. The pectolytic enzymes are thought to assist in intercellular hyphal penetration and killing of host cells during colonization.

The activity of β -glucanase and β -glucosidase increased with the age of infection. Their initial rise in activity coincided with fungal colonisation reaching the ovary base and the onset of honeydew exudation.

Fluorescence microscopy and microspectrofluorimetry demonstrated that β -glucanase from parasitic tissue and honeydew extracts of *C. purpurea* could degrade callose from 3 localities in the wheat ovary i.e. wounds, phloem and pollen tubes.

Interpretation of this degradative activity has led to the proposal that β -glucanases play an important role in the establishment of the sphacelial mycelium and in the maintenance of a nutrient supply to the developing sclerotium.

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1. INTRODUCTION

1.1. The ergot fungus

Ergot is indigenous to grasses and cereals in temperate areas. In Britain it does not normally pose a serious threat to grain yield, although ergot contamination of grain does reduce its value. In other parts of the world e.g. N. America, ergot is often severe on rye, durum wheat and Triticale, and, as in Britain, can be a considerable menace to male sterile varieties used in the development of F_1 hybrids (Platford & Bernier, 1970; Shaw, 1975).

Ergot is best known for its effects on animals and humans if they ingest it, since during sclerotial growth, alkaloidal secondary metabolites accumulate. The pharmacological activities of these alkaloids and related topics have, in the past, dominated the literature on ergot fungi.

In contrast, the approach of the present study is phytopathological, since this aspect has received relatively little attention. It includes a detailed microscopic study of the path of infection and subsequent development of C.purpurea. It then investigates some of the enzymes produced by the fungus during parasitism and in saprophytic culture, and explores the possible roles of these enzymes.

The following section gives a brief introduction to the ergot alkaloids, the life cycle of the parasite, aetiology and the little-studied resistance mechanisms of plants to this fungus. Then follow reviews of the literature concerning each topic studied in the present work.

1.2. Ergot alkaloids

The occurrence of ergot fungi on graminaceous hosts has been observed for centuries by different civilisations, many of which also utilised the properties of ergot sclerotia, mainly in medicine or rituals (Bové, 1970). However, the descriptions of plant diseases in ancient manuscripts are not always sufficiently explicit for it to be clear whether they are describing ergot.

The first generally accepted work in which the identification of ergot is not disputed is that of Adam Lonicer of Frankfurt in 1582. In his book he describes the use of ergot in midwifery. This usage is due to the occurrence in sclerotia of the alkaloids, which have since been shown to have a wide range of pharmacological activities (Chassar Moir, 1964; Bové, 1970; Mantle, 1968b, 1969a; Groger, 1972).

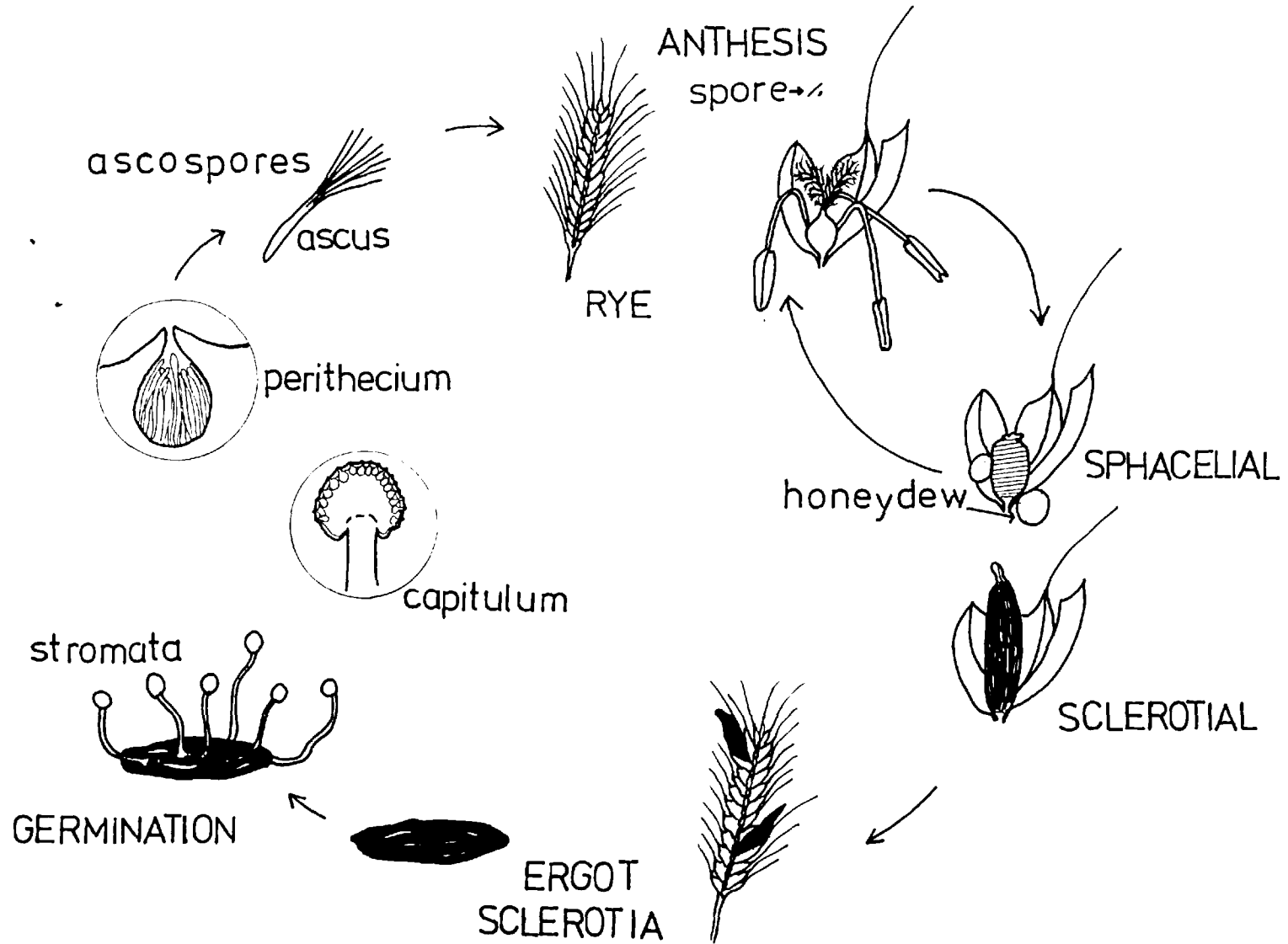
The biological effects of alkaloids include vasoconstriction, muscular contraction, antagonism to adrenalin and serotonins, and effects on the central nervous system. Although these effects can be used therapeutically, accidental ingestion of ergot has led to ergotism in both animals and man, which has been fatal in some cases (Barger, 1931; Wogan, 1966; Fuller, 1969). Symptoms associated with ergot poisoning include gangrene, convulsions and hallucinations. Alkaloids are presently used in medicine mainly for the control of post-partum haemorrhage and in the treatment of migraine.

1.3. Life cycle (Fig. 1.)

Ergot sclerotia in the soil germinate in spring and early summer to produce stromata. Within the capitulum of the stroma, numerous perithecia are embedded. These contain many elongated asci each containing 8 ascospores. The ascospores are forcibly discharged from the perithecia and are disseminated by air movement. They constitute the primary inoculum for infection.

If ascospores land on the stigma or ovary of a suitable host, usually during anthesis, they may germinate and penetrate the ovary. The ovary is destroyed within about 10 days and replaced by fungal mycelium (the sphacellium). This is characterised by profuse conidiation and secretion of a sweet sticky liquid, known as honeydew, in which the conidia are carried. This serves as secondary inoculum and can be disseminated by head to head contact, rain splash or insects. After about another week, mycelial differentiation into sclerotial tissue takes place. Sclerotial growth continues (while honeydew exudation subsides) and eventually a hard,

Fig.1 LIFE CYCLE OF ERGOT



compact, purple-brown ergot sclerotium develops. The sclerotium grows until the ear is mature and is often larger than seeds in the same ear.

Before and during harvest, ergots may be knocked out of the ears and fall to the ground where they overwinter, and remain viable in the soil for about a year. Alternatively, they may be harvested along with the grain, in which case they could be sown into the soil the following season.

The present study deals with the part of the life cycle during which the fungus is parasitic on the plant.

Knowledge of the life cycle has largely been responsible for the control measures of ergot, since these are mainly cultural (although the possibility of biological control has also been shown (Mantle, 1965; Puranik & Mathre, 1971; Mower et al, 1975)). Cultural control includes planting sclerotia-free seed, crop rotation, deep tillage and weed control.

1.4. Aetiology

The control of weeds is particularly important in a wheat crop since their role as potential reservoirs of inoculum has been postulated for some time, and recently has been clearly demonstrated (Shaw, 1975; Mantle et al, 1977). These authors confirmed the hypothesis that weeds play a part in the aetiology of ergot. This statement has to be qualified however, since not all grass weeds infected with ergot can provide inoculum which will successfully infect wheat, rye or barley crops. This is due to host restriction of certain strains but the mechanism responsible for host restriction is not known (Stager, 1903,1923; Loveless, 1971; Mantle, 1967,1969b).

Host restriction may be aided by differences in flowering dates because inoculum from weeds may not be available at the time of anthesis of the crop. Shaw (1975) and Mantle et al (1977) showed that blackgrass (Alopecurus myosuroides) posed the most serious threat of ergot infection in the wheat crop. Not only was the flowering date ideal for infection by ascospores and building up honeydew inoculum before wheat anthesis, but

also all blackgrass isolates from several localities readily infected wheat. By contrast, isolates from ryegrass (Lolium spp.) rarely produced sclerotia on wheat. Cross inoculation experiments (Shaw, 1975; Mantle et al, 1977) showed that ergot isolates from various other grasses were either 'highly' or 'weakly' infective on wheat. The infectivity of isolates was determined mainly by the formation of ergot sclerotia, but it was noticed that 'weakly infective' strains, yielding only a few sclerotia, progressed only as far as the sphacelial stage. Thus the plant-parasite interaction appeared to be unable to support those isolates which do not develop sclerotia, but it is debatable whether this constitutes a form of resistance.

1.5. Resistance

Resistance of various spring and durum wheats to C.purpurea was studied by Platford and Bernier (1970) who found that the more so-called resistant cultivars, Kenya Farmer and Carleton, produced fewer, smaller sclerotia and less honeydew. Further work involving testing of various ergot isolates against numerous Triticum spp. revealed a lot of variation in the degree of host 'resistance' and pathogen virulence (Bernier, 1976, 1978). There appeared to be a gradation in the symptoms from susceptible (in which large sclerotia were produced) through progressively smaller sclerotia (and less honeydew exudation) to no sclerotia in the 'resistant' varieties. However, even though resistance resulted in no sclerotia, the floret did not produce a seed either. The ovary was usually shrivelled and the early infection aborted. Thus resistance did not maintain seed yield, but in the field would affect the level of primary and secondary inoculum. The basis for this resistance has not been explained but a model system seems to be evident in T.zhukovskyi which failed to support sclerotium formation by any Canadian strain of C.purpurea (Bernier, 1978). Further investigation of this, as yet unexplored, approach to the study of ergot disease would surely reveal some very interesting aspects of the interactions between this parasite and its hosts.

1.6. MICROSCOPY OF INFECTION AND ERGOT DEVELOPMENT

The ergot fungus Claviceps purpurea has been the subject of much work with regard to its pharmacology, toxicology and history. Relatively little, however, has been published on the plant pathological aspects, particularly on microscopical studies of infection and the development of this parasite. Most of the existing literature on this topic is between 50 and 100 years old, and was written mainly in French and German. The first detailed work of this kind was carried out by Tulasne in 1853 and extended by Kuhn in 1863. By 1853 it was recognised that ergot was a fungus and not an aberrant seed, and that it took several different forms. Tulasne was the first both to show that these forms were different stages in the life cycle of the fungus, and also to illustrate the various stages of host colonization and ergot development. Since then, descriptions of different parts of the life cycle of ergot have been published intermittently (Carruthers, 1874; Wilson, 1875; Whetzel & Reddick, 1911; Brentzel, 1927; Stoll & Brack, 1944; Ramstad & Gjerstad, 1955; Kybal, 1964). Other publications which have included studies on infection and development have varied considerably in the attention given to detail (Engelke, 1902; Kirchhoff, 1929; Milovidov, 1954, 1956; Campbell, 1958; Luttrell, 1977). Unfortunately not all the accounts of the infection process and early development of the parasite are in agreement, and according to Luttrell: "the early stages in invasion of the ovary by C.purpurea have not been adequately documented" (Luttrell, 1977). Indeed, so far no electron microscopical observations of parasitic C.purpurea have previously been published. Ultrastructural studies, however, have been carried out on C.purpurea growing in submerged culture (Vorisek et al, 1974b; Rehacek et al, 1975), and on the paspalum ergot fungus, C.paspali (Vorisek et al, 1974a). Luttrell

(1977) published the first electron micrographs of an ergot fungus during parasitism in his study of C.paspali colonization of Paspalum dilatatum (dallisgrass).

The aim of the present work therefore is, to investigate the infection, early colonization and subsequent development of C.purpurea in cereal ovaries in much greater detail than before, by using both light and electron microscopy.

Since the ergot fungus infects the flowering parts of cereals, it is important to have a basic understanding of the structure of the cereal flower, how flowering, or more precisely anthesis, occurs, and what changes take place in the ovary tissues during and shortly after this process. On the basis of this understanding, the various stages of ergot development can more easily be appreciated in both anatomical and biochemical terms.

a) The structure of the flower and anthesis

The inflorescence of wheat, barley and rye is known as a spike and its main axis, the rachis, bears a number of spikelets (Plate 1). The axis of a spikelet is the rachilla and each spikelet has 2-5 florets contained within 2 glumes. The upper floret(s) of a spikelet may be sterile. A floret consists of the lemma and palea (flowering glumes) within which are found 3 stamens, one pistil and two lodicules (Fig.

2). The pistil consists of an ovary bearing 2 styles, each with a feathery stigma.

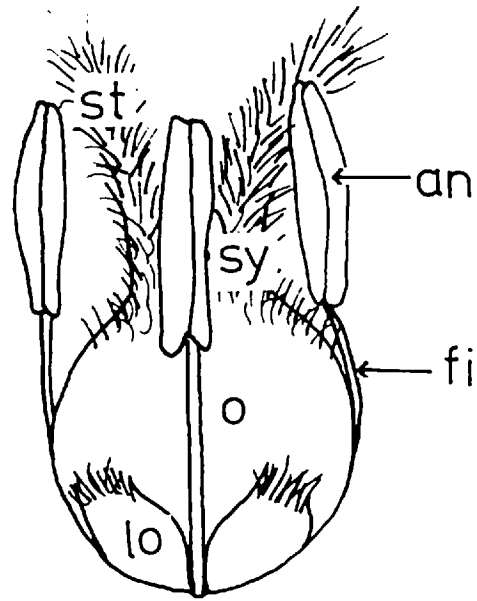
The flowering pattern of the cereals is broadly similar. At anthesis, the lodicules absorb water, swell and become turgid. This has the effect of pushing apart the lemma and palea, thus opening the floret. At the same time the filaments of the stamens elongate rapidly to about 3 times their original length, and the anthers hang outside the floret (Fig. 2 b). The anthers dehisce and release pollen.

FIG. 2. DIAGRAM OF THE WHEAT FLORET.

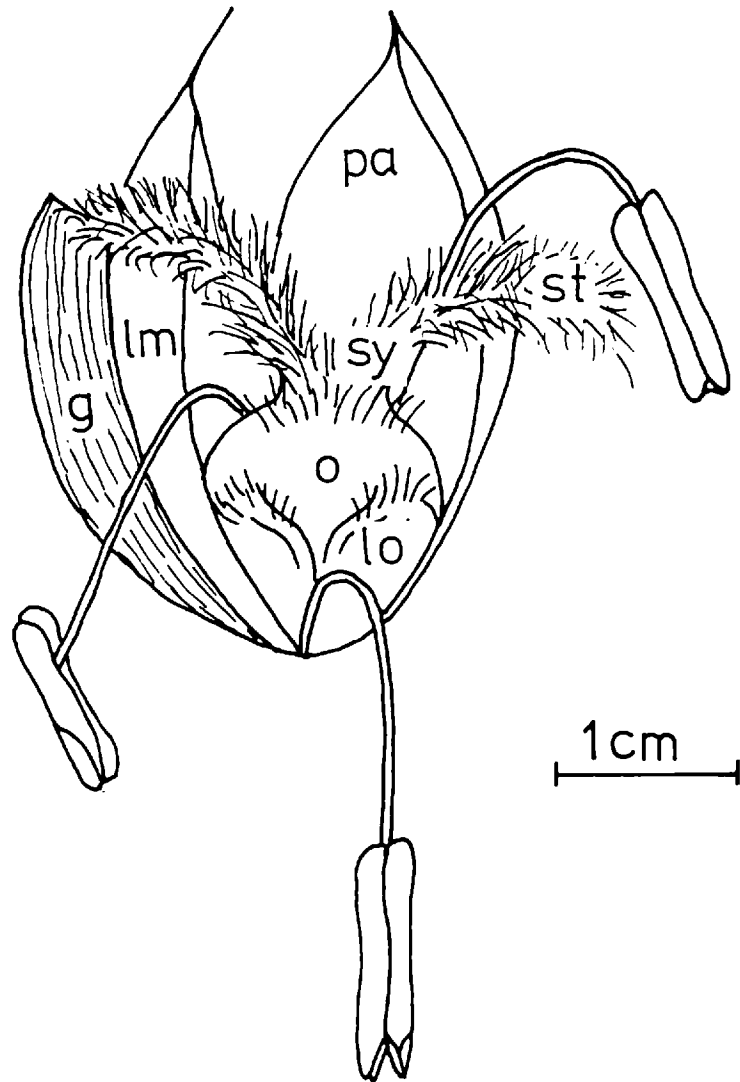
a) Ovary, dissected out from the protective glume, just before anthesis. The three stamens (anthers and filaments) are short and the stigmas erect. The lodicules are small and flattened against the ovary.

b) At anthesis. The ovary is shown in the normal position surrounded by the glumes. The lodicules swell and push apart the lemma and palea. The filaments elongate and the dehiscent anthers hang outside the floret. The stigmas curve away from each other and spread out.

an = anther; fi = filament; g = glume; h = hair; lm = lemma;
lo = lodicule; o = ovary; pa = palea; st = stigma; sy = style



a



b

Simultaneously the stigmas curve outwards away from each other, ready to receive pollen. After some time the lodicules lose their turgidity, collapse, and the floret closes, flowering having been completed (Percival, 1921; Peterson, 1965; Goss, 1968; Simmond & Campbell, 1976). This is a general description of the flowering process, and variations of it can occur. In wheat it is common for the flower to open after the pollen has been shed within the floret. Some varieties exhibit a closed flowering habit and in either event wheat, therefore, tends to be self-pollinated. Rye, however, is usually cross-pollinated and anthesis occurs much as in the above account. Infection by C.purpurea normally occurs during the flowering process, when the florets are open and exposed to the fungal spores. When the florets are closed, either before anthesis or, in the case of wheat, even at anthesis, infection may still occur if spores are carried into the floret by insects, honeydew or in water droplets (rain, dew etc.). The duration of opening of a floret can vary considerably, and is subject to environmental conditions as well as varietal characteristics. In wheat, many observations have been made of the duration of floret opening (Fruwirth, 1905; Obermeyer, 1916; Percival, 1921; Leighty & Sando, 1924) giving extreme values of 8 and 66 minutes in fertile florets. In male-sterile varieties however, and in florets containing sterile anthers in an otherwise fertile spikelet, the duration of floret opening is in the order of days rather than minutes (De Vries, 1971). Therefore, the period in which natural infection by C.purpurea is most likely to occur varies considerably with the conditions and the plant itself. If the infection is successful, the fungus develops in the ovary, but other parts of the flower can also be colonized e.g. lodicules, filaments (Kirchhoff, 1929; Milovidov, 1954).

b) The anatomy of the ovary

The wheat ovary is about 1-1.5mm long at anthesis and its apex is covered in long unicellular hairs (0.16 - 1.0mm long) (Percival, 1921). Distally, the apex of the ovary extends into two styles, bearing delicate stigmatic branches. Within the styles is found the conducting or transmitting tissue (Arber, 1937) for the pollen tube. The cells of this tissue are elongated and have a dense cytoplasm. This tissue continues down from the styles into the ovary and extends to the apex of the outer integument of the ovule. A diagrammatic representation of a longitudinal section through a cereal ovary at anthesis is shown in Fig. 3 a. Within the ovary is a single anatropous ovule, which is surrounded by two integuments. Each integument consists of a double layer of cells, the outer one being incomplete and forming an entrance to the ovule at the micropyle. The ovule contains the embryo sac which is surrounded by the nucellus. The nucellus is bordered by the nucellar epidermis, which lies immediately within the inner integument of the ovule (Fig. 4). External to the ovule is the parenchymatous ovary wall. It has an inner epidermis, adjacent to the outer integument, and an outer epidermis, which is covered by a thin cuticle.

The vascular system of the floret is of particular interest since, in the event of infection, it ultimately supplies the parasite with nutrients. The ovary has three vascular traces running longitudinally through the ovary wall (Fig. 3 b). The two lateral traces are continued into the styles while the finer dorsal trace terminates blindly. The three traces arise from the base of the floret, where there is a complex arrangement of various types of vascular tissue. The rachilla has 6 vascular bundles which are connected to those of the lemma and palea. In the upper part of the rachilla, just below the point of attachment of the ovary, there is a discontinuity in the

FIG. 3. DIAGRAMMATIC REPRESENTATIONS OF LONGITUDINAL AND TRANSVERSE SECTIONS THROUGH A CEREAL OVARY AT ANTHESIS.

a) Longitudinal section.

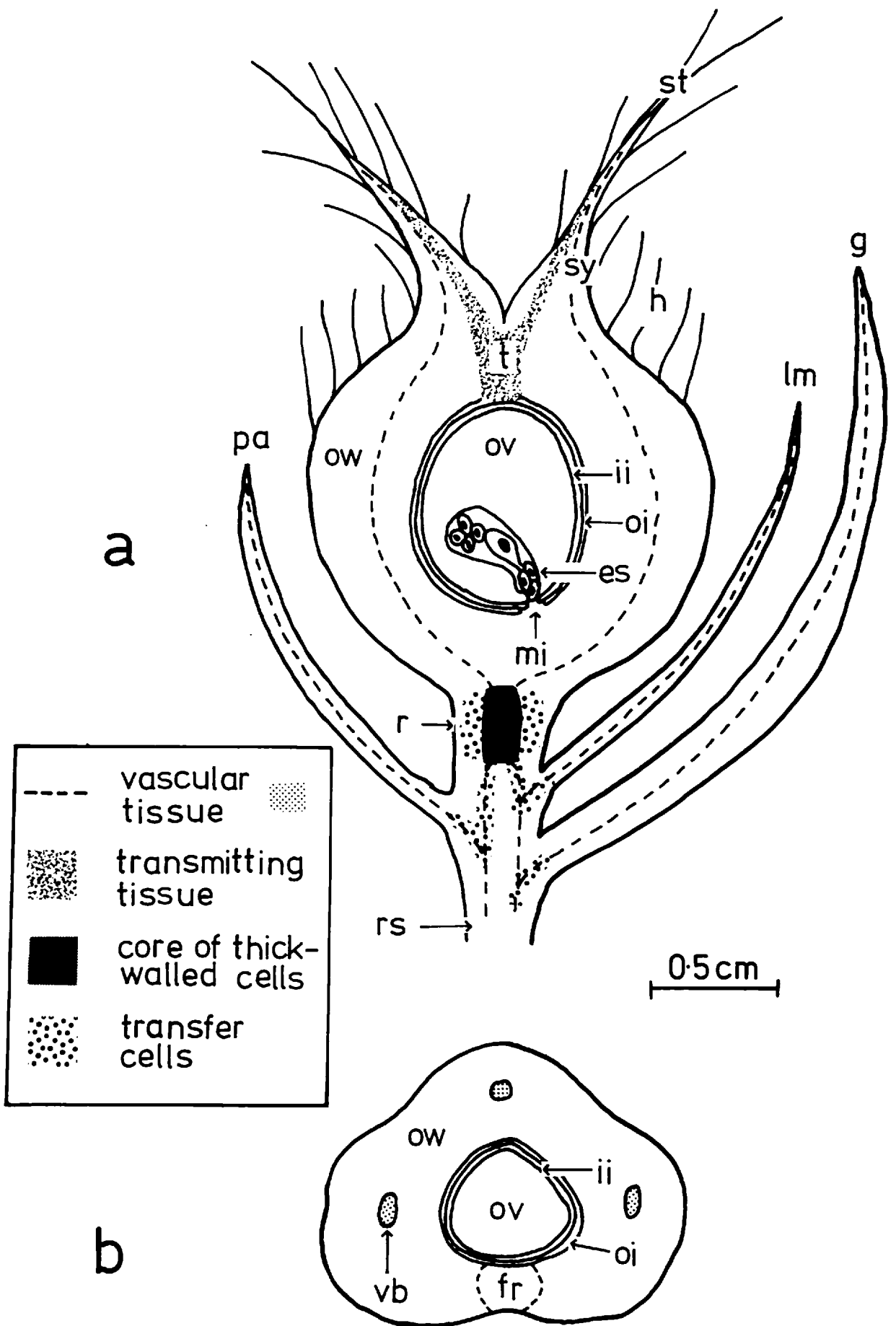
At the apex of the ovary there are two feathery stigmas, each containing a vascular trace and transmitting tissue. The latter is specialised for the passage of pollen tubes and extends to the outer integument of the ovule. The ovule is surrounded by the inner and outer integuments and contains the embryo sac.

The vascular system of the ovary consists of three vascular traces (seen clearly in b) originating from the base, above a core of thick-walled cells in the rachilla. Vascular transfer cells are found girdling the core and also at the junctions of the vascular tissue of the lemma, palea and glume with that of the rachilla and rachis.

b) Transverse section.

The two lateral and single dorsal vascular traces of the ovary are situated within the ovary wall. The dorsal trace ends blindly at the apex of the ovary while the two lateral traces continue up into the stigmas. There is little differentiation into distinct phloem and xylem in these traces.

es = embryo sac; f = furrow; g = glume; h = hair; ii = inner integument; lm = lemma; mi = micropyle; oi = outer integument; ow = ovary wall; ov = ovule; pa = palea; r = rachilla; rs = rachis; st = stigma; sy = style; t = transmitting tissue; vb = vascular bundle



xylem. This was discovered in wheat by Zee & O'Brien (1970,1971) to be an area of particularly thick-walled cells and was later found also in barley (Kirby & Rymer, 1975) and other grasses. This core of thick-walled cells consists of isolated portions of normal tracheary elements separated from each other by abnormally differentiated tracheary tissue. The core effectively interrupts the continuity of tracheary elements from the rachilla to the ovary and severely restricts the flow of material from the xylem in the rachilla to that in the ovary. However, sieve elements by-pass this core and these, together with their associated transfer cells, provide the only route for nutrients from the rachilla to the ovary. There is a network of transfer cells, associated with both the xylem and the phloem, where the glumes, lemma, palea and ovary join the rachis and rachilla. Transfer cells (Pate & Gunning, 1969,1972, ; Gunning & Pate, 1969,1974; Gunning & Steer, 1975) are cells specialised for absorptive and secretory functions and can occur in any anatomical position where there is intensive movement of solutes. They accomplish this by having a system of wall ingrowths which increase the surface area of the plasmalemma. A functional relationship exists between transfer cells and plasmodesmata, the latter allowing efficient diffusion of the increased quantity of solutes to or from surrounding cells. Transfer cells are important in these particular locations in the rachilla since it is thought that those associated with the xylem at the base of the core absorb solutes from the transpiration stream and transfer them to the sieve tubes symplastically. Other types of transfer cells particularly associated with the phloem may help to transport sugar synthesized in the glumes to those sieve tubes which encircle the core and supply the ovary. Therefore the xylem discontinuity has an important effect on xylem translocation to the ovary and later the developing seed (or ergot if infected) but solute transfer is

maintained from the rachilla to the ovary by the girdle of transfer cells and sieve tubes around the central core of thick-walled cells.

c) Changes in the ovary after anthesis

Immediately after anthesis and fertilization, several transformations take place in the ovary tissue (Fig. 4). The stigmas shrivel soon after pollen grains have germinated and pollen tubes have grown into them towards the ovule. Environmental conditions, particularly humidity and temperature, affect the onset and rate of stigma collapse. Once fertilization is accomplished (i.e. the male gamete has fused with the nucleus of the ovum) the zygote goes into a state of dormancy during which time it grows but does not divide (Batygina, 1974). This dormant stage lasts about 18 hours during which the endosperm has already begun to grow and divide. After the dormant period, the zygote divides and the young embryo rapidly develops. As the endosperm enlarges and starts to accumulate starch, the nucellar cells and its epidermis collapse and degenerate. The inner integument remains intact during the growth of the embryo and only when the seed has attained its maximum size does it collapse. Eventually it forms the testa. The outer integument however begins to degenerate at about the same time as the zygote begins to divide, and the cells shrivel up in a matter of days. The different layers of the ovary wall remain active for varying periods after fertilization (Fig. 4) but the largest part, i.e. the parenchyma of the ovary wall, begins to degenerate about 2-3 days after fertilization. The inner epidermis and inner layer of the ovary wall persist for about 7 and 9 days respectively after fertilization, and then their cell walls begin to thicken and contents disappear. These two modified layers can be located within the maturing seed. The cuticle and outer epidermal wall gradually thicken, and these, together with

FIG. 4 . PART OF A SECTION ACROSS A CEREAL OVARY ILLUSTRATING THE POSITION OF THE TISSUES AT ANTHESIS, AND THEIR FURTHER DEVELOPMENT DURING SEED FORMATION.

After anthesis, in the normal course of development from fertilized ovum to seed, the tissues in and around the ovule transform and take on different functions. Working from the interior to the exterior, i.e. from left to right of the diagram.

Within the ovule the embryo begins to develop in its embryo sac. The surrounding nucellar tissue gradually degenerates and makes room for the rapidly developing endosperm cells and aleurone cells. The nucellar epidermis begins to collapse 6-7 days after anthesis as the endosperm enlarges. The inner integument persists until the seed has reached its maximum size, and then the cells collapse and are crushed together to form the testa or seed coat. The outer integument however, begins to collapse soon after anthesis, and within 3-5 days has completely degenerated. The cells of the inner epidermis of the ovary wall start to change about 8 days after anthesis, when their walls thicken and the cells separate. At maturity they form the 'tube cells'. The cells of the inner layer of the ovary wall remain photosynthetically active until about 10 days after anthesis, when their walls thicken, their contents degenerate, and at maturity form the 'cross layer'. The parenchyma of the ovary wall begins to lyse 3-4 days after anthesis and at maturity forms a crushed layer between the 'cross layer' and the outer epidermis. The walls of the latter thicken, as does the cuticle, and these 3 tissues form the pericarp coat at maturity.

the crushed remains of the parenchyma of the ovary wall eventually form the pericarp coat (Percival, 1921).

An interesting transformation takes place in the vascular system of the ovary soon after fertilization. As the endosperm and, a little later, the embryo divide and grow, the vascular traces in the ovary wall become longitudinally stretched, distorted and progressively discontinuous particularly in the central and distal regions of the ovary. The lowest regions may persist and function for some time (Alexandrov & Alexandrova, 1939a,b). Simultaneous with this process, another conducting system, that of the furrow region (= placental-chalazal region) begins to differentiate, and replaces that of the ovary. This system has well defined xylem and phloem elements and solely supplies the developing endosperm and embryo (Frazier & Appalanaidu, 1965).

In flowering plants, the seeds are the biological end-products upon which the next generation depends, and since cereals are bred for their seed yield, grain filling is a particularly essential process in the ear. The plant is therefore geared physiologically and morphologically to the production of healthy viable seeds, and the vascular system of the ear is organised so as to provide the developing seed with an efficient nutrient supply. Since the ergot fungus replaces the ovary before the supplementary vascular system of the furrow region can differentiate and function, it must rely for its nutrition on the intact (Fig. 3 .) elements of ovary vascular traces, possibly together with the other parenchymatous tissue at the interface with the parasite. The extent to which the ovarian traces are important conducting elements to the ergot is open to speculation. Therefore, an appreciation of the anatomy of the host-parasite interface is essential in order to begin to interpret the mechanism of nutrient supply.

In conclusion, since infection by C.purpurea normally occurs during flowering, the events which occur at this stage are very significant. If pollination and fertilization do not succeed, for whatever reasons, and spores of C.purpurea are present, the chances of infection are relatively high. However, in the majority of cases, the fungal spores are probably present on the stigmas or ovary surface at the same time as pollen grains. The tissue changes resulting from pollen germination, growth and fertilization of the ovum are important determinants of the success of ergot infection, and their description here is particularly relevant since it has often been observed (e.g. Shaw, 1975), that ovaries become resistant to infection a few days after fertilization. The basis for this resistance has not been explained, but the speed and nature of the changes in the ovule and ovary following fertilization are presumably responsible for this in some way.

1.7. PECTOLYTIC ENZYMES IN PATHOGENESIS

In this study of the infection of cereals by C.purpurea and the role of some enzymes in parasitism, it is necessary to have a basic understanding of the composition and structure of plant cell walls and how various enzymes affect them. The following review outlines the current thinking on this topic, with emphasis on pectic substances and pectolytic enzymes.

a) The plant cell wall

The walls of plant cells are of fundamental importance in many aspects of plant morphology and physiology, and in plant-pathogen interactions (Esau, 1965; Albersheim, 1965). The wall can be considered as having three separate regions: the middle lamella, the primary wall and the secondary wall. The middle lamella functions as a cementing layer between plant cells. The primary wall is that part of the wall formed first by young, growing cells, and primary walls from different plant species have many features in common. The secondary wall, which is laid down after cessation of cell growth, strengthens the tissue structure and adds support to the whole plant. Lignin is a characteristic component of secondary walls and is often deposited after the secondary wall has formed (Lampert, 1970; Northcote, 1972; Talmadge et al, 1973).

The major components of plant primary cell walls are pectic substances (34-36%), cellulose (26-27%), hemicelluloses (34-38%) and structural protein (2-10%) (Northcote, 1963; Lampert, 1965; Keegstra et al, 1973). The pectic fraction is composed mainly of a high molecular weight polymer of α -1-4 linked galacturonopyranose whose carboxyl groups may or may not be methylated (Fig.5) (Wood, 1955; Kertesz, 1951; Bateman & Millar, 1966; Fogarty & Ward, 1974). The main chain is interspersed with 1-2 linked rhamnose which may some-

times be unevenly distributed and concentrated in certain parts of the chain causing kinks (Aspinall et al, 1968; Aspinall, 1970; Northcote, 1972), or its distribution may be non-random, as in cultured sycamore cell walls (Talmadge et al, 1973). The pectic fraction also contains neutral sugars bound, usually covalently, to the rhamnogalacturonide component. The exact nature of the neutral sugars varies between plant species but an arabinan and/or a galactan are usually present (Aspinall, 1970). In addition, some of the hydroxyl groups on C2 or C3 of the main chain may be acetylated (Deuel & Stutz, 1958) and the carboxyl groups can form ionic bonds with polyvalent cations e.g. Ca^{++} and Mg^{++} to yield rather insoluble salts. In the cell wall, these ions contribute to structural integrity by forming salt bridges between wall components.

Pectic acid is a term used for a pectic substance with no methyl groups. Pectinic acids are those substances which contain a variable quantity of methyl ester groups but less than pectins, which have 75% or more of their carboxyl groups methylated. Protopectin is an old term used for the insoluble parent pectic material which occurs in plants and which yields soluble pectic substances on restricted hydrolysis (Joslyn, 1962). The most striking property of pectic molecules is their ability to form gels and viscous solutions with water. This characteristic is important in their role within the plant wall where they act as a 'filler' substance between the cellulose microfibrils, thereby influencing the distribution of water in the wall.

Cellulose is a linear polymer of β -1-4 linked D-glucopyranose residues which form long, flat molecules, which are arranged into microfibrils. Within the microfibrils there are crystalline and amorphous regions depending on the orientation of the molecules, (Frey-Wyssling, 1969; Muhlethaler, 1967; Preston, 1971; Northcote,

1972). The microfibrils play an important part in the wall structure, where they constitute the basic skeletal elements. They are surrounded by non-cellulosic polysaccharides and glycoprotein which make up a continuous matrix.

The non-cellulosic polysaccharides or hemicelluloses include several polymers which vary between different tissues. Those commonly occurring include xyloglucans, xylans, mannans, glucomannans, galactoglucmannans, arabinogalactans, and arabinoxylans (Whistler & Richards, 1970; Bauer et al, 1973).

Primary walls also contain a protein which functions structurally. This is a glycoprotein rich in hydroxyproline (30%) which amino acid has not been found in any other plant protein. The carbohydrate moieties of the glycoprotein are arabinose and galactose (Lamport, 1965, 1970; Northcote, 1972; Keegstra et al, 1973).

Recent studies on cell walls from suspension-cultured sycamore cells (Keegstra et al, 1973; Talmadge et al, 1973; Bauer et al, 1973) have elucidated how these components are connected to create a functional wall matrix. According to these authors, many features of the sycamore cell walls are also found in cell walls of other plants. Although their evidence is incomplete, they suggest that the primary wall can be considered a single macromolecule in which all the components except cellulose are linked covalently. Cellulose is H-bonded to at least one hemicellulose (Blake & Richards, 1971). They have proposed a tentative structure for the cell wall (Keegstra et al, 1973) and this model has proved useful to workers studying cell wall degradation.

From this brief review on cell wall structure, the pectic polymers are revealed as forming a major component of plant cell walls. Their presence, however, is not restricted just to the primary cell wall since they also constitute much of the middle

lamella.

b) Cell wall degrading enzymes

Many plant pathogenic microorganisms are capable of producing extracellular enzymes which can alter or degrade the polysaccharide components of plant cell walls (Bateman & Millar, 1966; Albersheim et al, 1969; Bateman & Basham, 1976). However, many non-pathogenic and saprophytic organisms also produce polysaccharide-degrading enzymes (Bateman & Millar, 1966) which fact implies that ability to secrete these enzymes need not necessarily enable these organisms to be plant pathogenic (Bateman, 1976). Alternatively, in certain plant pathogens, ability to secrete these enzymes may be vital for pathogenesis (Basham & Bateman, 1976). These enzymes can play an important role in inter- and intracellular penetration and tissue maceration, and the ability of plant pathogens to secrete them represents just one aspect which may be significant in pathogenesis.

i) Pectolytic Enzymes

The pectic-degrading enzymes, which degrade the α -1-4 galacturonide bonds in the rhamnogalacturonide fraction of the cell wall (Fig.

5), are the key factors in the enzymatic degradation of the wall (Basham & Bateman, 1975a,b; Stephens & Wood, 1975). Their effects are three-fold: they solubilize the middle lamella, they weaken or loosen the structure of the primary wall, and in so doing allow other enzymes access to non-pectic wall polymers. These enzymes are the most studied of the wall-degrading enzymes, and knowledge of the role of the others is at present only elementary.

In the present study on C.purpurea, the production of pectolytic enzymes 'in vitro' and 'in vivo' were investigated. To help in appreciating which enzymes perform which transformation (Fig. 6), the classification of pectolytic enzymes will be described. Up to

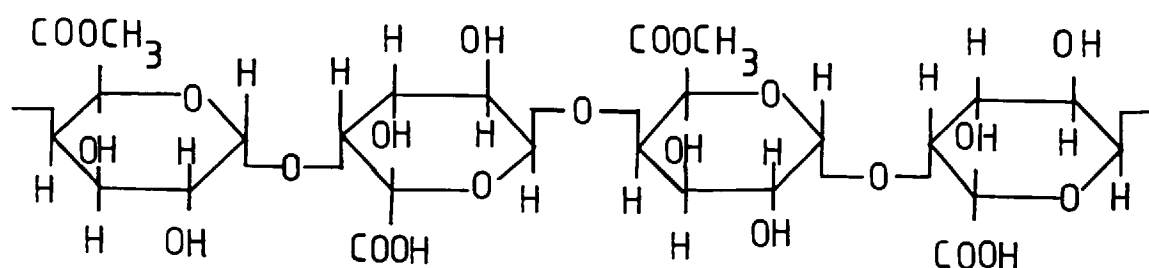


Fig. 5 Partially esterified chain of α -1-4-poly-D-galacturonic acid.

1960, hydrolytic cleavage of the α -1-4 galacturonide links in pectic substances was thought to be the only method of degradation. In that year, Albersheim et al discovered an enzyme which cleaved the chain transeliminatively, i.e. cleavage at C4 was accompanied by a simultaneous elimination of H from C5, and yielded a reaction product with an unsaturated bond between C4 and C5 which absorbs maximally at 235nm. The classification of pectolytic enzymes was modified after 1960 (Bateman & Millar, 1966), and the nomenclature (Table 1) used throughout the present work is based on that of Fogarty & Ward (1974). The three main criteria used in the classification are:

- 1) the method of cleavage of the α -1-4 glycosidic links (i.e. hydrolytic or transeliminative),
- 2) the substrate preference (i.e. pectin or pectic acid),
- 3) the position of cleavage in the chain (i.e. terminal = exo or random = endo).

Enzymes which cleave links other than the α -1-4 galacturonide bonds are also produced by plant pathogens and play an additional role in

TABLE 1. CLASSIFICATION OF PECTOLYTIC ENZYMES1. PECTINESTERASES:

These enzymes remove some or all of the methoxyl groups of pectin or pectinic acids to yield pectic acid or less methylated pectinic acids and methanol. Although their action does not affect the length of the pectin chain, they can alter its solubility and affect the rate at which it can be attacked by the depolymerising enzymes (Fig. 6). PE does not cause tissue maceration or cell death.

2. DEPOLYMERASES:a) Hydrolytic cleavage of α -1-4 glycosidic links

ai) Polymethylgalacturonase (PMG):

Pectin is the preferred substrate and is attacked randomly (by endo-PMG) or sequentially from the non-reducing end (by exo-PMG).

a ii) Polygalacturonase (PG):

Pectic or polygalacturonic acids are the preferred substrates and are attacked randomly (by endo-PG) or sequentially (by exo-PG).

b) Transeliminative cleavage of the α -1-4 glycosidic links

bi) Polymethylgalacturonate lyase (PMGL) = pectin transeliminase(PTE)*:

Pectin is the preferred substrate and is attacked randomly (by endo-PMGL) or sequentially from the non-reducing end (by exo-PMGL).

b ii) Polygalacturonate lyase (PGL) = pectic acid transeliminase(PATE)*:

Pectic or polygalacturonic acids are the preferred substrates and are attacked randomly (by endo-PGL) or sequentially (by exo-PGL).

* = Alternative names used by some authors.

Fig. 6 is a simplified illustration of the modes of action of these enzymes.

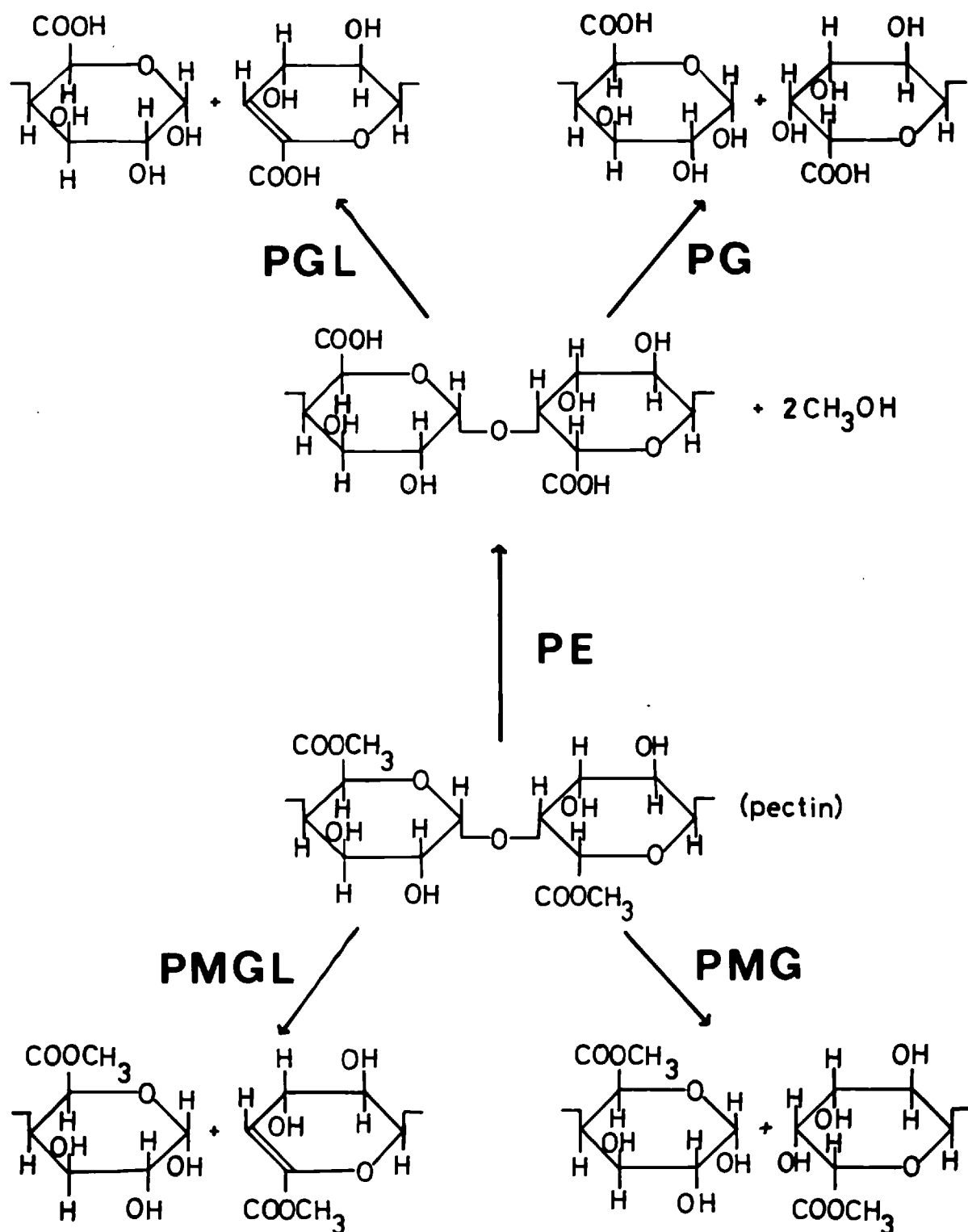


Fig. 6 . ENZYMATIC DEGRADATION OF PECTIN

(for details see text and Table 1)

wall degradation e.g. rhamnosidase (Kaji & Ichimi, 1973) and araban- and β -1-4 galactan - splitting enzymes (Byrde & Fielding, 1965; Bateman et al, 1969; Van Etten & Bateman, 1969; Bauer & Bateman, 1975; Hislop et al, 1974).

Some pathogens produce both pectic hydrolases and lyases, while others produce only one type (Goodenough & Maw, 1974; Talboys & Busch, 1970; Bateman, 1972; Mount et al, 1970). Also, some can produce different isozymes of certain pectinases (Garibaldi & Bateman, 1971). These properties may help to ensure at least some degradation of the host's wall polysaccharides in a variety of environmental conditions (Bateman, 1976; Albersheim et al, 1969).

Pectolytic enzymes also occur normally in plants and are produced by healthy plant tissue. In such circumstances their concentration is very low in comparison to that found in diseased tissue, but in healthy plants they are found in highest concentration in the fruits. There they are thought to play an important role in fruit maturation, ripening and abscission (Reymond & Phaff, 1965; Hobson, 1962; Hultin & Levine, 1963; Rouse et al, 1964; Morre, 1968). The two enzymes most commonly involved in these functions are PE and PG (McCready et al, 1955) and because of the nature of their function, they are subject to seasonal fluctuations. Reports of lyases in healthy plant tissue are rare although PMGL has been demonstrated in healthy pea seedlings (Albersheim & Killias, 1962). It is not clear how changes in pectolytic enzyme activity in healthy plants are controlled, although inhibitors, e.g. auxin, fatty acids, have been implicated (Albersheim & Killias, 1962; Miller & McCulloch, 1959; Bryan & Newcomb, 1954). Whether these factors have any influence over pectolytic enzymes produced by plant pathogens during infection and colonization is not known.

The control of the synthesis of polysaccharide degrading

enzymes by pathogens is an important factor in the outcome of an infection, i.e. whether the plant will be resistant or whether the pathogen will successfully colonize (Albersheim et al, 1969; Bateman, 1976). In some host-parasite relationships e.g. obligate parasites, the precise regulation of such enzyme production is far more necessary than in organisms causing, for example, soft rot, since in the former a delicate balance is required between tissue colonization by the pathogen and host cell vitality. The production of pectolytic enzymes by plant pathogens and the ensuing wall degradation is a complex phenomenon. The environment in which the pathogen finds itself greatly influences whether the pathogen produces a particular enzyme, and its subsequent activity and stability (Albersheim et al, 1969; Albersheim & Anderson, 1971; Albersheim & Anderson-Prouty, 1975). This applies to production in culture and also during plant parasitism. Enzyme production may be controlled by a variety of mechanisms, but it is clear that carbohydrates play a large part in this regulation. Polysaccharide degrading enzymes are produced by microorganisms either constitutively or inducibly (Keen & Horton, 1966; Mussel & Green, 1970; Patil & Dimond, 1968; Mandels & Reese, 1960). Induced synthesis is brought about usually by a monomer or dimer of the enzyme's substrate. It has been repeatedly demonstrated that different pectolytic enzymes are produced on different pectic substrates in culture, and the addition of, or substitution by, glucose results in an altered pattern of enzyme synthesis (Waggoner & Dimond, 1955; Hancock et al, 1964; Hancock, 1966). When work of this nature was extended to the pathogenic situation, again different enzymes or different proportions of the enzymes were produced (Bateman, 1966; Hancock et al, 1964).

The synthesis of these enzymes is also subject to catabolite repression. This is defined as a reduction in the rate of enzyme synthesis in the presence of glucose or other readily metabolized

carbon sources (Paigen & Williams, 1970; Cooper & Wood, 1975), and can apply to both induced and constitutive synthesis. It is brought about by the intracellular accumulation of certain sugars in the cells where enzyme synthesis occurs. These sugars repress further synthesis of the enzymes, but such repression is not specific since sugars released from polymers by certain enzymes may repress synthesis of different enzymes (Cooper & Wood, 1975). Enzyme synthesis is termed inducible if a greater enzyme activity is detected when the microorganism is grown on such substrates as mentioned above than when grown on glucose. However, sugars that serve as inducers also function as non-specific catabolite repressors when critical levels are exceeded. In some cases synthesis of an enzyme might appear to be inducible, where in fact it is constitutive but subject to severe catabolite repression. However, some constitutive enzymes appear not to be subject to catabolite repression (Ayers et al, 1969); Mussell & Strouse, 1972; Bateman et al, 1973). Most of the work done on enzyme induction has been performed on microorganisms grown in culture, but such regulation is important also in host-parasite relationships (Keen & Horton, 1966; Horton & Keen, 1966; Zucker & Hankin, 1971; Biehn et al, 1972; Fox et al, 1972; Weinhold & Bowman, 1974).

Another possible mechanism of regulating pectolytic enzyme activity is catabolite inhibition. This differs from catabolite repression in that glucose inhibits enzyme activity, rather than synthesis of enzymes involved in the utilization of other carbohydrates (Paigen & Williams, 1970). This mechanism would provide a rapid method of control over preformed enzyme molecules.

ii) Hemicellulases and Cellulases

These enzymes have received less attention than the pectolytic enzymes since, unlike the latter, none of them cause substantial

degradation of whole cell walls. Chronologically, the pectolytic enzymes are produced first by several pathogens when exposed to their host's cell walls, followed by hemicellulases and lastly, cellulase. This pattern of enzyme synthesis may reflect the extent of accessibility of the various wall polymers (Jones et al, 1972; English et al, 1971; Mullen & Bateman, 1975; Cooper & Wood, 1975). Generally hemicellulases degrade their substrates hydrolytically to the component sugars, mainly pentoses and uronides. Also, cellulases of certain pathogens are capable of hydrolyzing some hemicelluloses (Bauer et al, 1973; Mullen & Bateman, 1975b; Van Etten & Bateman, 1969; Jones & Bateman, 1972).

The enzymatic breakdown of cellulose involves a series of reactions. An enzyme known as C_1 loosens the cellulose chains, allowing greater accessibility for the sequential activity of the other enzymes: C_X (β -1-4 glucanase) and cellobiase (β -glucosidase). Several plant pathogens produce these enzymes both 'in vitro' and during parasitism (Bateman, 1964, 1969; Kelman & Cowling, 1965; Cooper & Wood, 1975; Keen & Horton, 1966). They are also subject to induction and catabolite repression as previously discussed for pectolytic enzymes.

c) Relationship between pectolytic enzymes, cell separation and cell death - a short review

The pectolytic enzymes have been implicated in tissue maceration for a long time (Brown, 1915; Jones, 1909). Maceration is brought about by degradation of the middle lamella region of the cell wall, and more recently pectolytic enzymes which split the α -1-4 bonds in the uronide fraction of pectic components of the wall have been shown to cause maceration (Bateman, 1963; Demain & Phaff, 1957; McClendon, 1964). The action of pectolytic enzymes on cell walls

can make non-pectic wall components more available to attack by other enzymes (Talmadge et al, 1973). These other enzymes attacking different wall constituents are not necessary for maceration to occur (Bateman, 1968; Sato, 1968). During maceration, plant cells are also injured and killed (Alberghina, 1973; Hall & Wood, 1973; Mussel, 1973; Mount et al, 1970; Tribe, 1955). Such injury and, ultimately, death is characterised by the inability of cells to retain neutral red stain (Tribe, 1955) as well as rapid loss of water and ions and inability to plasmolyse in hypertonic solutions (Hall & Wood, 1973; Mount et al, 1970; Wood, 1972). The neutral red method is based on the ability of a living cell to be plasmolysed in hypertonic solution and of its vacuole to retain neutral red stain, i.e. it tests the integrity of the plasmalemma and the tonoplast. However, the physiological basis of uptake of the stain is not clear. The tonoplast (the membrane delimiting the vacuole) has different properties from those of the plasmalemma, and can function in conditions in which the plasmalemma cannot (Robards, 1970; Stadelmann & Kinzel, 1972). Therefore, integrity of the tonoplast is not necessarily correlated with integrity of the plasmalemma. In diseased tissue the permeability of cell membranes can be altered (Wheeler & Hanchey, 1968), and this can be demonstrated by conductivity measurements (Hall, 1971). In some cases electrolyte loss (indicating plasmalemma leakage) may be detected when neutral red is still retained in the vacuole, suggesting that the tonoplast is still intact and functioning. Therefore, by relying solely on this test, an inaccurate impression of cell membrane integrity might be gained, since cell damage may occur before the neutral red method can detect it.

Recent work has shown that two types of endo-enzymes are responsible for macerating tissue and killing cells - namely polygalacturonases (PG) and pectic transeliminases or lyases (both

PGL and PMGL) (Mount et al, 1970; Garibaldi & Bateman, 1971; Byrde & Fielding, 1968; Hall & Wood, 1973; Bateman, 1972; Mullen & Bateman, 1971; Basham & Bateman, 1975,a,b). Several pectolytic enzymes have been reported not to macerate plant tissues (Byrde & Fielding, 1968; Garibaldi & Bateman, 1971) but the reasons for this are not understood. In several of these studies the enzyme preparations were not homogeneous. Basham & Bateman, (1975a,b) were the first to demonstrate that a homogeneous enzyme preparation (endopectate lyase from Erwinia chrysanthemi) could cause cell death and tissue maceration. So far there has been no report of these pectolytic enzymes causing tissue maceration without later causing cell death (Hall & Wood, 1973; Tribe, 1955). Ever since this phenomenon was first observed, numerous suggestions have tried to explain how cells are killed by pectolytic enzymes. Tribe made a significant discovery in 1955 showing that killing of protoplasts was delayed when the tissue was placed in a plasmolyticum containing pectolytic enzymes. In such circumstances cell separation still occurred. If the tissue was then deplasmolysed, electrolytes were rapidly released and cells lost their ability to retain neutral red stain. This finding has led several workers to consider the plasmalemma as a major target of pectic enzyme activity. Mount et al (1970) suggested that direct interactions between pectolytic enzymes and specific substrates in the host plasmalemma led to cell injury, and that sites of interaction in the membrane became masked or unavailable when the plasmalemma contracted during plasmolysis. This theory could be supported if the polysaccharide components associated with the plasmalemma surface were found to contain pectic substances (Roland, 1969; Roland & Vian, 1971). If pectolytic enzymes cannot penetrate the contracted plasmalemma (Garibaldi & Bateman, 1971), substrates in the cytoplasm will be equally unavailable. However, when tobacco protoplasts were subjected to osmotic stretching, (i.e.

protoplasts were allowed to expand in solutions of low osmotic strength), Basham & Bateman (1975a) found no significant increase in protoplast death. An increased death rate might have been expected since any previously masked sites would have been exposed, allowing enzymatic interaction. However, osmotic stress may be required before any such interaction causes detrimental effects. Although this theory has not been eliminated, so far there is no direct evidence to support it, and since isolated protoplasts are not killed by pectolytic enzymes, it seems an inadequate explanation (Tseng & Mount, 1974; Pilet, 1973; Stephens & Wood, 1975).

However, isolated protoplasts are killed by phosphatidase and proteinase (which are found in infected tissue (Tseng & Mount, 1974)), and contamination of pectolytic enzyme preparations by these two has been thought a possible explanation of cell death (Tribe, 1955; Brown, 1965). It has been demonstrated, however, that phosphatidases and proteinases from pathogens are non-toxic to intact plant tissue (Tseng & Mount, 1974) even after tissue has been macerated with a pectolytic enzyme (Stephens & Wood, 1975). This together with work on highly purified pectolytic enzymes (Basham & Bateman, 1975a) has eliminated these two enzymes as causes of cell death in host tissue.

Another possible cause of cell death is the toxicity of breakdown products from plant tissue following pectolytic enzyme degradation. The action of pectolytic enzymes on plant walls can release oxidase enzymes which generate hydrogen peroxide (Lund, 1973). Hydrogen peroxide may accumulate in the tissue, and, since it is reported to cause increases in plasmalemma permeability (Siegel & Halpern, 1965), may contribute to cell death in tissues treated with pectolytic enzymes. However, Bateman & Basham (1976) and Stephens (1974) found that catalase had no effect on cell death by pectic lyase. Therefore, hydrogen peroxide is apparently not involved in

cell injury or death caused by pectic lyase. In addition, Basham & Bateman (1975a) found no evidence of a low-molecular weight phyto-toxic breakdown product from pectic lyase-treated tissue. This was supported by Stephens & Wood (1975). Therefore it appears that cell injury or death is not due to toxic products from the action of pectolytic enzymes on plant tissue.

Another explanation involves the lysis of the plasmalemma by positively-charged protein molecules (Ruesink, 1971; Ruesink & Thimann, 1965). However further work on the ionic properties of the enzyme has not supported this (Basham & Bateman, 1975a).

Plasmodesmata have also been implicated in cell death, but alone these structures are not considered to account adequately for cell death (Hall & Wood, 1973; Wood, 1976).

The hypothesis which best fits the evidence (on cell death caused by pectolytic enzymes) is that membrane damage results from enzymatic injury to the cell wall. The loosened structure of the wall would then allow protoplasts to expand under turgor pressure. Stretching of the plasmalemma could then lead to increased permeability. Rupture of the plasmalemma would occur when the limit of elasticity was exceeded, and this would be the stage at which neutral red could be released from stained cells (Wood, 1967; Alberghina et al, 1973; Hall & Wood, 1973; Basham & Bateman, 1975a,b; Wood, 1976; Bateman, 1976). It has been shown (Basham & Bateman, 1975a,b), that mere contact between enzymatically treated cell walls and the plasmalemma did not cause cell death; only when these cells were osmotically stressed did any injury and cell death occur. It was concluded that membrane damage is an indirect phenomenon and is closely correlated with cell wall breakdown. Although electron microscopical studies (Stephens, 1974) have failed to contribute to the understanding of how pectolytic enzymes cause cell death, the biochemical evidence of

the structure of cell walls and their degradation by pectolytic enzymes supports the hypothesis that direct action of these enzymes on the cell walls leads to membrane damage and ultimate cell death.

1.8. β -GLUCANS AND β -GLUCANASES

β -1-3 glucans occur in the walls of fungal hyphae and are thought to predominate in the outermost region (Hunsley & Burnett, 1970). The wall consists of intermeshed microfibrils embedded in an amorphous matrix. In filamentous fungi the microfibrillar phase is composed of chitin or cellulose; in yeasts the β -glucans are non-cellulosic. The binding matrix consists of various polysaccharides and protein. Using enzyme dissection as a technique to identify and locate the various components of hyphal walls in Neurospora crassa, the innermost layer has been shown to consist mostly of chitin microfibrils possibly mixed with protein. External to this is a discrete layer of protein, then another protein layer containing strands of glycoprotein reticulum. The outermost layer contains glucans with β -1-3 and β -1-6 linkages. These regions tend to merge into one another without forming distinct layers (Hunsley & Burnett, 1970).

Bartnicki-Garcia (1968) has shown that fungi can be classified according to the main components of their walls because of the close correlation between the conventional classification and fungal wall chemistry. Claviceps spp. are in his category V, determined by the predominance of chitin and non-cellulosic β -glucans.

β -glucans also occur in the walls of yeasts along with mannans, chitin, proteins and lipids (Pfaff, 1971). The relative abundance of these constituents varies between species and between different forms of the same species. In Schizophyllum commune alkali-soluble and alkali-resistant forms of β -glucans occur in the wall depending on their attachment to chitin. Also, a water-soluble β -glucan (with a β -1-3 linked backbone and β -1-6 links at about every 4th unit) is excreted into the culture medium and surrounds the wall structure (Wessels, 1978).

Such extracellular secretion of β -glucans has also been observed in other fungi (Perlin & Taber, 1963; Clarke & Stone, 1963; Davis et al, 1965; Buck et al, 1968; Willetts, 1971, 1972; Archer et al, 1977;

Santamaria et al, 1978). Buck et al (1968) showed that submerged cultures of Claviceps fusiformis became increasingly viscous during growth, due to the production of extracellular glucan, having a β -1-3 linked main chain, and β -1-6 links at approximately every 4th unit. β -glucans with β -1-3 links also occurred in the walls of cultured mycelia and of parasitic sclerotia of this fungus. Sclerotium rolfsii, Sphacelia sorghi and C. litoralis were also shown to possess β -glucans having β -1-3 backbones and β -1-6 linkages of varying frequency. Perlman & Taber (1963) similarly found two β -glucans in C.purpurea, one associated with the mycelium and one in the culture filtrate. Their structures were closely related and probably differed only in molecular size. The glucan produced by C.fusiformis was remetabolised to a very limited extent after the first growth phase was complete (Buck et al, 1968). However, Dickerson et al (1970) found a variant of C.fusiformis (Strain 139/2/1G) which initially, in culture, produced a β -glucan which was later degraded by the subsequent production of β -glucanase. The viscosity of the culture was correspondingly reduced and the liberated glucose allowed a further increase in biomass towards the end of the fermentation. Similar β -glucanase activity was found in cultures of C.purpurea which apparently did not produce glucan during growth. This activity presumably was sufficient to prevent an accumulation of glucan, thereby keeping viscosity low. These enzymes were found to be constitutive (Dickerson et al, 1970). Other workers also noticed reductions in viscosity of various fungal cultures due to glucan breakdown, and speculated that β -glucanases were responsible (Davis et al, 1965).

The work of Santamaria et al (1978) and Archer et al (1977) demonstrates an interesting phenomenon in the production of fungal glucans. Both the above authors worked on Monilinia frutigena glucans. Santamaria et al (1978) found that this organism produced a glucan with a β -1-3 linked main chain and β -1-6 links on every second glucose unit. Archer

et al (1977) however, found the presence of both α - and β -linked glucans. The main linkage was 1-4 with some 1-2 links, and 1-3 type links were virtually absent. These differences are due probably to the difference in culture media used by the two sets of workers.

It is clear, therefore, that one fungus can produce structurally different extracellular polysaccharides under different conditions. It follows that these differences also occur in wall glucans. Buck et al (1968) suggested that there may be a structural connection between the cell wall glucans and those secreted extracellularly. This however has not been verified, nor whether such extracellular glucan production occurs during the growth of Claviceps spp. in their hosts.

Production of extracellular glucans is thought to be a mechanism by which sugars are set aside, to be remetabolised at a later stage (Archer et al, 1977.) In sclerotia of Claviceps spp., glucans may form a reserve polysaccharide, which together with the triglyceride oils, are used in sclerotial germination (Dickerson et al, 1970).

β -glucans also commonly occur as storage polysaccharides in several classes of algae (Haug & Larsen, 1973). Laminarin is a term for β -glucans found in brown algae (Phaeophyta) e.g. in Laminaria cloustoni. Laminarin is therefore a term describing a range of essentially linear β -1-3 glucans which vary slightly depending on their source. They may differ in the following: degree of branching, branch length, occurrence of β -1-6 links, presence of mannitol residues, degree of polymerization, which factors would affect their solubility (Wang & Bartnicki-Garcia, 1974). For instance, laminarin from L.cloustoni has been shown to contain both β -1-3 and β -1-6 links and mannitol residues (Nelson et al, 1963) whereas that from Chrysophyta does not contain mannitol (Beattie et al, 1961). Extracted laminarins are often used as substrates for testing the activity of various β -1-3 glucanases (Nelson et al, 1963; Reese & Mandels, 1959).

Polysaccharides very similar to laminarins have been isolated from

mycelium and zoospores of Phytophthora spp. and other Oomycetes, and have been termed mycolaminarins (Wang & Bartnicki-Garcia, 1974). These authors suggested that mycolaminarins may constitute distinctive storage polysaccharides in the Oomycetes since glycogen, the typical reserve polysaccharide in other fungi, is apparently absent. They also suggest that this may indicate a phylogenetic link between Oomycetes and the algae (Phaeophyta & Chrysophyta).

These mycolaminarins also have special properties in that they are phytotoxic (Keen et al, 1975). Plants treated with mycolaminarins extracted from the mycelium of various Phytophthora spp. produced symptoms similar to those observed in infected plants. Similar symptoms were produced by algal laminarin, pustulan ($\alpha\beta$ -1-6-glucan), carboxymethyl cellulose and mycolaminarin phosphate, and therefore this appears to be a non-specific effect. The mycolaminarins of Phytophthora became systemically distributed, without occluding vascular tissue, and produced widespread symptoms of toxicity. In culture however, very little mycolaminarin is produced which suggests that equally little may be produced extracellularly in the parasitic situation. Keen et al (1975) suggest that lysis of fungal cells could release the cytoplasmic laminarins into the tissue. In addition, since β -glucans are also found in the fungal wall, the activity of host enzymes might cause their release and produce toxic effects. The mode of such toxicity, however, is yet to be explained, although recent work has shown that β -1-3 glucans function as elicitors of phytoalexins (Ebel et al, 1976; Ayers et al, 1976a,b).

Phytoalexins are produced by plants in response to several different unrelated stimuli. They are toxic to different types of microorganisms (Abersheim & Valent, 1978) and structurally are very varied (Ward & Stoessl, 1976). Evidence has been increasing to indicate that phytoalexins produced during fungal infection may play an important role in resistance by curtailing fungal growth (Yoshikawa et al, 1978; Albersheim

& Valent, 1978). Incompatible reactions between fungus and plant usually result in a hypersensitive response, in which invaded cells rapidly die and phytoalexins are produced (Jones et al, 1975; Kiraly et al, 1972; Mayama et al, 1975).

The host-parasite system Phytophthora megasperma var. sojae (Pms) and soyabeans (Glycine max) has been studied extensively (Ebel et al, 1976; Ayers et al, 1976; Keen, 1978). This work has shown that the elicitor, present in both culture filtrates and mycelial walls, is a β -glucan. Partial hydrolysis of this polysaccharide yielded various oligosaccharides which also possessed elicitor activity. The smallest elicitor-active oligosaccharide so far found contains nine glucosyl residues (Albersheim & Valent, 1978). This has a β -1-3 linked backbone with β -1-6 and -3-6 linked residues. Such branching appears to be a prerequisite for elicitor activity since other β -1-3 linked glucans lacking or low in C6 branching (e.g. laminarin) were found to have little elicitor activity.

Other results from Albersheim's laboratory have shown that the glucan elicitors from 3 races of Pms appear structurally identical and have equivalent abilities to elicit accumulation of the phytoalexin glyceollin in soyabeans. They are equally effective in this elicitation in resistant and susceptible varieties (Ayers et al, 1976). Therefore, the ability to stimulate phytoalexin production cannot alone determine race specificity. In addition, β -glucan isolated from yeast walls (Saccharomyces cerevisiae, which is not a plant pathogen) has the same glucosyl linkages and elicits the same response (although stimulates lower maximum levels of phytoalexin) as that of the Pms glucan elicitor (Hahn & Albersheim, 1978). They, and other workers, have shown that a range of different fungi can stimulate phytoalexin production in one plant (Lisker & Kuć, 1977), which can then protect the plant from a broad spectrum of microorganisms (Albersheim & Valent, 1978). These authors also showed that different plants can respond, by phytoalexin production,

to a single elicitor.

In direct contrast to the results of Ebel et al (1976) and Ayers et al (1976), Keen (1975) found race specific elicitors in Pms as well as non-specific activity. The non-specific activity was due to β -glucans which were present in mycelial walls, intracellular mycolaminarins and culture filtrates of the pathogen. The race-specific elicitors appear to be glycoproteins which are probably located on the cell surface (Keen, 1978). Keen suggests that variation in the glycosyl part of the glycoprotein could account for the differences between races of the pathogen and thus for race specificity. Albersheim et al (1978) have also isolated factors from 3 Pms races which are race specific. The factors from incompatible races can protect a normally susceptible plant from infection by a compatible race. The exact nature of these factors, however, has not so far been reported.

Therefore, one current interpretation of this data (Albersheim & Valent, 1978) is that plants recognise the presence of microorganisms by molecules (elicitors) which originate from the microorganism. Since these elicitors are foreign to the plant, it responds by producing phytoalexins. β -glucans in fungal walls (Bartnicki-Garcia, 1968) are such elicitors, and it is likely that other structural types also act in this way. The phytoalexins are involved in resistance, although they do not entirely account for it. They do however prevent the growth of non-pathogens and incompatible races of pathogens, and in this way they appear to constitute a widespread defensive mechanism. Microorganisms which are pathogens have presumably evolved ways to overcome this mechanism. The question of elicitation as a determinant of host-specificity, however, is still unclear.

Interesting observations have been made on the antitumour activity of β -glucans in humans and other animals (Browder et al, 1976; Whistler et al, 1976). Generally, β -glucans with a predominance of 1-3 links are active in this way, whereas those with mainly β -1-6 links are less active.

Such glucans have been isolated from a variety of organisms including fungi and yeasts (Komatsu et al, 1970). These observations have prompted Hahn & Albersheim (1978) to suggest that β -glucans may function in recognition between different cell types, and their role as elicitors of phytoalexins is just one example of this.

Callose

Callose is a β -1-3 glucan which is thought to have a stable triple helix conformation (Kessler, 1958; Rees, 1973). It has a wide distribution in different cells and tissues of plants, and develops under various conditions (Mangin, 1892; Currier, 1957; Poovaiah, 1974; Fulcher et al, 1976). It is usually found mainly in 3 situations:

- a) as a wound response
- b) in phloem
- c) in pollen tubes.

The purity of callose found in these locations appears to vary and there is some disagreement in the literature. According to Currier (1957) callose might be a heterogeneous substance exhibiting source-dependent compositional differences. However, Crafts & Currier (1963) consider that callose deposits in diverse locations have a similar structure. It may be that callose has a basic β -1-3 linked glucan chain with slight variation in branching. Alternatively callose may occur together with other substances e.g. protein, lignin, cellulose which might interfere with the identification of callose, particularly when this involves histochemical staining.

- a) Callose is formed as a general host reaction to cell injury (Aist, 1976) be it mechanical (Nims et al, 1967; Walker, 1955; Currier, 1957; Lipetz, 1970), fungal (Bushnell, 1972; Kitazawa et al, 1973; McKeen & Rimmer, 1973; Kusano, 1936; Bushnell & Bergquist, 1975), viral (Shimomura & Dijkstra, 1975; Faulkner & Kimmins, 1975) or by nematode infection (Riggs et al, 1973). It has also been shown to occur in response to stress,

such as a small drop in temperature (Smith & McCully, 1977; Majumder & Leopold, 1967). Mechanical injury can be caused by cutting, inserting needles into tissue or single cells, ultrasonics and pressure. Pressure can be exerted by intercellular hyphae and may, in addition to the chemical stimuli which hyphae would provide, be a causal factor in papilla formation (Hanchey et al, 1968; Heath & Heath, 1971; Sargent et al, 1973; Skipp et al, 1974). Plant cells respond to the above types of injury in a similar manner (Mercer et al, 1974; Aist, 1976).

The initial response is usually a rapid aggregation of the cytoplasm at the wound site (Kusano, 1936; Walker, 1955; Nims et al, 1967) followed by the deposition of material between the plasmalemma and the wall, which seals or plugs the wound. This occurs more rapidly in young actively growing cells than in older, less active cells (Crafts & Currier, 1963). These wall appositions, when induced by fungal attack, are often termed papillae (Bracker, 1968; Smith, 1900). Although papillae are heterogeneous in structure and composition, callose is normally present in large proportion (Mangin, 1895; Aist & Williams, 1971; Sargent et al, 1973; Sherwood & Vance, 1976). Other reported constituents include lignin, cellulose, chitin, gums, silicon, protein, pectin and suberin (see Aist, 1976 for refs.), although these are not necessarily present in all cases.

In some virus infections resulting in local lesions, callose accumulation (+ other substances) in the veins or around the lesions, has been thought a factor in preventing the systemic spread of the virus (Shimomura & Dijkstra, 1975; Hiruki & Tu, 1972; Wu & Dimitman, 1970).

The main function of papillae, whether induced by mechanical or fungal injury, is thought to be to plug and seal the wound, thus preventing ion loss and the expulsion of the cytoplasm by turgor pressure. Papillae may thus also form a barrier impermeable to toxic materials which might damage the host cells (Skipp et al, 1974; Mercer et al, 1974). Relative impermeability of callose has been demonstrated by Heslop-

Harrison (1966). In addition to this wound healing role, it has been suggested that papillae act as a defense mechanism against fungal invasion (Bushnell & Bergquist, 1975; Heath, 1974), since papilla formation often correlates with failure of fungal penetration. Aist (1977) showed that mechanically induced papillae can prevent fungal ingress. However, some of the other results supporting this suggestion can have other interpretations (Kusano, 1939; Vance & Sherwood, 1976; Aist & Israel, 1977).

b) Callose is also found in the sieve tubes of phloem. This has been an area of study and contention for many years (Nageli, 1861; Esau, 1939) with claims that callose is found in sieve tubes only as a result of trauma during tissue preparation, and is not a normal constituent (Engelman, 1965; Zimmerman, 1960; Eschrich, 1963). It is now generally accepted that some callose is present in normal, healthy sieve tubes, but that further deposition, often massive, occurs upon injury, trauma or senescence (Engelman, 1965; Crafts & Currier, 1963; Eschrich, 1963). In contrast to that in papillae, callose in sieve tubes appears to be relatively pure (Ziegler, 1960; Esau & Cheadle, 1961; Lerch, 1960). In functioning sieve tubes, callose forms cylinders around the connecting strands in the sieve plate pores, and also in lateral pit areas (Currier, 1957; Crafts & Currier, 1963). Upon wounding, deposits of callose are found on either side of the sieve plate and also lining the pores, often constricting them (Engelman, 1963). More severe constriction and larger deposits are found in dormant or senescing sieve tubes (Crafts & Currier, 1963; Eschrich & Currier, 1964).

The functions of callose in sieve tubes include the classic concept of plugging and sealing. In normal sieve tubes, it appears to act as a valve in controlling translocation across the sieve plate, and since it is often deposited at plasmodesmata, it could affect cell to cell translocation (Livingston, 1935; Meeuse, 1957). Webster & Currier (1968) showed that heat-induced callose caused a reduction in lateral movement

of assimilates in phloem. In injured vessels, further callose deposition plugs the wound and prevents leakage of translocate (Crafts & Currier, 1963). It would also restrict movement between injured and healthy cells by 'blocking' the plasmodesmata. This suggestion is only valid if callose was relatively impermeable. Heslop-Harrison's work (1966) suggests that callose may be more impermeable than cellulose cell walls, and may restrict the passage of small molecules between tissues.

Alternatively, callose has been thought of as a by-product of disturbed metabolism without a defined function (Muller-Stoll & Lerch, 1957). Indeed the proposed function of plugging and sealing is based largely on the location of callose deposits rather than on translocation studies. However, the work of Webster & Currier (1968) showed that callose deposition has a real effect on translocation, by its plugging action.

c) The occurrence of callose in pollen tubes has been known for a long time. Strasburger (1878) first noticed plugs of unidentified material in pollen tubes of Gloxinia, which Mangin (1890) found to be callose (Brink, 1924). Further investigation has revealed that callose occurs also in the walls of pollen tubes. The main components of pollen tube walls are cellulose, pectin and callose (Rosen, 1968; Roggen & Stanley, 1971), callose predominating in the older part of the tube and pectin towards the tip (Linskens & Kroh, 1970). It has also been observed that when pollen tubes stop growing, callose accumulates in the tip (Roggen & Stanley, 1971; Tupy, 1959; Gorska-Bryllass, 1965). Pollen tube growth occurs only at the tip, and as growth proceeds, the older portion becomes disorganised and degenerates. Cresti & van Went (1976) recently made an electron microscopical study of callose deposition and plug formation. They showed that callose was deposited in two regions of the pollen tube, both outside the plasmalemma, closely appressed to the wall, and also as grains within the cytoplasm. Wall deposition resulted from callose formation within the rough endoplasmic reticulum cisternae which vesicles eventually

fused with the plasmalemma, thus secreting callose on to the inner surface of the wall. Callose grain formation occurred further back in the tube, and resulted from the disturbance of the normal process of wall deposition. Thus callose was freed into the cytoplasm and formed a plug. The disturbance arose from the degeneration and disorganisation of the cytoplasm in this region, portions of which often became enclosed in the callose grains. The zone of cytoplasm in which wall deposition took place appeared well organised and normal. Cresti and van Went's interpretation that plug formation is a reaction to trauma or damage is supported by the work of Tsinger & Petrovskaya-Baranova (1967) but the reason for this degeneration is unknown (Jensen & Fisher, 1970). Their explanation correlates with that for wound callose formation.

The consequence of plug formation is compartmentalisation of the pollen tube. The older i.e. distal parts of the tube gradually become isolated by successive deposition of plugs as the tip grows. Since most of the cytoplasm and two male gametes are in the tip, the older parts become obsolete. This 'active vegetative cell' (Brink, 1924) is capable of independent existence, since if the pollen tube is cut on the grain side of the plug, the portion of the tube containing the tip continues to grow. The plugs are therefore barriers to cytoplasmic flow. Thus, in this location, the function of callose appears to be one of sealing and plugging. However, the callose in the wall of the pollen tubes may have a different function. Cresti and van Went (1976) suggest that this extracellular callose may be involved in the imbibition of water, thus maintaining the osmotic conditions in the pollen tube.

It is interesting that incompatible reactions between stigma and pollen lead to large depositions of callose in the pollen grain protoplasts and pollen tube (Knox, 1973). If the pollen tube grows at all, it does so very slowly and eventually stops, while callose is deposited as unusually long plugs, thus occluding the tube, and at the tip (Tupy,

1959; Heslop-Harrison et al, 1974). Often the stigma cells adjacent to the incompatible grains also produce callose, unlike those further away (Knox et al, 1972; Knox, 1973; Heslop-Harrison et al, 1974). This does not occur in unpollinated stigmas or compatible pollinations (Heslop-Harrison, 1975). According to Knox (1973), this callose deposition plays an important part in arresting pollen tube growth. Cresti & van Went (1976) however, supported Tupy (1959) in contending that the initial effect of the incompatible reaction is inhibition of growth of the pollen tube which leads to premature degeneration of the cytoplasm. This in turn causes deposition of callose as plugs. Therefore, callose deposition in this situation results from a specific interaction between the stigma surface and the pollen grain, in which pollen wall proteins are involved. The way in which callose formation is started and its functions are, however, yet to be elucidated.

β -glucanases

β -1-3 glucanases have a wide distribution, occurring in algae, fungi, bacteria, higher plants and lower forms of sea-living animals, e.g. snails (Chesters et al, 1956; Huang & Giese, 1958; Currier, 1957; Reese & Mandels, 1959; Moscatelli et al, 1961; Villaneuva et al, 1976). This short review will be confined to fungal and plant β -1-3-glucanases.

In fungi, these enzymes are commonly constitutive (in contrast to β -1-2 and β -1-4 glucanases which are adaptive or inducible) and usually have an endohydrolytic (i.e. random) action. However, a highly active exo- β -1-3 glucanase is produced by Basidiomycete QM806, which was selected out of 140 organisms as the best source of this enzyme (Reese & Mandels, 1959). This enzyme was therefore used in the present study, to compare the β -glucanase activity of various Claviceps extracts. β -1-3 glucanases are secreted extracellularly and can therefore be found in culture filtrates. They also occur intracellularly, and in Candida utilis and Geotrichum lactis both intracellular and extracellular enzymes

show similar properties, suggesting that both activities correspond to a single enzyme (Villanueva & Gacto, 1973).

Several possible functions have been suggested for β -1-3 glucanases. They may digest β -glucans in their environment to smaller, more readily utilisable units. Internally, they may hydrolyse or help in synthesising food reserves with β -1-3 glucosidic links. This activity may be affected by growth and nutritional conditions since these would influence the level of β -glucan reserves.

The role most commonly ascribed to β -1-3 glucanases is in hyphal morphogenesis (Burnett, 1968). Bartnicki-Garcia (1973) has suggested a model for apical growth and branching in filamentous fungi, which involves a delicate balance between cell wall lytic and synthesising enzymes. The lytic enzymes (from cytoplasmic vesicles) are thought to be secreted into the wall where they attack the microfibrillar skeleton and create regions for the insertion of new wall materials. Synthesising enzymes (also from vesicles) rebuild the microfibrils after new material has been added. The participation of β -1-3-glucanases in wall growth has not been conclusively demonstrated, although there is much circumstantial evidence to imply it. There is a positive correlation between the levels of β -1-3 glucanase (and other lytic enzymes) and apical growth and branching in Neurospora crassa (Mahadevan & Mahadkar, 1970) and, more recently, has also been shown in Saprolegnia monoica Pringsheim (Fèvre, 1977a,b). This type of function, therefore, refers to intracellular β -1-3 glucanases whose activity would necessarily be controlled. In order to be effective as wall 'softeners', they would have to be endo-enzymes. Kritzman et al (1978) have made an elegant immunofluorescence study of the location of β -1-3 glucanase activity in the mycelium of Sclerotium rolfsii. They found that activity was confined to specific points i.e. hyphal tips, branching zones, clamp connections and new septa. They also suggested, however, that more β -1-3 glucanase was present along the hyphal walls, but in an inactive state,

and only becomes activated when exposed to the substrate. Branch initiation may be a consequence of this process. This is supported by Rosenberger (1978) in Aspergillus nidulans where the lytic enzymes are thought to be bound to the older parts of the wall, but are normally prevented from acting by an unknown mechanism. Branch formation is presumed to occur after activation of the enzymes by a suitable stimulus. Other workers have also provided evidence of β -glucanase association with the cell wall (Fleet & Phaff, 1974). Since β -glucans occur in fungal and yeast walls, β -glucanases and other enzymes have been used successfully to obtain protoplasts, and thus to help elucidate wall structure (Hunsley & Burnett, 1970; Peberdy, 1972, 1976).

In yeasts the main function of β -1-3 glucanases appears to be related to cell wall growth and budding, since in synchronous culture, these enzymes are produced maximally coincident with the budding process (Maddox & Hough, 1971). In yeasts, both intra- and extra-cellular β -glucanases have been discovered, although in some cases only the intracellular form is known (Brock, 1965). Normally β -glucanases from yeasts are highly specific for either β -1-3 or β -1-6 links. However, recent studies suggest that some β -glucanases exist which can hydrolyse both β -1-3 and β -1-6 links (Farkas et al, 1973; Villanueva et al, 1976). In addition different molecular forms of β -glucanases can be produced by one organism, suggesting that each form is responsible for the hydrolysis of specific linkages (Villanueva et al, 1976). Activity of β -glucanases can also be affected by molecular size of the polymer and degree of branching (Parrish & Perlin, 1960). In many cases it has been difficult to ascertain the specificity of β -glucanases because of the lack of homogeneous water-soluble substrates (Pegg, 1976). Laminarin has been widely used, but the presence of β -1-6 links in this polysaccharide may affect the activity of some β -1-3-glucanases. In addition, some β -glucosidases show a high affinity for oligosaccharides e.g. gentiobiose, laminaritriose, and therefore, if the

β -1-3 glucanase preparation is contaminated with such enzymes, estimation of enzyme activity may be inaccurate. In such cases it may be difficult to characterise the β -1-3 glucanase as an endo- or exo-type enzyme.

β -1-3 glucanases also occur in healthy plants (Whistler & Smart, 1953; Clarke & Stone, 1961; Abeles & Forrence, 1970; Pegg, 1976, 197) although their exact function has not been elucidated. It has been thought however, to be connected with the occurrence of callose which is produced quickly and can be removed with equal rapidity (Currier, 1957; Heslop-Harrison, 1966). In such cases β -1-3 glucanase activity need only be localised. In sieve tubes its formation and destruction could regulate flow of assimilates, and in lateral pit areas, could control cell to cell translocation. In Vitis vinifera, β -1-3 glucanase may remove dormancy callose from sieve plates (Clarke & Stone, 1961). The transient nature of callose deposits has also been shown in differentiating angiosperm meiocytes (Knox & Heslop-Harrison, 1970) and in the cross walls of actively dividing meristematic cells (Waterkeyn, 1967) and may be a common feature of early wall formation in cytokinesis (Fulcher et al, 1976). Whether this transience is only apparent, due to the masking of callose by other substances, or real, due to the action of β -1-3 glucanases, is still not clear.

However, β -1-3 glucanase has been found constitutively in healthy tomato plants (Pegg, 1976) although its function is obscure. After infection with Verticillium albo-atrum, this β -1-3 glucanase activity increased in both susceptible and resistant varieties, although activity in the former was substantially higher than that in the latter. The increase was proportional to the overall quantity of the pathogen (or its metabolites) in the plant, and was not considered to be primarily associated with resistance. Other authors have observed similar increases in host β -glucanase activity after infection with viruses and fungi (Moore & Stone, 1972; Abeles et al, 1971; Wargo, 1974). The wall polymers of certain fungal

hyphae are attacked by these enzymes, which are thus thought to be involved in hyphal lysis, since this has been observed in many cases. Also, since ethylene is produced by the host when infected by V.albo-atrum, this gas has also been implicated in the observed symptoms. Exposure of healthy plants to ethylene caused an increase in their β -1-3 glucanase activity (Abeles & Forrence, 1970). Treatment of plants with ethylene at or just before inoculation with V.albo-atrum significantly reduced the symptoms, and mycelial lysis was also observed (Pegg, 1977).

Therefore, plant β -1-3 glucanases may, in some cases, constitute or contribute to a defense mechanism, although further work is necessary in order to substantiate this suggestion.

2. MATERIALS AND METHODS

2.1 General

a) Host plants

Host cereals were grown under glasshouse conditions or in open plots at the Chelsea Physic Garden, London. These cereals included Svalof's Fourex Rye (Swedish Seed Association, Svalof, Sweden); Maris Dove and Sirius spring wheats (Plant Breeding Institute, Cambridge) and two dwarf millet (Pennisetum typhoides) varieties, n^o 16363 and BB-144-F12. These millets were kindly supplied by the Director, Centre de Recherches Agronomiques, Senegal, W. Africa. They were grown in the glasshouse during the summer, where temperatures reached 90°F and did not fall much below 55°F. They grew to about 1 ft. in height (Plate A) in contrast to the 5-6 ft. of conventional varieties.

b) Ergot fungi

- i) Claviceps purpurea: Strain 29/4D was originally isolated from an ergot on blackgrass (Nisbet, 1975).
- ii) Sphacelia sorghi was isolated from sclerotia on Sorghum in Nigeria (Mantle, 1968a, 1973).
- iii) C.fusiformis: Strain 139/2/1 had been obtained from sclerotia on bull-rush millet (P.typhoides) collected in Senegal and supplied by the Director, Centre de Recherche Agronomiques, Senegal, W. Africa. (Szczyrbak, 1972).

Strain 139/2/1G arose spontaneously as a variant of 139/2/1 during a fermentation study and was distinct from parent strain by its ability to autolyse the extracellular glucan and yield more alkaloid (Dickerson et al, 1970; Banks et al, 1974). C. fusiformis 'ex Bajra' was isolated from freshly harvested ergots growing in a naturally infected crop of millet in N. India (bajra is the local Indian name for millet). These ergots

were supplied by Dr. D.N. Roy, National Institute of Nutrition, Jamai-Osmania P.O., Hyderabad, India. The sclerotia were surface sterilised with 0.1% mercuric chloride for 5 mins, washed and aseptically cut into segments. These were plated out onto medium T agar containing 50µg/ml aureomycin and subsequently replated until the fungus was obtained in pure culture. The plates were incubated at 27°C.

c) Culture maintenance

Cultures were stored in 10% glycerol in sealed ampoules which were kept at -196°C under liquid nitrogen.

d) General media

i) Medium T (modified from Stoll et al, 1957).

Sucrose, 100g; L-asparagine, 10g; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25g; KH_2PO_4 , 0.25g; KCl, 0.125g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.033g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.027g; L-cysteine hydrochloride, 0.01g; Yeast extract (Oxoid), 0.1g; per litre distilled water pH 5.2 adjusted with 10% NaOH. Sterilised at 5 psi for 20 mins. at 121°C. Dispensed 100ml into 500ml Erlenmeyer flasks. For solid media, 15g Oxoid Agar No.3 per litre was included.

ii) Medium for production of β -glucanase by Basidiomycete QM806

(modified from Reese & Mandels, 1959)

Glucose, 10g; $(\text{NH}_4)_2\text{SO}_4$, 1.4g; urea, 0.3g; $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1.9g; CaCl_2 , 0.3g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3g; Yeast extract (Oxoid), 0.1g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5µg; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 2µg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3.5µg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2µg; per litre distilled water, pH 6.2, sterilised at 5 psi for 30 mins. at 121°C. Dispensed 100ml into 500ml Erlenmeyer flasks.

2.2. MICROSCOPY OF INFECTION AND ERGOT DEVELOPMENT

a) Inoculation

Inoculum in the form of a spore suspension was obtained by diluting honeydew collected from infected plants grown specifically for this purpose. Florets of wheat and rye, grown in outdoor plots, were inoculated at anthesis by injecting the spore suspension with a hypodermic syringe to fill the floral cavity (Plate 1a). Florets on only one side of the spike were inoculated, leaving the other side as healthy controls.

b) Sampling

Samples of ears were harvested daily during the first week after inoculation and thereafter at approximately 3 day intervals. Infected ovaries were carefully dissected from the floret. Any adhering plant tissues (lemmas, paleas and lodicules) were removed since it was found that if these remained, inefficient infiltration of the fixatives and resin led to bad polymerization, and section cutting was impossible. Samples of tissues therefore, were kept as small as possible and, in later stages of infection, they were trimmed down.

c) Fixing

Tissues were treated at room temperature and were:

- i) fixed in 2.5%(v/v) glutaraldehyde in 0.1M cacodylate buffer pH 7.2.
- ii) washed in four changes of 0.1M cacodylate buffer pH 7.2, 30 min. each.
- iii) post-fixed in 1%(w/v) osmium tetroxide in 0.1M cacodylate buffer pH 7.2.

iv) washed in four changes of 0.1M cacodylate buffer pH 7.2,
30 min. each.

v) stored in buffer at 4°C.

(Care was taken when using the above reagents as osmium tetroxide is especially harmful to the eyes and cacodylate buffer is carcinogenic. Gloves and a fume cupboard were used during the fixation.)

d) Embedding in resin

A low-viscosity epoxy resin (Spurr, 1969) was used and the tissue was first:

i) dehydrated in

a) 25% ethyl alcohol in water for 40 min.

b) 50% " " " " " " "

c) 90% " " " " " 1 hour.

d) 100% ethyl alcohol for 1 hour.

e) 100% " " overnight.

ii) infiltration: The tissue was processed through the following solutions with continuous agitation:

a) 25% resin in 100% ethyl alcohol for 3 hours.

b) 50% " " " " " " "

c) 75% " " " " " overnight.

d) 100% " " " " " for 9 hours.

e) Fresh 100% resin for 4 hours.

(Since resin is removed from glassware by acetone, which solvent facilitates penetration of resin into the skin, gloves were worn for all operations to protect from unpolymerized resin which is carcinogenic.)

iii) Polymerization: Tissues and resin were transferred to either 'Beem' capsules or polythene cups and orientated for subsequent longitudinal or transverse sectioning. The resin polymerized

at 70°C for 8 hours in an oven. These tissue segments in cups were sawn out with a small hack-saw and glued to short wooden dowels with araldite.

e) Cutting sections

The tissue blocks were coarsely trimmed as usual with a razor blade and trimming was finished using a glass knife on the microtome. Sections were cut with a Cambridge Huxley microtome and a LKB Ultratome MK II.

i) Light microscopy

Sections were cut in the range 0.2-2.0 μ and collected from the water in the boat of the glass knife by a small platinum loop. They were then placed carefully onto a clean glass slide which was heated briefly on a hotplate, when the sections stretched and adhered to the slide as the water evaporated.

ii) Electron microscopy

Silver sections, in the range 600-900Å were cut on a LKB ultramicrotome using glass knives. Ribbons of sections were collected on copper grids.

f) Staining

i) For light microscopy:

General stain

Several stains were tested with a view to obtaining differential staining of plant and fungal tissue. This is quite feasible in conventionally fixed and embedded material, but was found to be more difficult with resin-embedded tissue. The following stains were rejected due to their poor staining ability:

- 1% safranin + picro-aniline blue,
- 1% safranin + lactophenol-cotton blue,
- 0.5% basic fuchsin in acid alcohol,

1% periodic acid + Azure II/methylene blue in borate buffer pH 10.

Good staining, though not strictly differential, was obtained with:

1% toluidine blue in borate buffer pH 10,

1% safranin in borate buffer pH 10 followed by toluidine blue in borate buffer pH 10,

0.5% basic fuchsin in borate buffer pH 10 followed by either Azure II/methylene blue or toluidine blue, both in borate buffer pH 10.

From these, 1% toluidine blue in borate buffer pH 10 was selected, since it was simplest to use. Drops of the stain were placed onto the sections on the slide, which was heated for approximately 1 min. on the hot-plate until steam was evident. The slide was then washed with distilled water and dried in hot air. Permanent preparations were made using Canada Balsam in xylene. Both plant cells and fungal hyphae stained blue.

Cutin

Ammoniacal basic fuchsin was used as a stain for cutin in the ovary cuticle. A 10% solution of basic fuchsin in absolute alcohol was added dropwise to strong ammonia solution until a pale yellow colour was obtained. Sections were immersed in this solution for 10-15 min. in a stoppered jar and then transferred to absolute alcohol several times. They were then mounted in Canada Balsam in xylene (Jensen, 1962).

The cuticle, however, was not selectively stained, since cellulose cell walls, starch grains and hyphae also stained red. In fresh or formalin fixed tissue, cutin and lignin should stain red while the rest of the section remains colourless.

Apparently, resin embedding has profound effects on the results of this staining procedure, and must account for this unselectiveness. The cuticle, as well as other structures were clearly stained.

Lipid

Lipids in tissue were stained by a combination of two stains, which were found, during preliminary stain testing, to give good results. Sections were stained for 10-15 min. in a filtered alkaline solution of Sudan IV in 70% alcohol (70% alcohol was made up with borate buffer pH 10 instead of water). They were washed in 70% alcohol and brought down through an alcohol series to water. They they were stained for about 1 min. with 0.1% filtered toluidine blue in borate buffer pH 10 on a hot-plate until steam was evident. This was followed by washing in distilled water and mounting in glycerol. Plant cell walls, nuclei and starch grains stained blue while lignified tissue stained dark blue. Hyphal walls and cytoplasm stained pale blue and lipids stained deep red.

Neutral red method for distinguishing between live and dead cells

Fresh infected (5 days after inoculation) and healthy wheat ovaries were hand sectioned, and the tissues were placed into a plasmolysing agent containing the vital stain neutral red (Tribe, 1955). This solution contained: 1M KNO_3 , 0.1% neutral red and 0.1M sodium phosphate buffer pH 7.5 (in the ratio of 85:15:5 by volume). The tissue remained for 20 minutes^{in the} solution, which was then replaced by a solution containing 0.9M KNO_3 and 0.1M sodium

phosphate buffer pH 7.5.

The stain was retained by the vacuoles of live cells but not by dead cells (Hall & Wood, 1973; Cooper, Rankin & Wood, 1978).

ii) For electron microscopy:

Sections were stained on their grids with uranyl acetate followed by lead citrate in the usual manner (Reynolds, 1963; Kay, 1967).

g) Microscopical observations

Optical sections were observed using a Leitz Ortholux microscope, with both bright field and phase contrast optics. Micrographs were taken on the Leitz Orthomat microscope camera using Ilford Pan F film.

Electron microscopical observations were made using AEI EM6, AEI EM6B and AEI Corinth 275 microscopes.

2.3. PECTOLYTIC ENZYMES IN PATHOGENESIS

a) Preparation of pectin: Pectin and sodium polypectate were washed in acidified alcohol to remove any contaminating hexoses. Although there was a tendency for pectin to form clumps in solution if not dispersed, a homogeneous solution was obtained by adding the pectin in very small quantities to the buffer while stirring vigorously. The viscous suspension was left overnight to allow air bubbles to escape before being used.

When autoclaving pectin media, the pressure was kept at 5psi for 10 minutes. These precautions were necessary since pectin is degraded above this pH and pressure by β -elimination cleavage of glycosidic links (Albersheim, 1959; Pope, 1972; Fogarty & Ward, 1974).

Pectin and sodium polypectate were obtained from Sunkist Growers Pharmaceutical Products, Ontario, California.

b) Culture Media1. For growth of C.purpurea on pectic substratesi) Pectin Medium Type A

Pectin (acid-washed) 5g, 10g, 25g or 50g per litre

KH_2PO_4 0.25g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25g

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.033g

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.027g

Asparagine 5g

Yeast extract 0.1g

$\pm$ Sucrose 5g

pH 5.5 with NaOH. Dispensed 100ml into 500ml Erlenmeyer flasks and sterilised at 5psi for 10 minutes.

ii) Polygalacturonic acid medium

As for Pectin Medium Type B (below) substituting 25g/l polygalacturonic acid for 35g/l pectin, and pH adjusted to 5.2 and also 7.5 with NaOH.

Glucose, (2.5g/l) was not added until after 5 days growth.

2. For production of pectolytic enzymesi) Pectin Medium Type B

Pectin (acid washed) 35g per litre

KH_2PO_4 0.25g

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25g

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.033g

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.027g

Asparagine 5g

$\pm$ Glucose 2.5g

pH 5.5 with NaOH. Dispensed and sterilised as Pectin Medium Type A above.

ii) Media, Type C

As for Pectin Medium Type B substituting 50g/l glucose for 35g/l pectin, and pH adjusted to either 5.2 or 7.5 with NaOH. The media were autoclaved at 15psi for 20 minutes and dispensed as above.

c) Assay techniques for pectolytic enzyme activity

1. Pectinesterase

i) Titration of free acid groups

This method is based on that used by Talboys & Busch (1970) who modified an original procedure of Kertesz (1951). A 1.2% pectin solution, 5ml, was adjusted to pH 5.5 with 0.1N NaOH and a drop of chloroform was added to prevent microorganism contamination. The test solution, 5ml, was added, mixed and the pH of the mixture was measured immediately. It was then incubated at 30°C. After 30 minutes the pH of at least 3 replicates was measured. If the pH had decreased, the mixture was titrated back to the original pH with 0.01N NaOH. This was repeated at regular intervals up to 6 hours and the volume of 0.01N NaOH required at each sampling time was noted. Results were expressed as mls of 0.01N NaOH required to neutralise the acidic groups released/hour/g dry weight of mycelium. Boiled test solution was used as a control.

ii) Cup-plate method

This method is based on that first used by Dingle et al (1953) who adapted it from the diffusion assay technique for antibiotics. The procedure consisted of incorporating 1% pectic substrate into a 2% buffered agar solution, pouring into petri dishes and allowing to set. Wells were then cut out by means of a cork borer (6mm), and the test solution was introduced by a pipette. A well containing water was included on every plate as a control. After a certain period of incubation (24-30 hours) the plates were sprayed

Table 2 . Protocol for pectolytic and cellulolytic enzyme assays by the cup-plate method

ENZYME	PE	PG	PMGL	CELLULASE (Cx)
SUBSTRATE	1% Pectin (+ Calcium acetate, 0.5%)*	1% Sodium polypectate	1% Pectin	1% Sodium Carboxymethyl-cellulose
pH/BUFFER	0.1M Walpoles acetate, pH 4.5	0.2M phosphate, pH 5.2	0.2M phosphate/citrate, pH 5.2	0.1M Walpoles, pH 4.5
DEVELOPER	a) 10% copper acetate* b) Hydroxamic acid reaction** (McComb & McCready, 1958)	5N HCl (Dingle et al, 1953)	100% isopropanol McClendon, 1964; Weste, 1970)	10% copper acetate (Dingle et al, 1953; Weste, 1970)
POSITIVE REACTION	a) Clear zone on opalescent background. b) Clear zone on red background.	Clear zone with white opalescent halo.	White zone on opalescent background.	Clear zone on opalescent background.

When testing solutions for pectinase activity, aureomycin (0.005%) was incorporated into the agar.

* This assay is suitable only in the absence of PG, since PG attacks the calcium pectate formed (Dingle et al, 1953).

**Hydroxamic acid reaction: This method can be used for PE whether PG is present or not. The plate was flooded with 3ml hydroxylamine hydrochloride solution (3.75%) and 3ml NaOH (9.4%) was added while agitating the plate. It was left to stand for 10-15 minutes. Then 3ml HCl was added, followed by 3ml fresh FeCl_3 solution (10% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 0.1N HCl). After a few minutes enzyme activity was indicated by clear zones on a red background.

or flooded with a developing agent, after which positive reactions were evident from zones around the wells (Table 2). The diameter of the zone of activity is logarithmically related to the enzyme concentration. In a variation of this method, the fungus was inoculated onto agar plates glucose-free Medium B in which the pectic substrate was incorporated at 1%. After growth for one week, the plates were developed as above.

2. Polygalacturonase (PG) and 3. Polymethylgalacturonase (PMG)

i) Since the depolymerising pectinases produce reducing groups on substrate hydrolysis, (Fogarty & Ward, 1974), the following method, which involves measuring these groups was adopted for assaying both polygalacturonase and polymethylgalacturonase activities.

A 0.1% solution of polygalacturonic acid (for PG assay) and pectin (for PMG assay) was made up in M^CIlvaine's buffer at pH 4.5 and 5.6 respectively. (Fungal PG's have pH optima in the region of 4-6, and the lesser studied PMG's pH optima lie around 4-7 (Fogarty & Ward, 1974; Seegmiller & Jansen, 1952). To 8ml of the 0.1% substrate solution was added 2ml of the test solution and the mixture was incubated at 30°C. Samples (1ml) were taken immediately after mixing and then at regular intervals up to 5 hours. (When parasitic extracts were tested for these enzymes, 1ml of extract was added to 5ml of substrate solution). At least three replicates were taken at each sampling time. Boiled test solution was used as a control. An additional control using water instead of test solution was also included in order to test whether any substrate degradation occurred other than that caused by the test solution. The samples were tested for reducing groups by the Nelson-Somogyi method using galacturonic acid as the standard, (Kurowski & Dunleavy, 1976; Spiro, 1966).

Nelson-Somogyi method for measuring reducing groups

The sample (1ml) was mixed with 1ml of the copper reagent and capped with glass bulbs. After boiling for several minutes the mixture was cooled in a water bath and 1ml of the arsenomolybdate reagent was added. After mixing the solution was diluted with 3ml distilled water and mixed by inversion. The absorbance was read at 540nm.

Copper reagent: Solution I

Potassium sodium tartrate 15g

Na_2CO_3 anhyd. 300g

NaHCO_3 20g

Distilled water 300ml

A solution of 180g anhyd. Na_2SO_4 in 500ml of water was heated to expel the air. When it had cooled, it was added to the above solution and made up to 1ℓ with water.

Solution II

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (5g) and anhyd. Na_2SO_4 (45g) were dissolved in water and made up to 250ml. Just prior to use, 4 volumes of Solution I were added to one volume of Solution II.

Arsenomolybdate reagent

Ammonium molybdate (25g) was dissolved in 450ml water and 21ml conc. H_2SO_4 was added while the solution was stirred. Then 3g $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 25ml water and mixed with the molybdate solution. It was incubated at 37°C for 36 hours and stored in a brown glass-stoppered bottle. Just prior to use, one volume of the solution was added to 2 volumes of 1.5N H_2SO_4 .

ii) Cup-plate method: See p.74

4) Polygalacturonate lyase (PGL) and Polymethylgalacturonate lyase (PMGL)

i) The assay for these two enzymes is based on the fact that

the unsaturated reaction products formed by transeliminative cleavage of polygalacturonic acid and pectin absorb maximally at 235nm, (although some workers have claimed 230nm and 240nm as peak absorbance wavelengths) (Albersheim & Killias, 1962; Byrde & Fielding, 1968; Kurowski & Dunleavy, 1976; El Goorani et al, 1976; Talboys & Busch, 1970; Fogarty & Ward, 1974).

Solutions of polygalacturonic acid, 0.1% (for PGL assay) and pectin, 0.05% (for PMGL assay) were made up in McIlvaine's buffer at pH 7.8 and 5.5 respectively. (The optimal pH of PGL's fall in the range 7-10 while that of PMGL vary between 5 and 8.6 (Fogarty & Ward, 1974).)

The substrate solution (4ml) was incubated with 1ml of test solution at 30°C and its absorbance at 235nm was measured at regular intervals in a quartz cuvette. Each reading was replicated at least three times and boiled test solution acted as control. The concentration of unsaturated product was calculated from the increase in absorbance at 235nm by using the molar extinction coefficient of $46001 \text{ mol}^{-1} \text{ cm}^{-1}$ (Anon.1965).

ii) Cup-plate method: See p.74

d) Preliminary investigation of growth of C.purpurea on pectic and cellulose substrates 'in vitro' and pectinase and cellulase enzyme production

Agar plates containing Medium B in which the pectic or cellulose substrates were incorporated at 1% instead of 3.5% were inoculated with either a) a small mycelial disc (3mm) taken from the edge of a 10 day old colony growing on Medium T, or b) about 1×10^4 spores taken from the same colony and applied to the centre of the Medium B plate. The pectic and cellulose substrates included:

pectin (for PE and PMGL tests), sodium polypectate (for PG test) or

sodium carboxymethylcellulose (for cellulase, C_X , test).

e) Growth of *C.purpurea* on pectic substances 'in vitro' (liquid culture)

i) On pectin

Flasks containing Type A pectin media, with and without sucrose, were inoculated with a spore suspension (1ml) of *C.purpurea* (approx. 10^7 spores/ml) taken from a 10 day old culture growing on Medium T agar. They were incubated at 24°C on a rotary shaker. The progress of growth was observed daily and after 9 days, the mycelium was filtered off and the filtrate was tested for residual pectic material as follows:

Detection of pectic material in solution (Kertesz, 1951)

1) Formation of yellow colour with alkali: A few drops of 2% KOH were added to 0.5ml culture filtrate. After development of the yellow colour (about 15 min.), acid was added and a white flocculent precipitate indicated a positive result.

2) Colour formation and fluorescence with potassium permanganate:

The culture filtrate was boiled with 0.25% potassium permanganate solution and the formation of an intense magenta colour with slight green fluorescence indicated the presence of pectic materials.

3) Precipitation with ethyl alcohol: Equal volumes of culture filtrate and 95% ethyl alcohol were mixed and a translucent gelatinous precipitate indicated a positive result.

ii) On polygalacturonic acid

Flasks containing polygalacturonic acid medium at pH 5.2 and pH 7.5 were inoculated with a spore suspension (1ml) as above, and were incubated at 24°C . They were examined daily, and after five days glucose was added (2.5g/l) to half of the flasks. After a further five days, the mycelium was filtered off, washed and dried to constant weight. The filtrate was centrifuged and dialysed as

previously described and its protein concentration measured.

f) Production of pectolytic enzymes by *C.purpurea* 'in vitro' (liquid culture)

i) On Pectin Medium Type B

Five flasks of pectin medium Type B containing glucose and five without glucose were inoculated with 1ml spore suspension (approx. 10^7 spores/ml) of *C.purpurea* taken from 10 day old cultures grown on Medium T. The flasks were incubated at 24°C on a rotary shaker. After five days, the mycelium was filtered off, washed and dried to constant weight. The filtrate was bulked and centrifuged at 18,000 ^(38,000g) rpm at 4°C for 30 minutes, and the supernatant was dialysed at 4°C against 1.5mM M^cIlvaine's buffer pH 5.5 for 40 hours with two changes of buffer. Its protein concentration was measured and it was then tested for the five pectinase enzymes.

ii) On Type C Medium

Five flasks of this medium at pH 5.2 and five at pH 7.5 were inoculated and incubated as above. After five days, the mycelium was filtered off, washed and dried to constant weight. The filtrate was bulked and centrifuged at 18,000 ^(38,000g) rpm at 4°C for 30 minutes and tested for PE, PG and PMGL activities.

g) Pectolytic activity of parasitic extracts of *C.purpurea*

Parasitic extracts of *C.purpurea* were prepared as described on p.84. Of the following extracts, a selection was tested for PE, PG, PMG and cellulase activity: 7,12,14,20,22,26 days old. One healthy control, 12 days old (12c) and dialysed honeydew were also included. PE was assayed by the cup-plate method. The extracts were randomised on the plates and at least 4 replicates were used. The plates were incubated for 27 hours, after which they were developed using both developers.

2.4. β -GLUCANASE AND β -GLUCOSIDASE ACTIVITY OF CLAVICEPS Spp.

a) Inoculation

Wheat and rye plants were inoculated at anthesis as already described for microscopy (p.67).

Millet ears were inoculated at anthesis by (when only the stigmas were exposed) spraying a suspension of saprophytically grown spores and mycelial fragments onto the ears with a Shandon laboratory spray gun. When infection was evident, honeydew was collected and diluted to give an opaque spore suspension and this used to inoculate plants subsequently to be used for analysis. This procedure eliminated carry over of any factors from axenic culture. (Plate A).

b) Sampling

Infected millet ears were collected 10 days after inoculation, and also at maturity (10 weeks).

Infected and healthy ears of wheat and rye were sampled at intervals up to 60 days after inoculation. Infected ovaries or developing ergots (approx. 40 in earlier samples; approx. 20 in later samples) were dissected out and pooled. From about 14 days after inoculation, when sclerotial tissue began acropetally to displace sphacelial mycelium, ergots were divided into younger (basal) and older (apical) tissues and analysed separately.

Honeydew was collected, in all cases, throughout the period of its production.

c) Enzyme Preparation

i) Preparation of exo- β -1-3-glucanase from Basidiomycete QM806

Basidiomycete QM806, initially isolated by Reese & Mandels (1959), was obtained from the Commonwealth Mycological Institute, Kew, London, (Accession No. 155711) as a lyophilised culture. It was maintained on

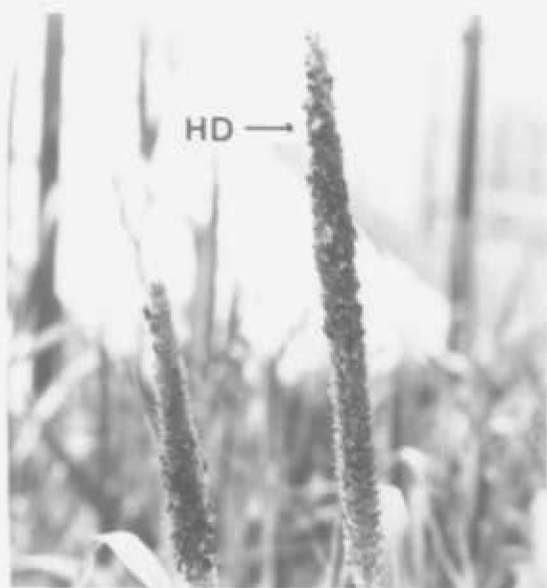
Plate A. INFECTION OF DWARF MILLET (Pennisetum typhoides) BY
 C.fusiformis (ex Bajra)

- a) At anthesis. Stigmas emerge (central ear) prior to anthers, which emerge a few days later from the apex downwards (ear on left).

- b) Honeydew exudation from infected florets.

- c) Collection of honeydew into wide mouth of hypodermic syringe. Anthers usually adhere to the honeydew, hindering collection.

- d) Development of millet seeds. Some honeydew is evident at the apex of the ear, indicating infection in that region.

**a****b****c****d**

malt agar pH 6 at 24°C and subcultured about every 8 weeks. A specific exo- β -1-3 glucanase was prepared by growing this organism for 14 days in shake flasks of the culture medium described previously (p.66). The sparse mycelium was filtered off and the filtrate was dialysed for 40 hours against 1mM phosphate buffer pH 6.2 with 3 changes of buffer. It was centrifuged at 30,000rpm for 30 min. to clear the solution, which was then freeze dried. The protein content of the product was estimated as 60% (w/w) and this material was used as a reference with which to compare the β -glucanase activity of parasitic tissue extracts and honeydew.

ii) From parasitic tissues and honeydew

Ergot tissue was extracted for about 2 hours in 0.01M acetate buffer pH 5.2 using a tissue grinder standing in ice; the ratio of buffer to tissue was roughly 5:1 (v/w). The extract was centrifuged at 18,000 rpm for 20 min. at 4°C. The supernatant (6-8 ml) was dialysed at 4°C against 0.01M acetate buffer (2x1) over 24 hours. The dialysate was freeze dried and assayed immediately or stored for a short time in a refrigerated desiccator. Healthy (control) ovaries were treated in a similar manner.

Honeydew was diluted with 0.01M acetate buffer pH 5.2 (about 1ml honeydew: 7ml buffer) and centrifuged at 18,000 rpm^(32,000g) for 20 min. at 4°C. The supernatant was dialysed as above and freeze dried.

d) Growth and β -glucanase production in liquid cultures of *C.purpurea*

Spores from 13-day old cultures of *C.purpurea*, grown on Medium T agar, were suspended in sterile water (approx. 2×10^5 spores/ml) and 1ml inoculated into flasks of Medium T. The flasks were incubated at 24°C on a rotary shaker and replicate pairs harvested at 4,6,8,10 and 12 days. Macroscopical and microscopical details of growth were observed. The mycelium was filtered off, and the filtrate was centrifuged, dialysed and freeze dried as previously described (in c)ii). Its β -glucanase activity

was then determined.

e) Growth and glucan and β -glucanase production in liquid cultures of *Claviceps fusiformis* Strain 139/2/1

Flasks of Medium T were inoculated with a mycelium-spore suspension of *C.fusiformis* Strain 139/2/1 of about 10^6 spores/ml, taken from a 10-day old culture growing on Medium T agar. Flasks were incubated at 27°C on a rotary shaker. They were sampled at intervals of 6, 8, 11 and 13 days after inoculation and the mycelium was filtered off. The filtrate was centrifuged and dialysed as for *C.purpurea* cultures above. After freeze-drying, exactly half of the product was suspended in distilled water and glucan was precipitated out by addition of twice its volume of absolute alcohol. The glucan was collected on a glass rod and dried at 70°C to constant weight. Part of the remaining half of the freeze dried dialysate was suspended in 0.01M acetate buffer and assayed for β -glucanase activity.

f) Growth and glucan production by *C.fusiformis*(α -Bajra) 'in vitro'

Once a pure culture of this isolate was obtained, growth characteristics were observed for 15 days on Medium T agar. Then spores from 10-day old cultures of *C.fusiformis*(α -Bajra) grown on Medium T agar were suspended in sterile water (approx. 2×10^6 spores/ml). Flasks containing 100ml Medium T were inoculated with 1ml spore suspension and incubated at 27°C on a rotary shaker.

Growth was observed for 20 days after which time a little absolute alcohol was added to the diluted culture, to test for the presence of polysaccharide (glucan).

g) Protein estimation

Protein in solution was estimated by determining the extinction of the sample at 260nm and 280nm and reading the concentration from a

nomograph compiled by Adams (California Corporation for Biochemical Research) from the data of Warburg and Christian (1942).

h) Preparation of β -glucan

Purified β -glucan was prepared from Sphacelia sorghi by the methods described by Buck et al (1968). Laminarin (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) was also used as another type of β -1-3-glucan substrate.

i) Determination of enzyme activity

1. β -glucanase determination

Purified β -glucan (S.sorghi) (7.5mg) was ground to a powder and solubilised in 0.5ml 0.01M acetate buffer pH 5.2 overnight. Laminarin was similarly treated but did not require grinding. The freeze dried tissue extracts to be assayed were dissolved in the same buffer and 1ml was added to the solubilised glucan substrate. The concentration of the tissue extract was adjusted to give a final protein concentration in the incubate of 1mg/ml (with glucanase from Basidiomycete QM806, protein concentration was 0.1mg/ml). The final concentration of glucan in the incubate was 5mg/ml. The mixture was incubated at 30°C and two 0.1ml aliquots were taken at intervals over 5 hours. The reaction was stopped by boiling briefly and the volume was made up to 1ml with distilled water. The liberated glucose was estimated by the following glucose oxidase method based on Fleming and Pegler (1963).

Glucose oxidase method for estimation of glucose

a) Glucose oxidase reagent: Glucose oxidase (90mg), horseradish peroxidase (9mg) and O-dianisidine hydrochloride (60mg) were dissolved in 300ml Tris-glycerol buffer pH 7.0. The solution was filtered through non-absorbent cotton wool and stored in an amber bottle at 4°C. Tris-glycerol buffer pH 7.0 was prepared by dis-

solving 61g Tris (hydroxy methyl) methylamine in 85ml 5N HCl.

It was diluted to 900ml with distilled water and the pH was adjusted with 5N HCl. It was then made up to 1 litre and 660ml glycerol was added. Excess buffer solution was stored at 4°C in an amber bottle.

- b) Method: Glucose oxidase reagent (2ml) was added to 1ml samples containing up to 50µg glucose/ml (the colour developed is linearly related to glucose concentration only up to 50µg glucose/ml), and was well mixed. It was incubated at 37°C for 1 hour after which the reaction was stopped by the addition of 2ml 18N sulphuric acid. The solution was mixed and its extinction was read at 540nm. Standard glucose solutions in the range 0-50 µg/ml were treated in the above manner and were used to construct a calibration curve.

2. β-glucosidase determination

Gentiobiose (7.5mg) was dissolved in 0.5ml of 0.01M acetate buffer pH 5.2 and 1ml of tissue extract (see above) was added. Subsequent procedure was as for glucanase determination.

j) Chromatography of reaction products

The action of C.purpurea honeydew, B.QM806 glucanase and C.fusiformis (ex Bajra) honeydew and tissue extracts was investigated on both laminarin and S.sorghi glucan by descending paper chromatography of the reaction products. Samples (between 20-30 µl) were taken at intervals during the reaction and spotted onto Whatman 3MM paper. Standard solutions (10µl) containing 150µg each of glucose, fructose, sucrose, maltose and raffinose, and another containing gentiobiose were used as markers. Chromatograms were run in a solvent containing n-propanol:ethyl acetate:water in the ratio of 7:2:1^(v/v/v) for 30 hrs. They were dried and then dipped in aniline-hydrogen phthalate reagent as modified from Partridge (1949), which contained: phthalic acid 1.7g; aniline 1ml; ethyl alcohol 90ml; glacial

acetic acid 5ml; TCA 40%, 5ml. The products were visualised as brown spots by heating in an oven at 110°C for 5 min.

k) Polyacrylamide gel electrophoresis

Several of the Claviceps parasitic extracts and B.QM806 extract were subjected to polyacrylamide gel electrophoresis in order to investigate their proteins. Polyacrylamide gel (8% w/v, with 1% cross linkage) was prepared by the method of Weber, Pringle & Osborn (1972) and included sodium dodecyl sulphate. (SDS dissociates proteins into their constituent polypeptide chains.) Microgels were obtained by using smaller tubes than usual (2.5mm int.diam. x 7cm). Electrophoresis was performed in the following buffer: equal volumes of 0.1M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.01M diNaEDTA + 0.1% w/v SDS, pH7.2. The gels were pre-run for 30 minutes with a tracking dye (0.05% bromophenol blue in 50% sucrose), after which the buffer was replaced. Each sample contained 0.2-2 mg protein/ml + 1% w/v SDS + 1% w/v 2 - mercaptoethanol + bromo phenol blue in 50% sucrose, and 10µl was carefully layered on top of the gel. In co-electrophoresis, equal volumes of the two samples were mixed and applied to the gel. When samples were to be denatured, the tubes were stoppered and heated at 100°C for 3 minutes and then cooled. The gels were run for 1.5 hours with the current adjusted to 7 mA/gel. After running, the gels were removed and stained with Coomassie blue for 2-3 hours (0.1% Coomassie blue, 10% 5-sodium salicylic acid, 10% TCA). They were destained in 5% acetic acid after which only the polypeptide bands remained stained.

2.5. FLUORESCENCE STUDIES OF CALLOSE (β -1-3-GLUCAN) HYDROLYSIS BY

C.purpurea

The following methodology was developed as a result of a series of trials in which conditions of experimentation were varied. Preliminary tests (see Appendix I) using whole ovaries and two different stains for

gross visualization of the effects of wounding led to a method using light and fluorescence microscopy. The latter was ultimately chosen for experimental work.

a) Wound callose induction

At about the time of anthesis, attached ovaries of wheat and rye were wounded by inserting into them a thin wire needle (0.125mm diameter) three times vertically to a depth of about 2mm. Infected ovaries, 5-10 days after inoculation, were treated similarly. The glume, lemma and palea were held apart to facilitate wounding, but were not removed as their removal was found to cause dehydration of the ovary. After wounding, the ears were placed with their stems in water in a humid atmosphere in the light at room temperature for about 4 hours to stimulate callose deposition. In control ovaries wounding was omitted.

b) Sectioning wounded ovaries

Wounded and control ovaries were carefully dissected from the ears after incubation and mounted in 2% gum acacia on a Peltier type freezing stage. When the ovary was frozen, longitudinal sections were cut in the range 60-70 μ on a Reichert sledge microtome. After the sections were cut, they remained in a drop of melted gum acacia on the knife, and were picked up with a small wire loop. Sections from the central wounded region of 6-7 ovaries were combined. They were then either stained directly or first incubated in various solutions and subsequently stained.

c) Treatment of sections for fluorescence microscopy

Sections were incubated in a solution of exo- β -1-3-glucanase from Basidiomycete QM806 (see 1.8.) under a variety of conditions and the resulting fluorescence was compared with the controls. The conditions are shown in Table 10 together with the results. About 12 sections per treatment were used in the incubations. The combination of parameters giving the most consistent results from these experiments was selected, and used subsequently to test the effects of dialysed honeydew and

extracts of parasitic mycelia of C.purpurea of three different ages. (For tissue extract preparation see 2.4c.ii). Between 20 and 25 sections were used per treatment. Fluorescence was assessed visually on an arbitrary scale of + (weak) to +++++ (strong). One of these parasitic extracts was chosen on account of its high β -1-3-glucanase activity (Fig. 11), and used together with dialysed honeydew and B.QM806 glucanase in the quantitative measurement of fluorescence by microspectrofluorimetry.

d) Treatment of sections for microspectrofluorimetry

Sections were placed in one of the following and incubated at 45°C for 2 hours:

- a) distilled water (1ml)
- b) β -glucanase (5mg/ml) from Basidiomycete QM806
- c) dialysed honeydew from C.purpurea (freeze dried; 5mg/ml)
- d) freeze dried extract from the apical portion of 30 day old ergots (top 3mm; 5mg/ml) selected on account of its high β -glucanase activity (see 3.3.)

Approximately 20 sections were used in each treatment. These were selected randomly so that not all sections from an individual ovary constituted one treatment.

e) Staining of sections

Sections were stained in excess 0.01% w/v aniline blue in M/15 K_2HPO_4 pH8 for 10-15 min. (Concentrations greater than this tended to overstain the sections (Eschrich & Currier, 1964). They were placed on glass microscope slides (0.8-1mm thick) in a drop of the stain, and heated briefly on a heating block to stretch them and ensure adherence to the slide. They were not mounted in any mounting medium nor covered by a coverslip.

f) Fluorescence microscopy

Aniline blue stained sections were examined with a Leitz Ortholux fluorescence microscope in a darkened room. The microscope was equipped

with a darkfield condenser (Tiyoda) fitted with a toric lens, which transmitted light from a 200W mercury vapour lamp. A drop of distilled water was placed between the condenser and the lower surface of the slide. The UV exciter filter UGI (2mm thick) was used having a peak transmittance at 365nm, as well as a BG38 suppression filter (4mm thick) to absorb unwanted red light. Barrier filter K530 was used. Low power objectives (4x and 10x) were used with numerical aperture (N.A.) of 0.1 and 0.25 respectively to scan the sections. The magnification and N.A. of the objectives are important since image brightness is inversely proportional to the square of the objective magnification, and directly proportional to the square of the N.A. Eyepiece magnification is also important and those of a relatively low power (8x) were used. When fluorescence of sections was assessed visually, the same objective and eyepiece were used for all sections so that variation in microscope components could not be responsible for differences in fluorescence.

Micrographs were taken with the Leitz Ortholux camera attachment using Ilford Pan F (b/w) film ASA50 and Kodak High Speed Ektachrome daylight (colour transparency) film ASA 160 but speed set at ASA 400 (see Appendix III).

g) Microspectrofluorimetry

Quantitative measurements of fluorescence emitted were obtained using a microspectrofluorimeter. This instrument is based on the Leitz microspectrograph and important features include monochromatic epi-illumination and photon counting. The optical part of the instrument consists of three main sections: the illuminating system, a microscope and the spectrometer. It was built by Pearse and Rost and is described and illustrated by them in detail (Pearse & Rost, 1968). Briefly, exciting radiation from the xenon arc is monochromated and is directed at the specimen through an epi-illuminator. This contains a beam-splitting mirror which reflects light through the objective onto the specimen. Fluorescent light is

collected by the objective and passed through a barrier or suppression filter and can be observed in the microscope, or diverted and measured in the spectrometer.

Trial sections were scanned at wavelengths ranging from 330-620nm to establish the optimum exciting wavelength and maximum emitting wavelength. These were found to be 400nm and 540nm respectively, and were selected by the excitation and emission monochromators. The size of the area from which fluorescence was to be measured was controlled by an adjustable slit which served as both the measuring field diaphragm and the entrance slit to the spectrograph. Its size was adjusted while viewing through an observation tube. Measurements were taken in two different ways (Fig. 7).

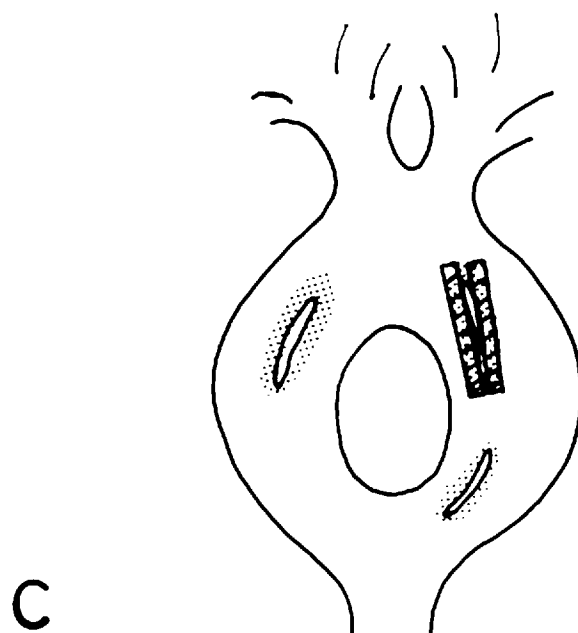
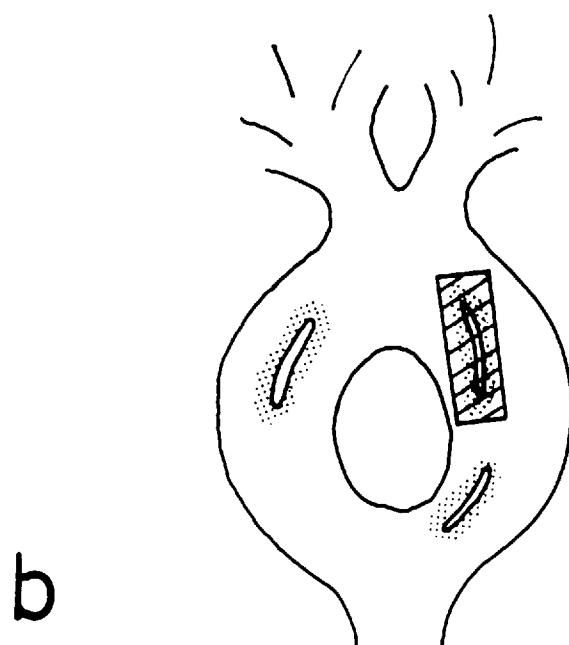
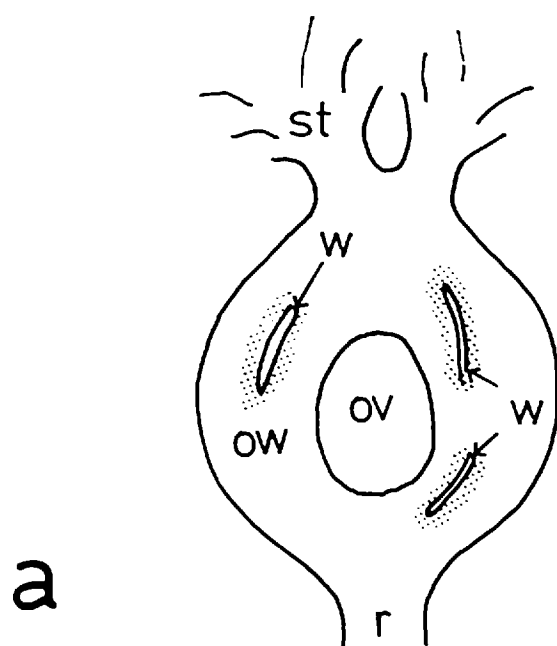
1) First, the measuring field diaphragm was adjusted to give a rectangle of size 0.415mm x 0.269mm, giving an area of 111635 sq. μ . This selected area was adequate for visualising the whole of any one wound region of a section (Fig. 7 b) (i.e. the wound itself and the area around it). In these measurements the eyepiece and objective magnifications were 10x and 4x respectively, the objective having a N.A. of 0.1. Each wound area was measured twice for ten seconds, and between 5 and 13 wounds were measured in each treatment. In this method, measurement of fluorescence was not restricted to that produced by callose, but also included other fluorescing materials e.g. cellulose in stray ovary hairs, chlorophyll etc. In addition non-fluorescing regions such as the wound itself would lead to further inaccuracies in measurement. This might be expected since the area being measured was relatively large. To reduce the possibility of such measurement errors, an improved method was devised.

2) This method involved measuring the fluorescence emitted from each side of a wound, parallel to its long axis (Fig. 7 c). The measuring field diaphragm was adjusted to give a rectangle, 0.14mm x 0.014mm, giving a total standard area per wound of 3920 sq. μ . Within this area only the fluorescing regions immediately around the wound could be vis-

FIG. 7. MICROSPECTROFLUORIMETRY OF WOUND-INDUCED CALLOSE IN SECTIONS
OF OVARY TISSUE.

- a) Digrammatic representation of longitudinal section through
an ovary, with 3 wounds in the ovary wall.
- b) First method of fluorescence measurement. The area selected
by the measuring field diaphragm was 111635 sq. μ . and
covered one wounded region. Owing to the relatively large
size of the area, errors in fluorescence measurement were
inevitable since the following may have been included:
- (i) fluorescing material other than callose,
 - (ii) large areas of non-fluorescing material.
- c) Second method of fluorescence measurement. The measuring
field diaphragm was set to give a small rectangle (1960 sq. μ .)
which covered one side (parallel to the long axis) of the
wound. Both sides of the wound were measured giving a
standard area per wound of 3920 sq. μ . Errors in measurement
were reduced since stray fluorescence and non-fluorescing
regions were largely eliminated.

ov = ovule; ow = ovary wall; r = rachilla; st = stigma;
w = wound; ☼☼ = fluorescence due to callose around the wound.



ualised. Each strip of fluorescing wound tissue was measured twice for 10 seconds, and between 10 and 15 different wounds were measured in each treatment. The eyepiece and objective magnifications were both 10x and the N.A. of the objective was 0.25. The eyepiece magnification does not affect the intensity of fluorescence measured, only that seen by the observer.

In both methods dark current readings were taken at each measurement, which was corrected accordingly. The dark current readings accounted for any background light e.g. from scatter, that could be detected by the instrument when the section was not illuminated by UV light. With this instrument, the intensity of fluorescence emission was measured by the photomultiplier, whose output was measured by photon counting. This has several advantages over D.C. measurements including greater sensitivity, higher accuracy since a direct digital output is obtained without having to read from a scale, it is unaffected by low frequency noise e.g. mains hum, and a truer result is obtained since the light is quantized (Pearse and Rost, 1968).

h) 'in vitro' germination of pollen

i) Free Pollen grains

Pollen grains of rye, wheat and daffodil (Narcissus spp.) were collected at anthesis and incubated at 24°C for 22 hours in darkness, on cavity slides and petri dishes in the following media:

Rosen medium (Rosen, 1961)

10% sucrose

0.01% Difco yeast extract

0.001% boric acid (+ 1% Oxoid No.1 agar for solid media)

Loo & Hwang medium (Loo & Hwang, 1944)

10^{-5} M manganese sulphate

0.6M sucrose (+ 1% Oxoid No.1 agar for solid media)

In some tests mixtures of equal volumes of the two media were used and temperature and light conditions were varied.

ii) Pollen germination on stigmas

Pollen grains from dehiscing rye anthers were sprinkled over excised rye stigmas floating in a mixture of equal volumes of the two liquid media and incubated for 5 hours at 24°C in darkness.

i) Treatment of pollen tubes with β -glucanases

Free germinated pollen grains of daffodil were incubated for 2 hours at 50°C in the following:

- a) B.QM806 β -glucanase (1mg/ml)
- b) freeze dried extract from 14 day-old parasitic ergot tissue (5mg/ml)
- c) crude undialysed extract from 14 day-old parasitic ergot tissue (5mg/ml)
- d) distilled water (control)

Rye stigmas bearing long pollen tubes were incubated for 16 hours at both 37°C and 50°C in the following:

- a) B.QM806 β -glucanase (1mg/ml)
- b) ergot honeydew (about 1ml diluted with 20ml acetate buffer (0.01M pH 5.2) and spores removed by centrifugation)
- c) freeze dried extract from 14 day-old parasitic ergot tissue (5mg/ml)
- d) distilled water (control)

After incubation, they were stained with 0.01% (w/v) aniline blue in M/15 K_2HPO_4 pH 8 for 10-15 minutes, dry mounted on glass slides and observed using fluorescence microscopy as previously described. Fluorescence intensity was assessed visually.

3. RESULTS

3. 1. MICROSCOPY OF INFECTION AND ERGOT DEVELOPMENT

The following description of the path of infection and subsequent development of the fungus is a summary of the findings from about seven infected ovaries at each stage, which were observed by light microscopy. Electron microscopical observations were made only of selected stages of infection.

Observations of ovary sections made one or two days after inoculation revealed no microscopic signs of infection. No hyphae were seen and the ovaries appeared perfectly healthy with cell division continuing apparently normally in the ovule and integuments. Environmental conditions influence the speed of germination and growth of fungal spores and may partly account for the apparent lack of infection at this early stage.

The first microscopic sign of infection occurred on the third day after inoculation, although there was no macroscopic evidence of this (Plate 1b). Hyphae were localised mainly in the upper half of the ovary, in the region between the stigmas and the ovule, in the ovary wall and around the ovule. However, there appeared to be very little disruption of structure and function of the ovary at this stage (Plate 2a). Several were observed growing on different parts of the external surface of the ovary (Plate 2b) and then penetrating towards the ovule. Transverse sections cut equatorially revealed hyphae growing along the surface of the ovary and penetrating from there into the ovary wall, especially in the furrow region (Plate 2c). Hyphae were sometimes observed growing down the side of an ovary hair and into the ovary wall. In some cases the hyphae appeared to be growing sub-cuticularly. Electron

microscopy revealed the cuticle of the ovary as being about 0.05μ thick and it appeared to be composed of several layers, typical of plant cuticles (Sargent, 1976). Sub-cuticular hyphae (Plate 3a,b) were located within the relatively thick layer of middle lamella material between the cuticle and epidermal cells. Areas of the middle lamella adjacent to the hyphae were less electron dense and its fibrils appear to have been loosened and degraded (Plate 3b). In other instances, where there appeared to be direct penetration, the cuticle was broken, presumably by the invading hyphae. Phase contrast microscopy of these hyphae yielded results similar to those of light microscopy although without improved clarity. These observations suggest that the fungal hyphae are able to break the cuticle and then penetrate directly between the underlying cells, or grow beneath the cuticle for a distance before penetrating inwards between the cells towards the ovule and vascular strands. Growth was always intercellular.

Some hyphae were also found between the cells of the tissues surrounding the ovule (i.e. the inner layer of the ovary wall, inner epidermis and the integuments) but never within the ovule itself (Plate 4). The cells of the ovule and integuments were seen in different stages of cell division even where fungal hyphae were adjacent to or in close proximity to them (Plate 4a,b,c). The cells of the outer integument were beginning to collapse, as is usual at this stage after anthesis, and hyphal penetration was probably helped by this natural degeneration (Fig. 4).

On the fourth day after inoculation, hyphal growth had extended to the lower part of the ovary, but the density of hyphae in the upper part had not increased. Again inter cellular hyphae were found in the ovary wall and outer and inner integuments (Plate 5). Hyphae were not observed within the ovule, where

nucellar cells were dividing much as in control ovaries (Plate 5c). Cell division was also seen in the inner integument (Plate 4d) even though fungal hyphae were very near to these cells. Several hyphae were observed near the vascular traces at the base of the ovary, and this was especially clear in transverse sections, where fungal hyphae appeared more concentrated around the three vascular traces. However, there was still little apparent disruption of the integrity of the ovary, and most cells in the ovary wall appeared healthy. In control ovaries, fertilization was imminent or had recently occurred. Electron microscopy of tissues at this stage showed intercellular hyphae as having a granular cytoplasm containing mitochondria, vesicles, small lipid bodies, several nuclei and complex membranous structures often within the vacuoles. The middle lamella of the ovary wall tissues appeared as lamellate fibrils in both healthy ovaries and the uninvaded regions of infected ovaries (Plate 6a,b). However, lamellae in the immediate vicinity of hyphae were more loosely woven and appeared to be disorganised (Plate 6c) as though in the process of degradation. The physical pressure of intercellular hyphae appeared to cause only slight distortion of the shape of the plant cells. In several ovaries there was evidence of stigma infection with fairly heavy hyphal growth down the stigmas into the ovary, around the top of the ovule and in the integuments (Plate 7a,b). The rest of the ovary was very sparsely infected, indicating that primary infection had occurred in the stigmas. Electron microscopy showed many hyphae growing between the cells of the stigma and style and in the transmitting tissue (Plates 8,9). This tissue is specialized for the passage of pollen tubes, and its cells are elongated and rich in cytoplasm (Arber, 1937; Jensen & Fischer, 1969) which is very electron dense. In many plants, the protoplasts of the transmitting tissue contract and die after the

pollen tube has grown within the tissue. This appears to be the case also when hyphae of C.purpurea grow in this tissue (Plate 9). The protoplasts contracted and their walls were easily invaginated by the growing hyphae (Plate 10b). The walls often appeared softened and the layers separated (Plate 9) and were easily pushed into folds by the growing hyphae (Plate 10a). Cell wall separation was also apparent in the ovary wall tissue (Plate 12b). It is possible that the hyphae obtain nutrients from the transmitting tissue in a manner similar to that of pollen tubes in other plants (Kroh & Helsper, 1974; Linskens & Esser, 1959). The transmitting tissue continues down the style to the apex of the ovule, and from there pollen tubes grow usually between the outer integument of the ovule and the inner epidermis of the ovary wall. Again, hyphae appear to take advantage of this route since they were seen within the outer integument of the ovule (Plate 11). The cells of the outer integument appeared collapsed and degenerating both in the presence and absence of hyphae (Plate 11, 12a). The decreased physical obstruction of these naturally degenerating cells (possibly aided by fungal invasion) appears to be utilized by the pathogen since colonization of the outer integument became fairly extensive before further invasion of the ovule occurred.

By day five after inoculation, the mass of hyphae had increased and colonized all parts of the ovary, including the ovule. At the ovary base, hyphae were found very close to the vascular strands but did not penetrate the rachilla (Plate 13b). Transverse sections revealed a predominance of hyphae around vascular traces and the ovule (Plate 13a) with a fairly heavy growth on the external surface (Plate 14b). This external mycelium now included not only invading hyphae but also hyphae which, having colonized the interior of the ovary, had grown out onto the surface and were about to

produce conidiophores. There was extensive colonization between the ovule and the integuments (Plate 13b,c) such that the ovule was becoming isolated. This isolation developed so that the fungal hyphae appeared to be concentrated in major channels in the ovary (Plate 14a). These channels presumably formed in areas of relatively low mechanical resistance through which hyphae could penetrate and grow rather easily, thus separating large areas of the ovary and facilitating the disruption of the tissues. Hyphal growth was still intercellular although ovary cell walls were often markedly invaginated and appeared to be softened by the invading hyphae (Plate 14b,c,d). Proliferation of hyphae occurred also in the intercellular spaces (Plate 14c,d). Electron microscopy indicated erosion of both the middle lamella and the wall, as demonstrated respectively by the disorganization of the middle lamella and separation of the fibrils within the thin wall (Plate 15,16). This degradation caused weakening of the wall, allowing hyphae to invaginate the cells (Plate 15a,b;16a). In such invaginated cells, disorganization was evident since the plasmalemma was incomplete and appeared to be pulled inwards (Plate 15b), as if contracting, leaving the inner surface of the wall bearing loose wispy fibrils (Plate 16b). Although the wall structure was degenerating and cell disorganization was apparent, hyphae did not directly breach the cell wall itself. Such features are typical of those caused by the action of pectolytic enzymes, and absence of direct wall penetration suggests little cellulase activity. The secretion of pectolytic enzymes by C.purpurea is shown later in this study. In addition, when fresh ovary sections of healthy and infected tissue (5 days after inoculation) were stained with neutral red in a plasmolyzing solution, healthy cells retained the stain in their vacuoles

(Plate 17a). However, in infected tissue, cells surrounded by hyphae were not stained, whereas adjacent tissue which was not hitherto colonized retained the stain (Plate 17b). The neutral red method tests the integrity of the plasmalemma and the tonoplast. It is commonly used to show the effects of pectolytic enzymes on cells i.e. cells treated with such enzymes are unable to retain the stain. In the present case the test showed that those cells in contact with the hyphae were no longer viable, whereas those only a few cells away, but not in direct contact with the hyphae were still functioning. It is possible therefore, that pectolytic enzymes play a part in the death of cells adjacent to the fungal hyphae.

Six days after inoculation, the fungal mass had increased further leaving fewer intact ovary cells. The ovule was completely surrounded and penetrated, and the integuments were separated by fungal hyphae. Hyphae grew very close to the vascular tissue at the base of the ovary but there was no penetration into the rachilla. The first sign of sporulation occurred at this stage, on the external surface of the ovary wall. Unless the ovary was dissected out or the glume and lemma removed, the floret appeared perfectly healthy (Plate 18a).

By day seven (Plate 19a) the hyphal mass was so extensive that no plant cells remained intact except in a 5-cell thick layer at the periphery of the ovary, where intercellular hyphae had not yet disrupted all the host tissues. Freely sporulating conidiophores were found all over the external surface and in internal pockets. Hyphae were found very close to the vascular tissue at the base of the ovary (Plate 19b), but hyphal penetration did not extend beyond the ovary base. The infected ovary had a white exterior and a soft consistency. This is known as the sphacelial stage (Plate 18b)

since the hyphae are of the sphacelial type. At about this stage honeydew exudation (Plate 20) began from the infected florets and continued for at least 10-15 days, gradually declining towards the end of the period. Honeydew exudation is the first external visible indication that infection has occurred.

Ten days after inoculation, there was no sign of the ovary as a recognisable structure. It had been completely replaced by a mass of hyphae which conformed to the shape of the floral cavity and was partly organised both externally and internally into sphacelial fructifications (Plate 21). It appeared white to the naked eye (Plate 18b) although green tissue was still visible when the tissue was sliced in two. The few remaining islands of ovary cells containing chloroplasts accounted for this. A few host cells were also found at the apex of the structure and these can be recognized by the hairs, as the upper part of the ovary (Plate 21). Host colonization stopped abruptly at a layer of cells just above the entrance to the rachilla (Plate 22a). This layer extended across the width of the rachilla to form a kind of 'plate'. This 'plate' was 4-6 cells thick and lay immediately above the core of thick-walled cells (seen better in Plate 26) which were described by Zee & O'Brien (1970, 1971). At the junction between host and parasite i.e. the upper cell layer of the 'plate', the cells were disrupted and cell contents were replaced by fungal hyphae. The cup-shaped remains of the host cell walls filled with hyphae are seen in Plate 22b. Below the disrupted cells of the 'plate', only plant cells were found. The host-parasite interface often appeared as a brown line to the naked eye, possibly due to localised oxidation of phenolic compounds. Honeydew exudation continued, its viscosity varying depending on the moisture content. Electron microscopical observation of tissue near the base of the sphacelium showed that

the hyphae contained large vacuoles and numerous lipid droplets which frequently seemed to coalesce and form larger deposits, often bulging into the vacuole (Plates 23,24 & 25). The lipid droplets tended to occur together rather than being distributed evenly throughout the cytoplasm. The hyphae varied in size and shape, some being long and occasionally branched (and typically sphacelial) while others were short and ovoid in shape (Plates 23a,25). Often hyphae were divided up into short lipid-containing segments by frequent septa, and possibly were the beginnings of a sclerotial-type morphology (Plate 25). The cells contained several nuclei. Conidia were also present at this stage and had dense cytoplasm containing lipid droplets. One or several rounded, electron-dense bodies, about 0.17μ in diameter, were often associated with the septa (Plate 24) of the hyphae. These bodies were usually seen on either side of a septum and often a membrane was visible around them (Plate 24b). They were also observed in the septal pore, as if plugging it (Plate 24b), often when the septum was separating a dense section of the hypha from one less dense. From their location, appearance and size, it is considered that these structures are Woronin bodies, and if so, this is the first time that they have been reported in C.purpurea. Although they were also seen in hyphae 4 days after inoculation (Plate 8), they occurred with more frequency at the 10-day stage, possibly correlating with the increased frequency of septa. Occasionally other material was seen in the septal pore either plugging or passing through it (Plate 23b).

Another interesting feature of hyphae at this 10-day stage was the presence of a coating meniscus on their surface. This meniscus surrounded the hyphae and conidia, and connected them to neighbouring cells (Plate 25a,b). Sometimes there appeared to be material interposed between the hyphal walls and this coating (Plate 25b),

and this meniscus was not observed at earlier stages of infection. Its origin is uncertain although one possibility is that it is the remains of the viscous honeydew which exudes freely at this stage. The hyphae are in effect bathed by the honeydew, whose concentrated sugars the fungus utilises. It is possible that the washing process during tissue preparation did not completely remove all the honeydew, and a thin layer remained surrounding the hyphae. Its absence at the 4 and 5 day stages support this explanation. Another possibility is that it is the remnants of extracellular glucan produced by the fungus. However, although this glucan is produced 'in vitro', it is not known if it is also produced 'in vivo', but the adhesive appearance of the coating is suggestive of such a substance. Around the surface of other hyphae of this age occurred a fine tangle of fibrils (Plate 24a). This may be similar to the capsule of fine polysaccharide fibrils noted by Vorisek et al (1974a) seen in C.paspali growing in submerged culture, although no such capsule was observed in C.purpurea grown in culture (Vorisek et al, 1974b). However, differences in growth form and ultrastructure are to be expected between hyphae from culture and those in parasitic conditions.

Twelve to thirteen days after inoculation (Plate 26) the fungal growth had filled the floral cavity and sporulation and honeydew exudation continued (Plate 27a). The first purple pigmentation was observed as the tissue was dissected from the floret and was located in a small superficial area in the lower part of the sphacelium (Plate 27a). Microscopically this proved to consist of cells morphologically different from the sphacelial hyphae and were short, fat, frequently septate and stained brown rather than the blue of the sphacelial cells with toluidine blue (Plate 28b). They also appeared to contain much more lipid than sphacelial cells and

tended to lie closely compressed together to form a prosenchyma. Adjacent to these sclerotial hyphae were conidiophores, and nearby were hyphae which appeared to have characteristics of both sphacelial and sclerotial hyphae - perhaps a transition type. These appeared fairly long, fat, frequently septate and stained less darkly than sclerotial hyphae. The sphacelial hyphae were long, thin, infrequently septate, with small vacuoles which seemed to be empty (Plate 28a). There was no marked boundary between these types of hyphae and sclerotial seemed to blend in with sphacelial via this apparent transition type (Plate 28b). The 'plate' of cells continued apparently to prevent further penetration into the rachilla (Plate 28a,c).

By day fifteen the purple pigmentation had extended to practically all the fungal tissue except the tip and the extreme base. The tip consisted mainly of remains of ovary cell walls, stigmas and loosely packed hyphae of the sphacelium (Plate 27b). The small sclerotium was much firmer than the sphacelium and protruded above the lemma, palea and glume. Its medulla consisted of closely packed sclerotial hyphae staining blue with toluidine blue, and separated by small empty channels. The cortical hyphae were brown, lay parallel to each other and bore a thin layer of small conidiophores on their external surface. When these sections were stained for lipid it was found that the hyphae at the fungal-plant interface were completely devoid of lipid, and instead had large empty vacuoles (Plate 29). This lens-shaped area of hyphae extended for about 150 μ into the sclerotium and consisted of fungal cells without the predominantly longitudinal hyphal orientation of those above. Fungal cells immediately distal to this region were roughly the same width as those above but contained much more lipid and were organised into distinct closely-packed septate hyphae all growing acropetally. The 'empty' hyphae were delimited from the fat-filled hyphae fairly

sharply (Plate 29b) and probably form the natural breaking-point of the sclerotium. In toluidine blue-stained sections the non-lipidic area stained a much paler blue than the rest of the fungal material. These interfacial hyphae form the youngest fungal tissue and grow rapidly. They are continually displaced acropetally and probably within a day or two develop large lipid reserves. No internal pockets of conidiation were found within these lipid-containing sclerotial hyphae. At the fungal-plant interface, the walls of the upper plate cells often stained yellow-green with toluidine blue instead of the blue of host parenchyma cells. This may be due to the interaction of the stain with the browned tissue. The interface between plant and fungus at the base was regular and constituted a definite line of demarcation between fungus and host. Vascular tissue was found just below the interface extending down into the healthy rachilla. Control ovaries at this stage had developed into young seeds and were soft, green and were being filled with starch (Plate 30a).

Twenty-one days after inoculation the parasitic tissues had developed into a small ergot sclerotium (Plate 30b). It was completely purple externally with plant debris and the sphacelial cap remaining on its tip. It had become harder and this caused problems in preparation and cutting of the sections due to the difference in hardness of ergot and plant tissues. The medulla tissue of the ergot stained blue-purple with toluidine blue and was very compact, while its outer cortical layers were brown (Plate 31a). These outer layers are naturally pigmented and the blue staining of toluidine blue was masked. The hyphae of the cortical layer (Plate 32a) appeared to be elongated and flattened or crushed, and were similar to the internal sclerotial cells except that they seemed to contain less lipid and hence were less bulbous. The outermost hyphae bore

a very thin layer of conidiophores and conidia, which appeared to extend over most of the external surface. A brown pigmentation similar to that of the cortical tissue was observed at the cut surface of the sclerotium, which had to be trimmed during preparation. This suggests that sclerotial cells may form brown oxidation products on exposure to air and this may account for the existence of the brown cortical layer. The medullary tissue of the ergot was not uniform but contained different types of hyphae (Plate 32b). At the plant-fungus interface, the 'lens' of young, non-lipidic hyphae was still evident and measured about 150μ long. (In rye this region measured about 90μ , 19 days after inoculation.) Distal to these lay fat-filled hyphae arranged in a prosenchyma, which were about 6.5μ wide. Further up in the sclerotium fat bulbous cells, whose average width was about 8μ , were found in plectenchymatic arrangement. Other much narrower and longer hyphae (about 1.3μ wide) were also located within the sclerotium. Variation in the lipid content and extent of vacuolization was also evident within these hyphae. Electron microscopy of tissue near the base of the ergot showed that the hyphae had large lipid deposits, fewer vacuoles and were more electron-dense than at the 10-day stage. They were also usually short with frequent septa (often accompanied by Woronin bodies), thus morphologically typifying sclerotial cells (Plate 33). The hyphal walls were of a thickness similar to those of the 10-day old hyphae, but the adhesive meniscus was not as prominent, and instead loose fibrillar material was more commonly seen around the hyphae (Plate 33).

The youngest fat-filled hyphae near the base of the ovary appeared to grow vertically in a definite close-packed hyphal arrangement, especially in the centre of the sclerotium. These probably correspond to the vertically orientated generative hyphae

observed by Dickson (1947). Towards the periphery however, the hyphae often appeared to be compressed and forced to curve round as if rapid growth from the base was pushing them up into a confined region above. The weight of the fungal material bearing down on the interface region must be considerable. The width of the plant-fungus interface is narrow relative to the width of the sclerotium , and rapid growth at the interface would cause horizontal development of the fungus which is restricted by the rigid glumes. The net effect is a rapid production of fungal material from the base upwards. This material must encounter a certain confining pressure, which results in a structure of highly compacted hyphae. At the base, the 'plate' of host cells persisted, apparently preventing rachillar penetration (Plate 31a). The base of the ergot was in close proximity to the host's vascular traces. The ergot at this stage was roughly $1\frac{1}{2}$ -2 times the size of a seed of corresponding age, and protruded outside the floral cavity (Plate 31b). At maturity the ergots can be considerably larger than the seeds (about eight times), and especially in rye where the long thin sclerotia may even twist themselves around the ear (Plate 34).

LEGEND FOR PLATES

Small letters refer to plant parts, capital letters to fungal parts.

a	anther
c	cuticle
cl	chloroplast
cw	plant cell wall
f	fibrils
fr	furrow region of ovary
g	glume
h	hair
i	integuments
ic	intercellular space
ie	inner epidermis of ovary wall
ii	inner integument
is	inter-stylar region of ovary
iw	inner layer of ovary wall
lm	lemma
m	meniscus of unidentified material coating hyphae
ml	middle lamella
mp	middle parenchyma of ovary wall
n	nucleus
nc	nucellus
ne	nucellar epidermis
o	ovary
oe	outer epidermis of ovary wall
oi	outer integument of ovule
ov	ovule
ow	ovary wall
p	plant cell
pa	palea
pd	plasmodesmata
pl	plasmalemma
pt	'plate' of cells at base of ovary
r	rachilla
s	starch grain
st	stigma/stigma cell(s)
t	transmitting tissue
tw	core of thick-walled cells in the rachilla
v	vacuole

vb vascular bundle
 vt vascular tissue
 x xylem vessel

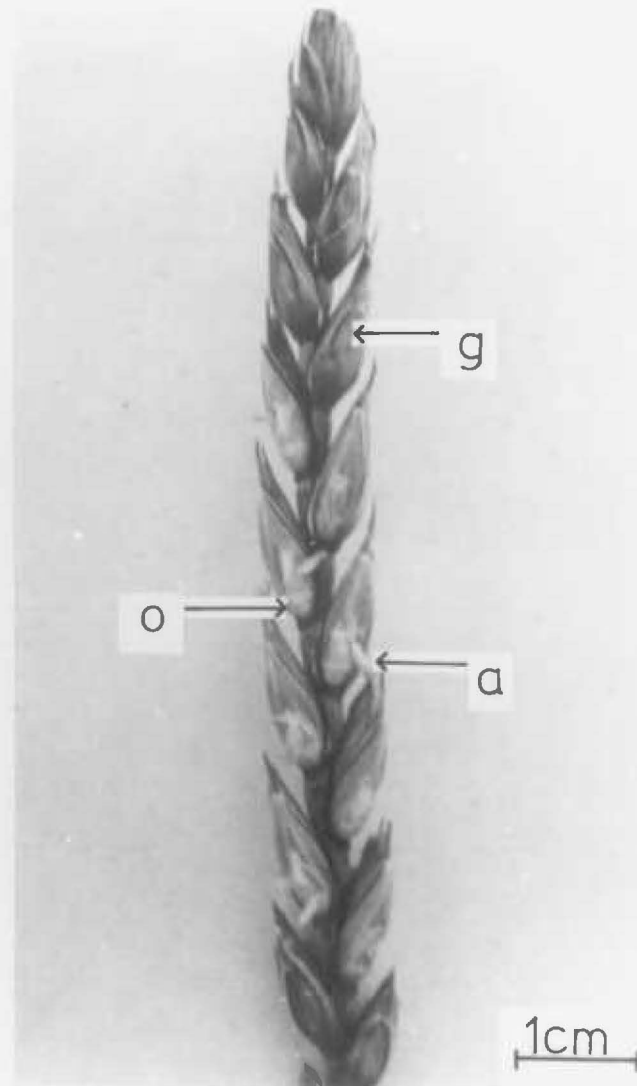
CO conidium/conidiation
 CH channels of hyphae
 CX cortex of sclerotium
 H fungal hypha(e)
 HD honeydew
 HW hyphal wall
 I host-parasite interface
 IV invaginated vacuole
 L lipid droplet
 LCH lipid-containing hyphae
 LFH lipid-free hyphae
 MD medulla of sclerotium
 N nucleus
 PC pocket of conidiation
 SCL sclerotial cells/sclerotium
 SM septum
 SP septal pore
 SPH sphacelium
 SPHC sphacelial cap
 TR possible transition type of fungal cell between sphacelial
 and sclerotial types
 V vacuole
 WB Woronin Body

PLATE 1. MACROSCOPIC STAGES OF ERGOT DEVELOPMENT I.

- (a) Inoculation of wheat ear at anthesis using a hypodermic syringe to inject a spore suspension into the floral cavity.
- (b) Wheat ear 3 days after inoculation with glumes and lemmas dissected away to reveal ovaries. Anthesis has occurred and the ovaries appear normal. There are no visible macroscopic signs at this early stage to indicate that infection has occurred.



a



b

PLATE 2. INITIAL STAGES OF INFECTION.

(3 days after inoculation)

- (a) Longitudinal section through a wheat ovary showing little sign of disruption at this early stage of infection.
- (b) External hyphae growing along the outer epidermis of the ovary wall.
- (c) Transverse section through an ovary near the furrow region showing invasion of the outer epidermis by an external hypha and inter-cellular growth in the ovary wall.

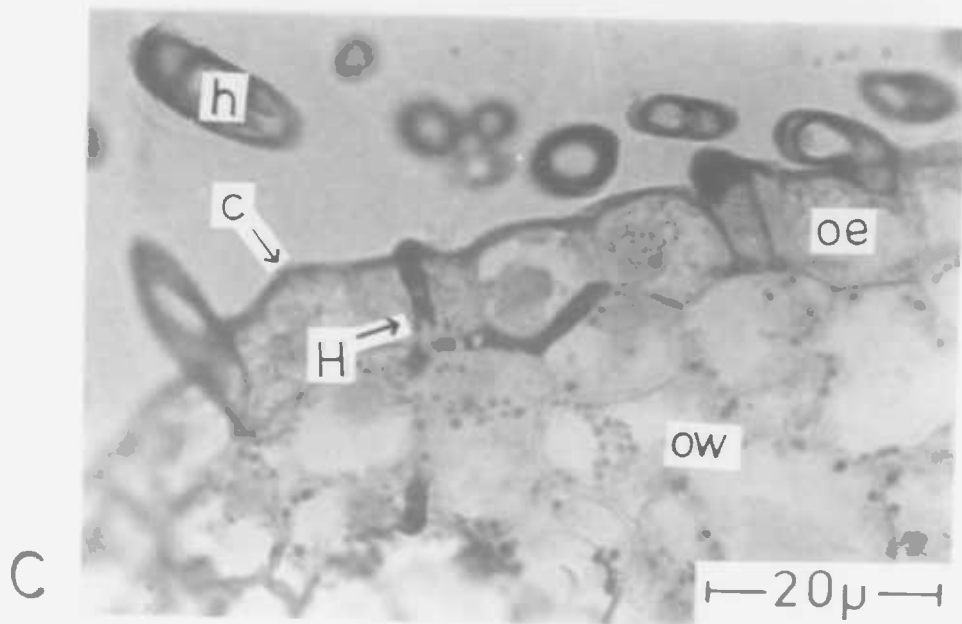
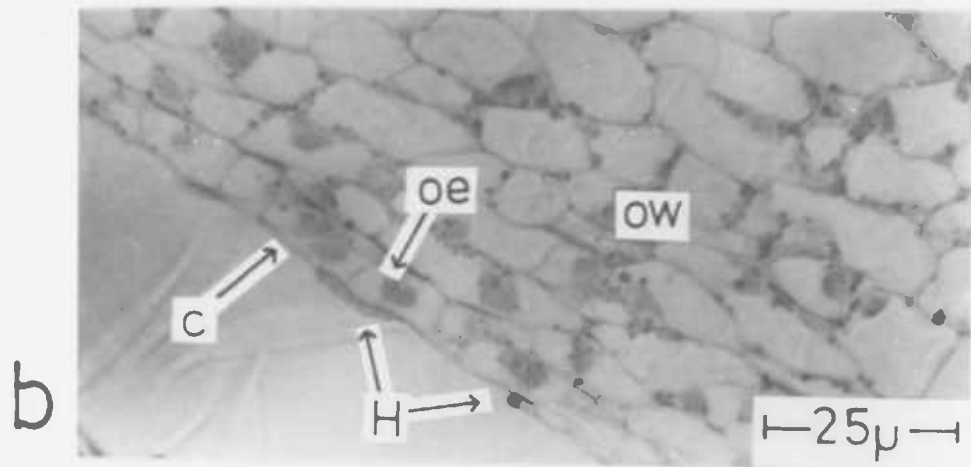
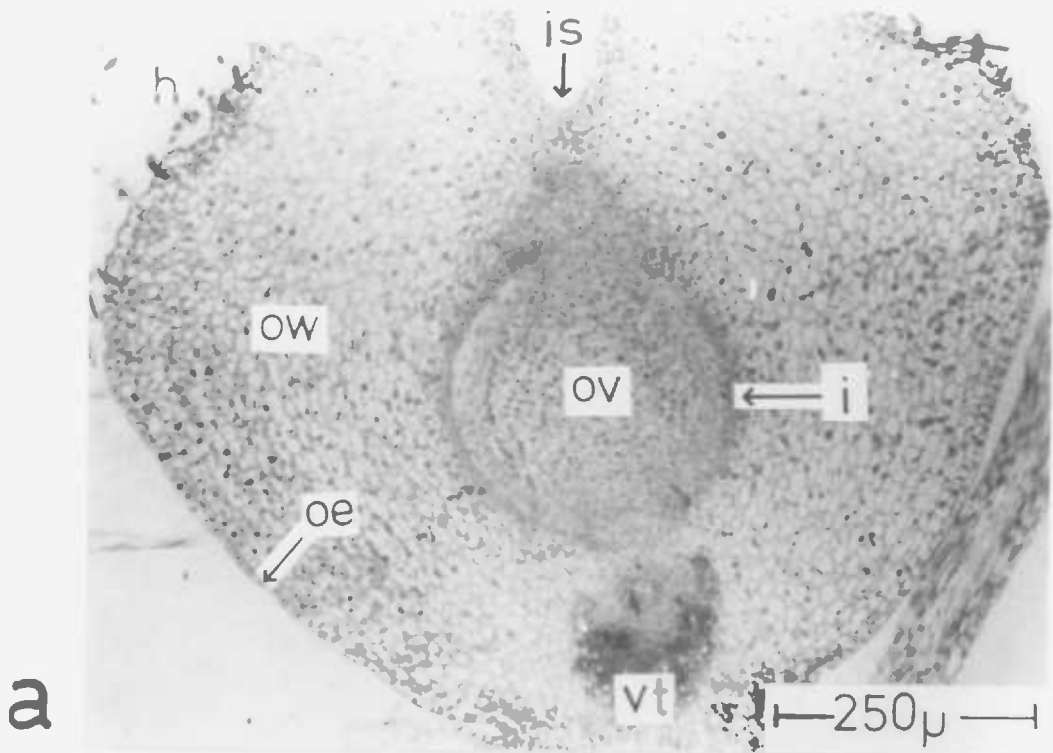


PLATE 3. INITIAL STAGES OF INFECTION (T.E.M.)
(3 days after inoculation).

- (a) Sub-cuticular hyphae in the inter-stylar region at the apex of the ovary (for location see Plate 2a).

- (b) Erosion of middle lamella by sub-cuticular hyphae. Degraded areas (arrows) are less electron dense and have a loose fibrillar appearance.

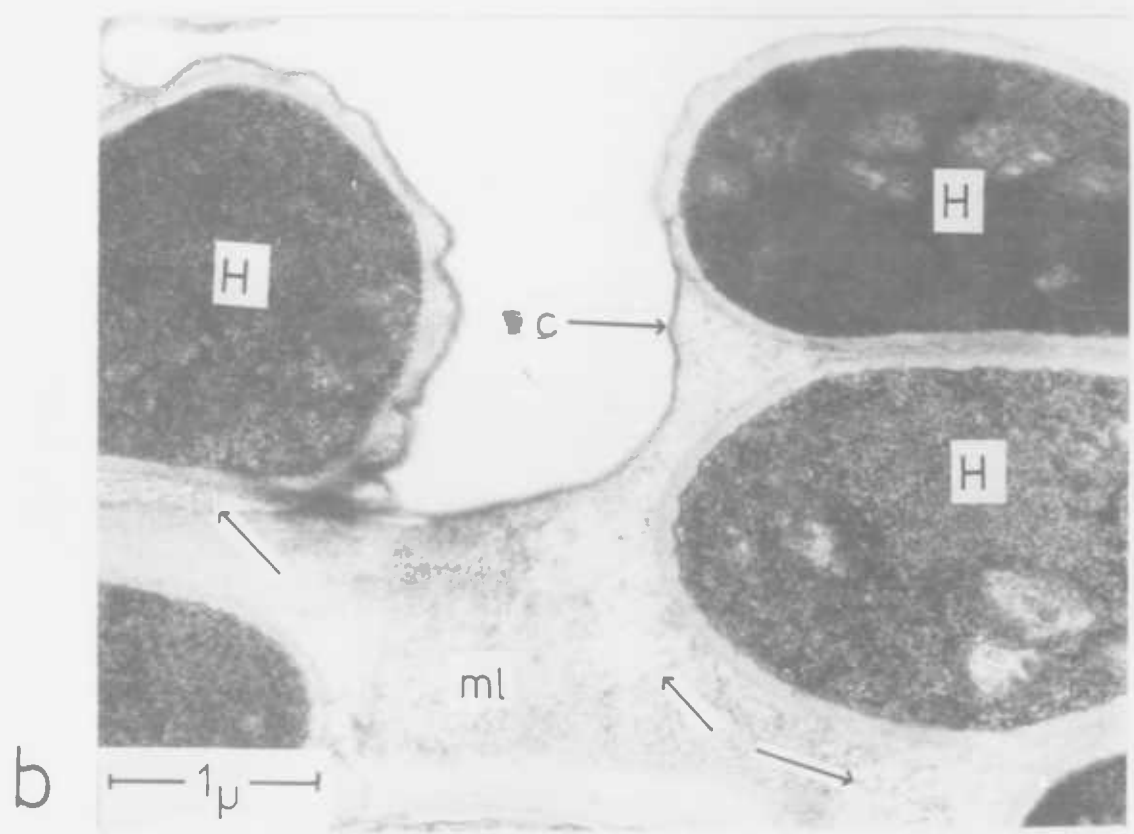
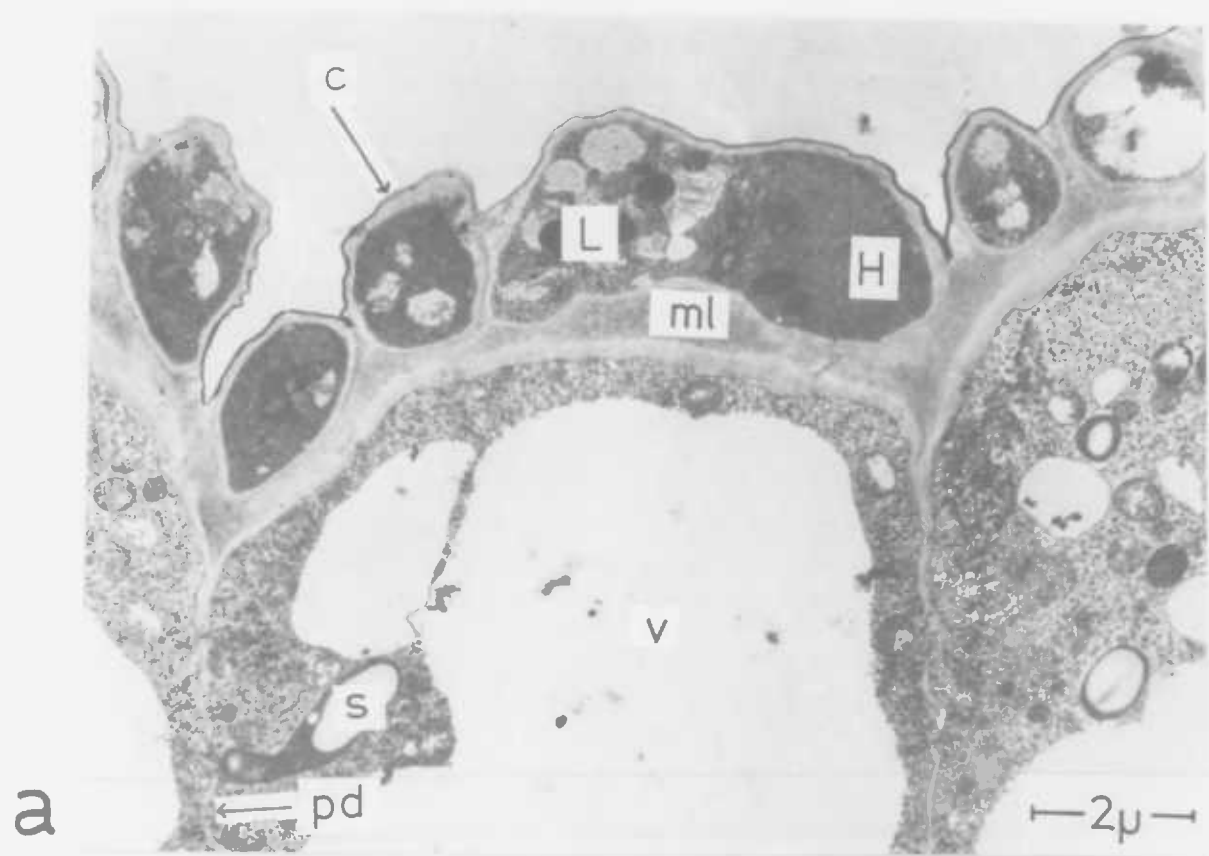


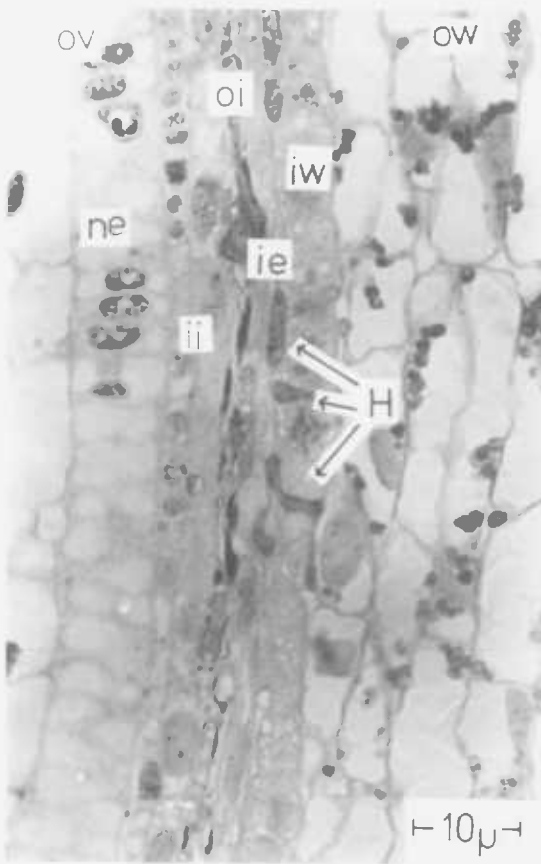
PLATE 4. HYPHAL GROWTH AROUND THE OVULE.

(3 days after inoculation).

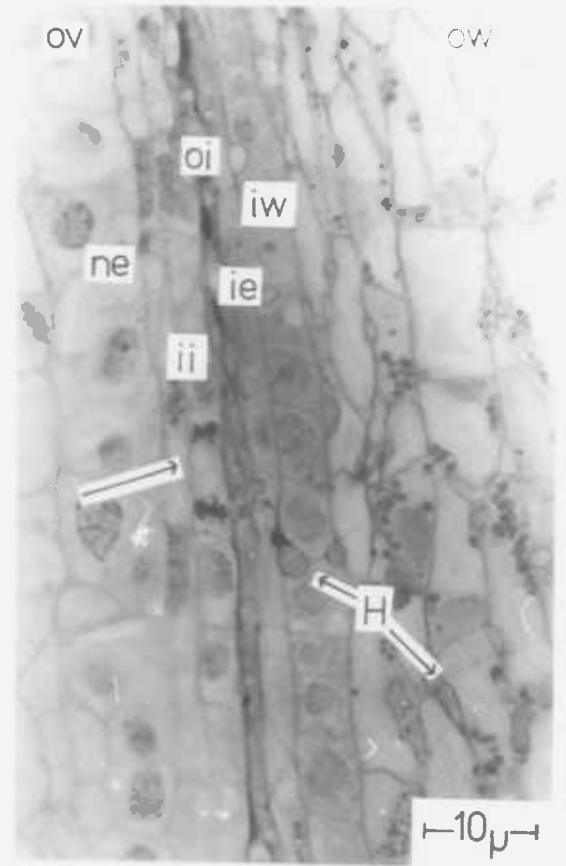
(a) Intercellular hyphal growth in the inner layer of the ovary wall, inner epidermis and outer integument. Outer integument cells appear collapsed and degenerating.

(b) Cells of the inner integument continue to divide (arrow) although hyphae are only one or two cells away.

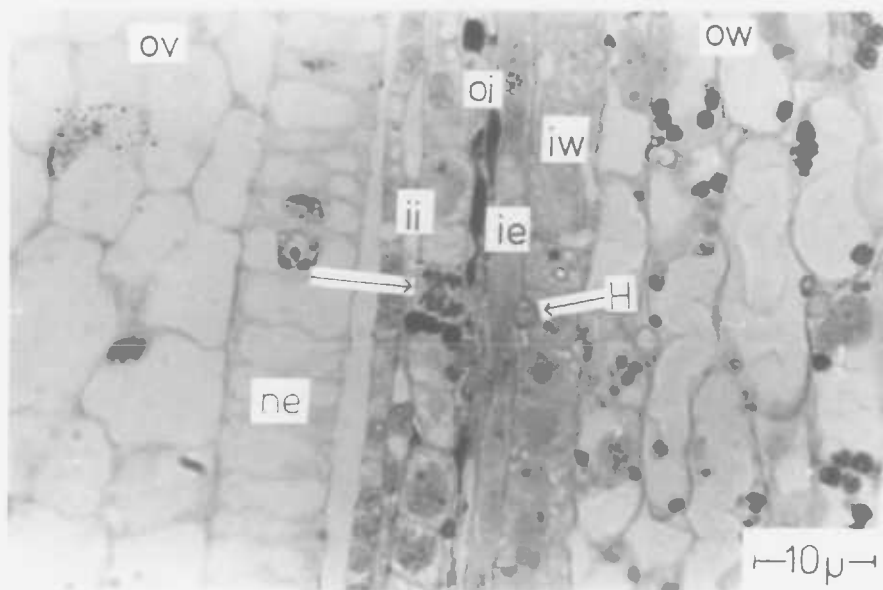
(c) Cell division (arrow) in the inner integument continues while cells in the outer integument collapse and hyphae proceed to invade.



a



b



c

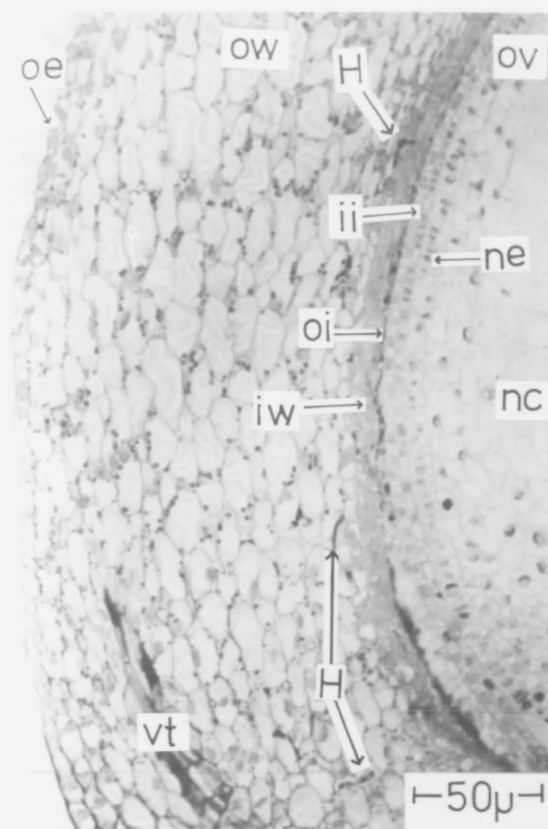
PLATE 5. HYPHAL GROWTH AROUND THE OVULE AND IN THE OVARY WALL.
(4 days after inoculation).

- (a) Part of the ovary wall and ovule showing location of various tissues in the ovary, and extent of fungal penetration. Nucellus and nucellar epidermis of the ovule are still uninfected.

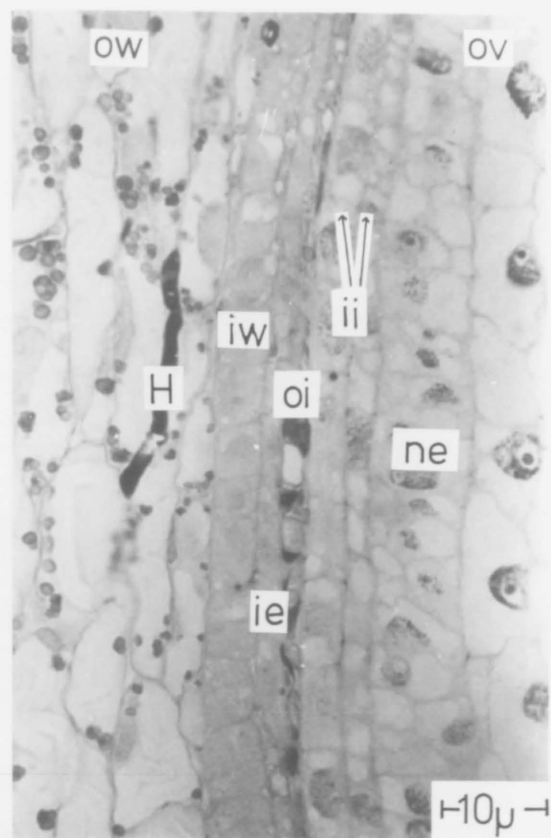
- (b) Hypha between the cells of the ovary wall.

- (c) Intercellular hyphae in the inner layer of the ovary wall, outer integument and now also in the inner integument. Cell division continues in the nucellar epidermis (arrow).

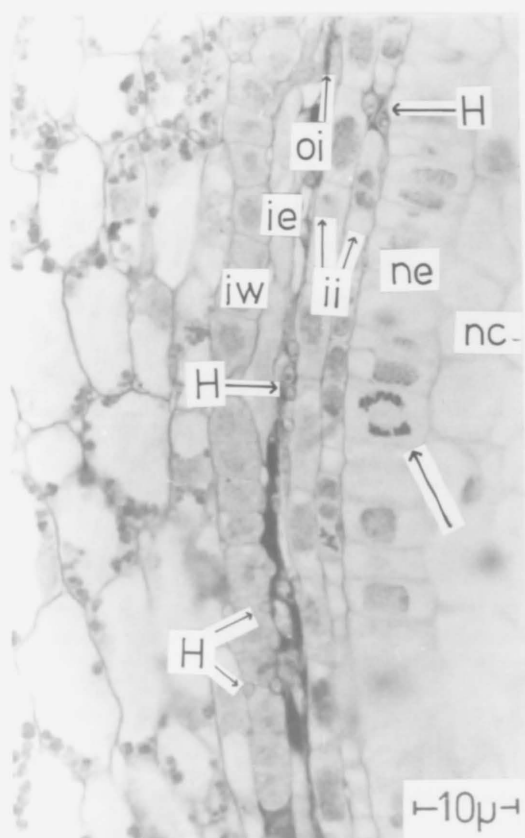
- (d) Cell division continues in the inner integument despite the close proximity of the fungal hyphae.



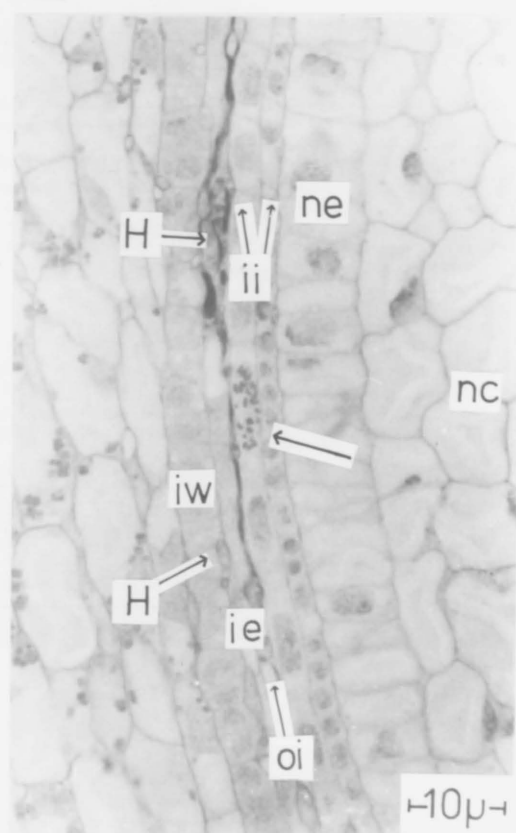
a



b



c



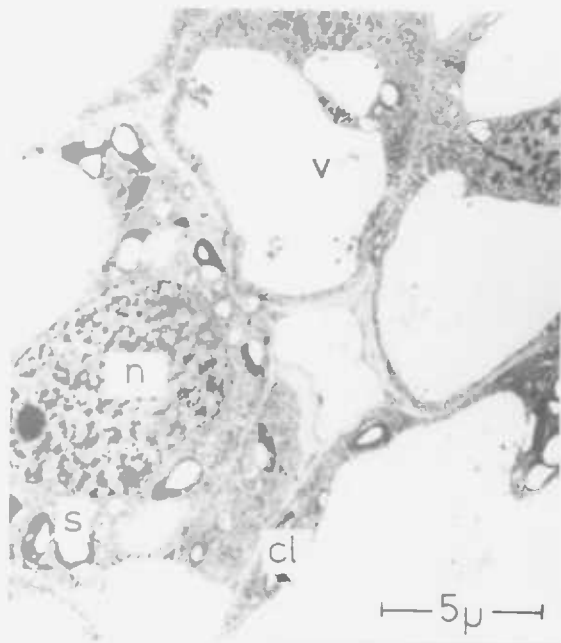
d

PLATE 6. INTERCELLULAR HYPHAE IN THE OVARY WALL (T.E.M.)
(4 days after inoculation).

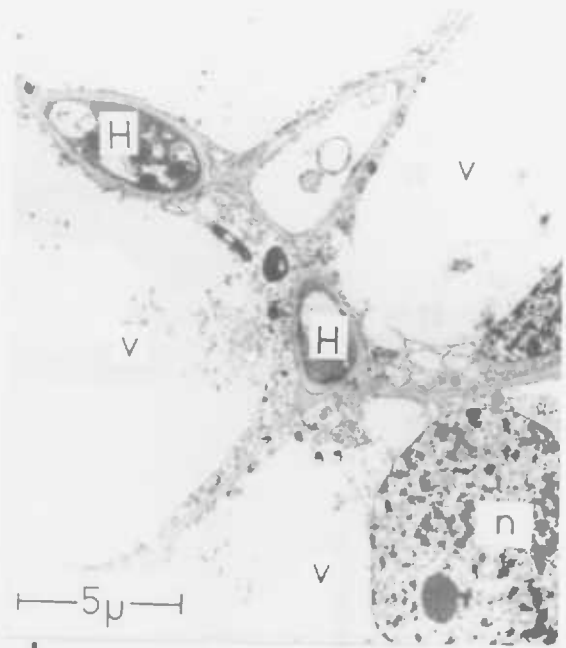
- (a) Healthy ovary wall tissue.

- (b) Intercellular hyphae in the ovary wall tissue of an infected ovary.
Plant cell wall distortion is relatively slight at this stage.

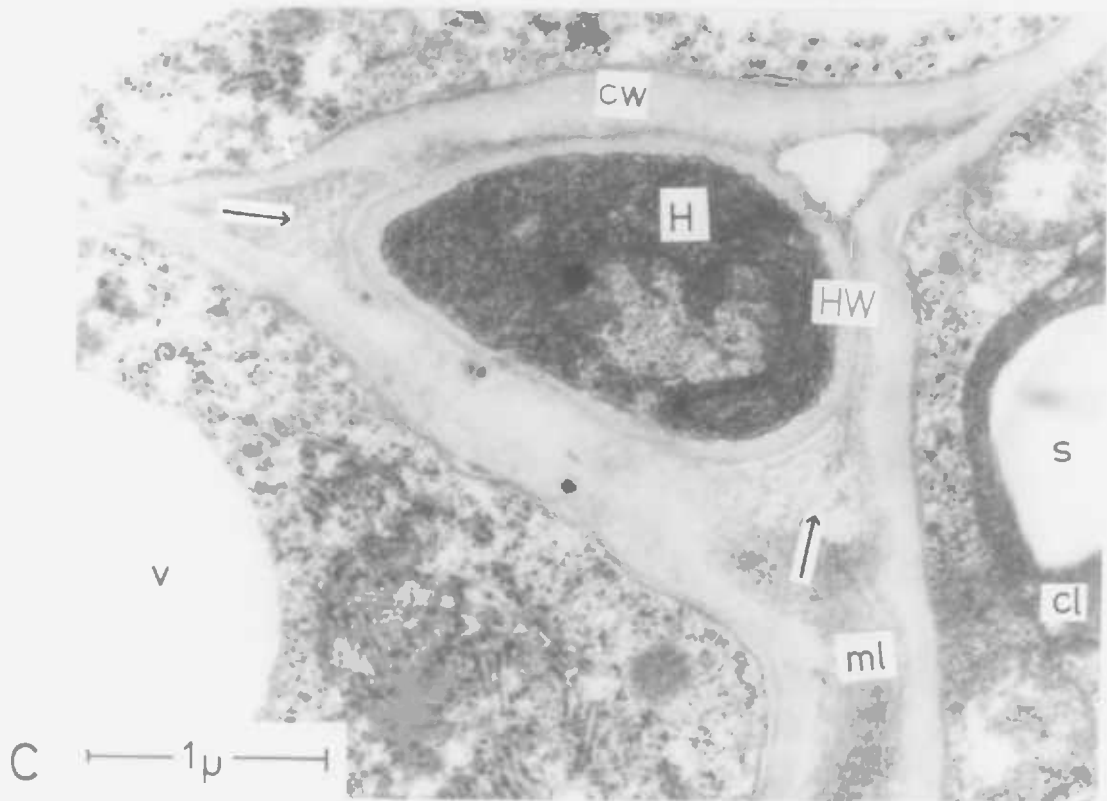
- (c) Erosion (arrows) of middle lamella by intercellular hyphae.
Eroded regions have loosely woven fibrillar appearance.



a



b



c

PLATE 7. STIGMA INFECTION.

(4 days after inoculation).

(a) Longitudinal section through an ovary showing dense hyphal growth down the stigmas and stylar region to the top of the ovule. There is little evidence of infection in other parts of the ovary, indicating that primary infection took place via the stigmas.

(b) Enlarged view of hyphal growth down the stigma to the top of the ovule and invasion of the outer integument.

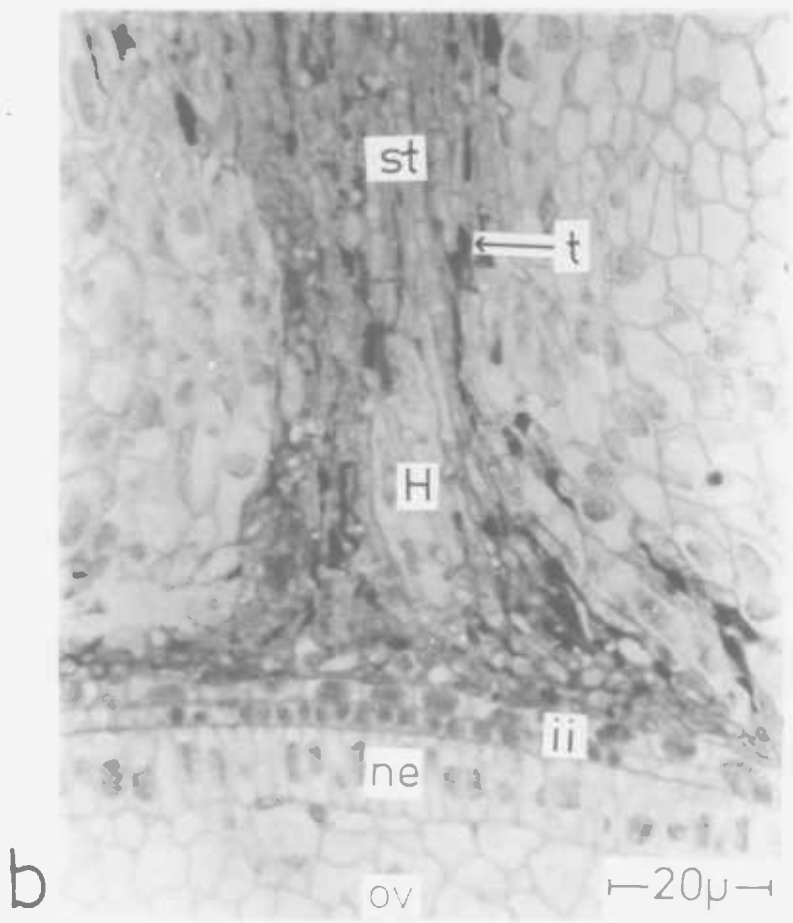
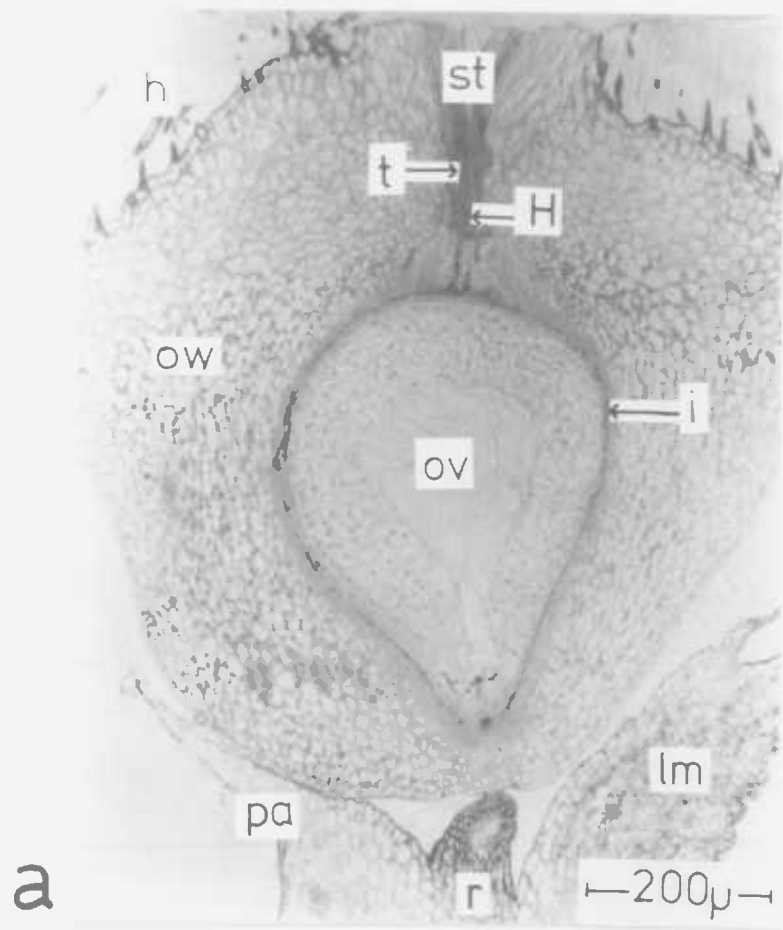


PLATE 8. STIGMA INFECTION (T.E.M.)
 (4 days after inoculation).

Low power electron micrograph of an infected stigma, in which hyphae are growing longitudinally between the transmitting tissue (whose cells have collapsed and are very electron dense).

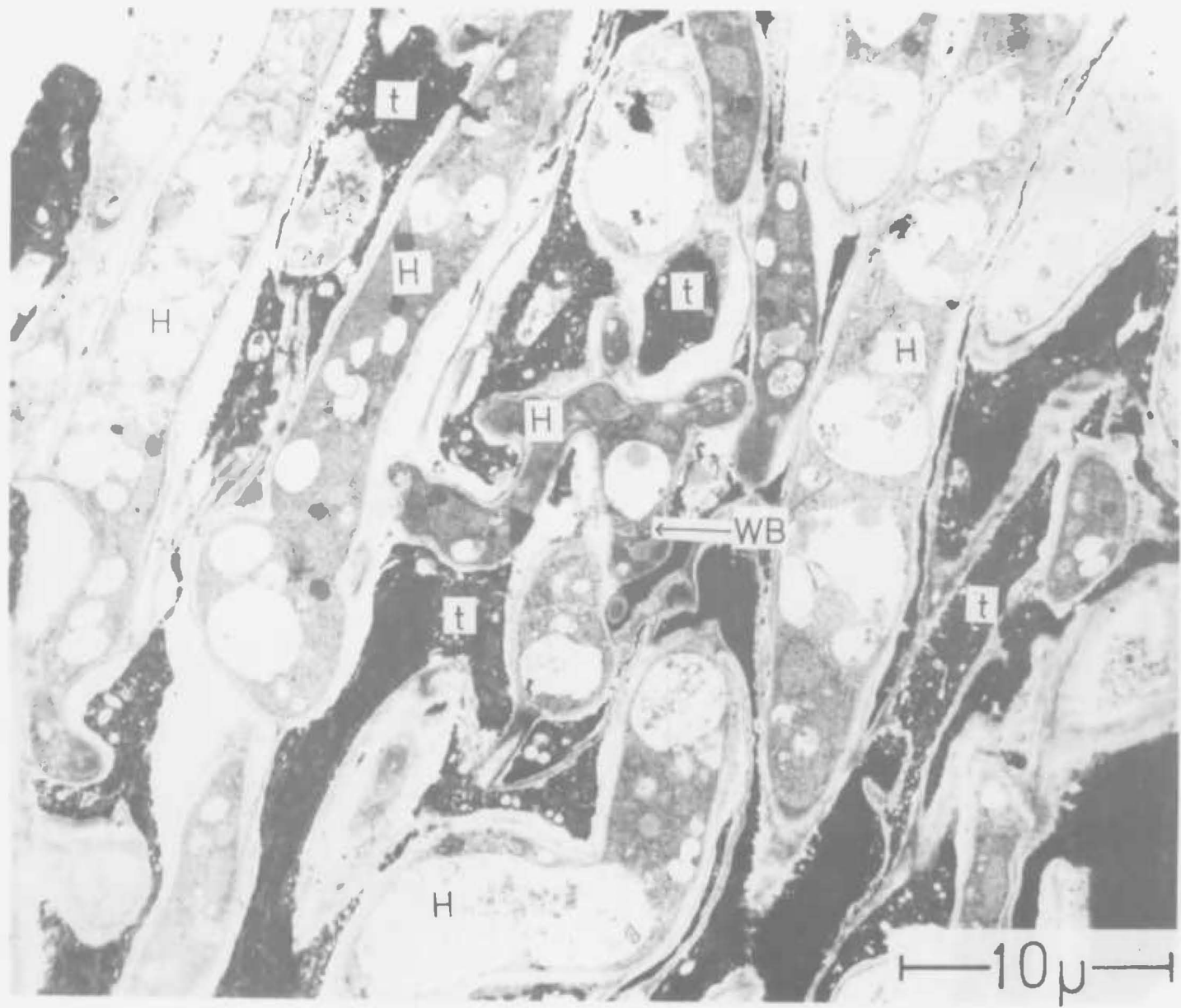


PLATE 9. STIGMA INFECTION (T.E.M.)
 (4 days after inoculation).

Longitudinal section across an infected stigma, showing hyphae growing down between the stigma cells and the transmitting tissue. Cells of the transmitting tissue often die as a result of infection, their protoplasts becoming very electron dense, in a manner similar to that during the passage of a pollen tube. The cell walls of the transmitting tissue appear softened and partially degraded (arrows) and their layers separated.

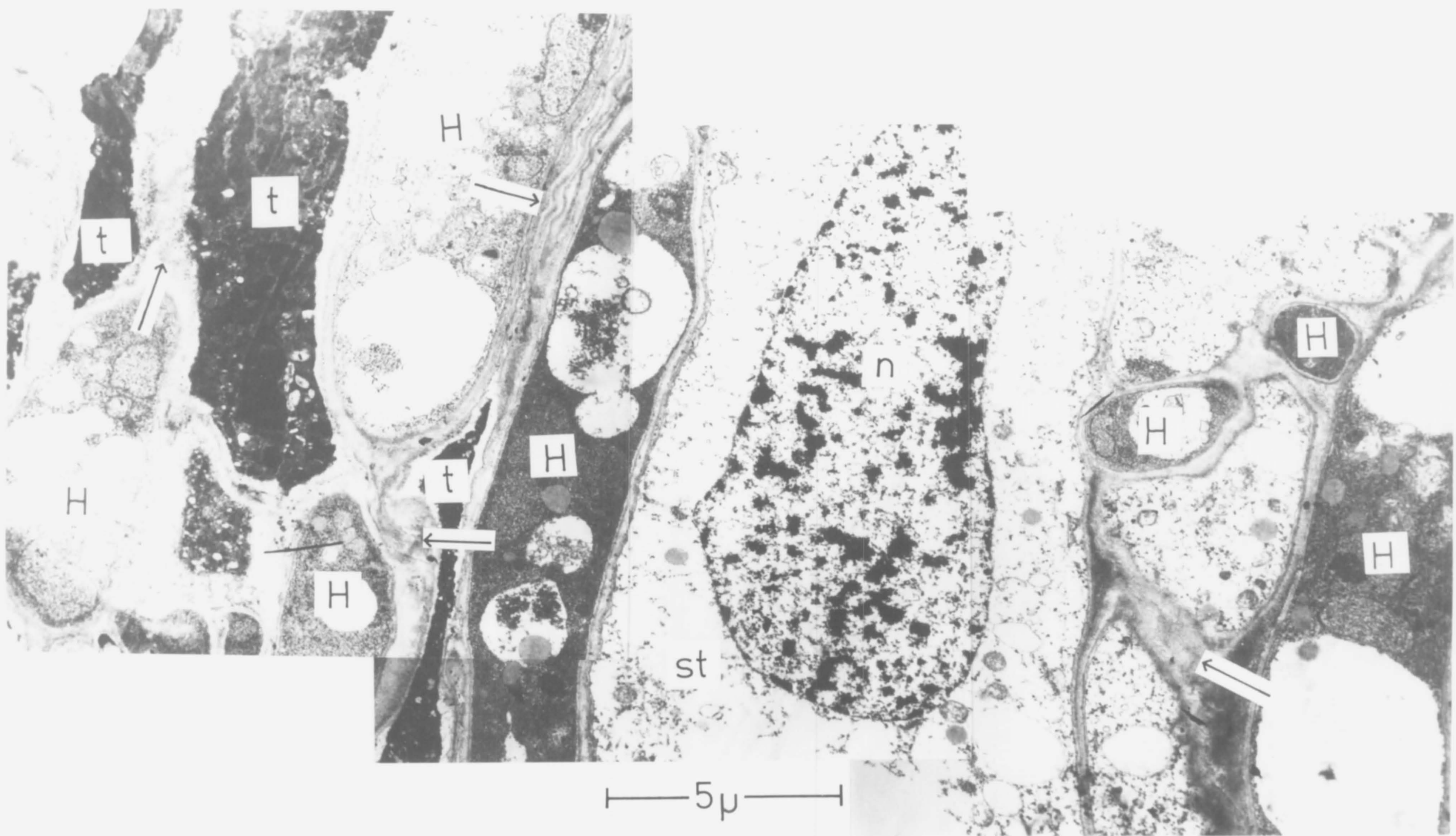


PLATE 10. STIGMA INFECTION (T.E.M.)
 (4 days after inoculation).

- (a) The cell walls of the transmitting tissue appear to have been solubilized (arrows) by the invading hyphae, and pushed into folds.

- (b) Detail of a cell of the transmitting tissue showing the cytoplasm contracted inwards away from the cell wall. The cell walls are partially degraded (arrows) and invaginated by the invading hyphae.

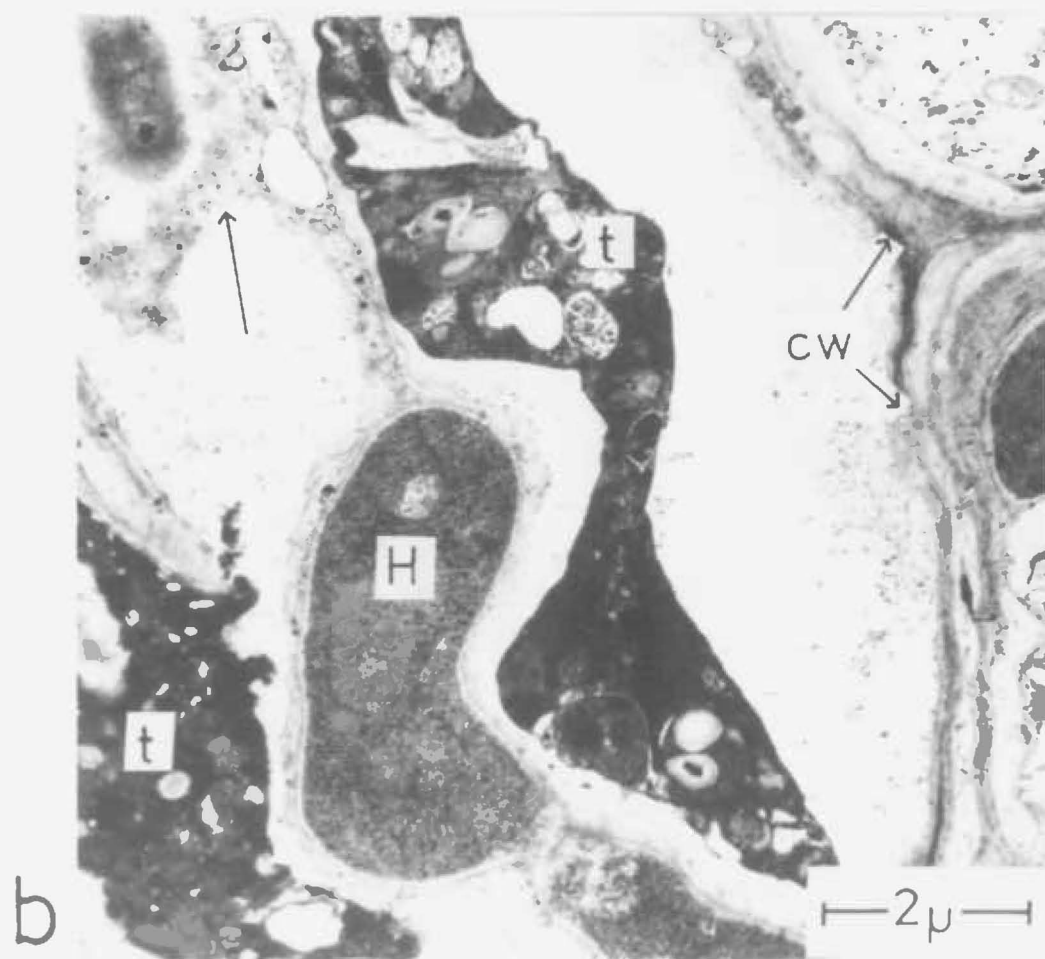
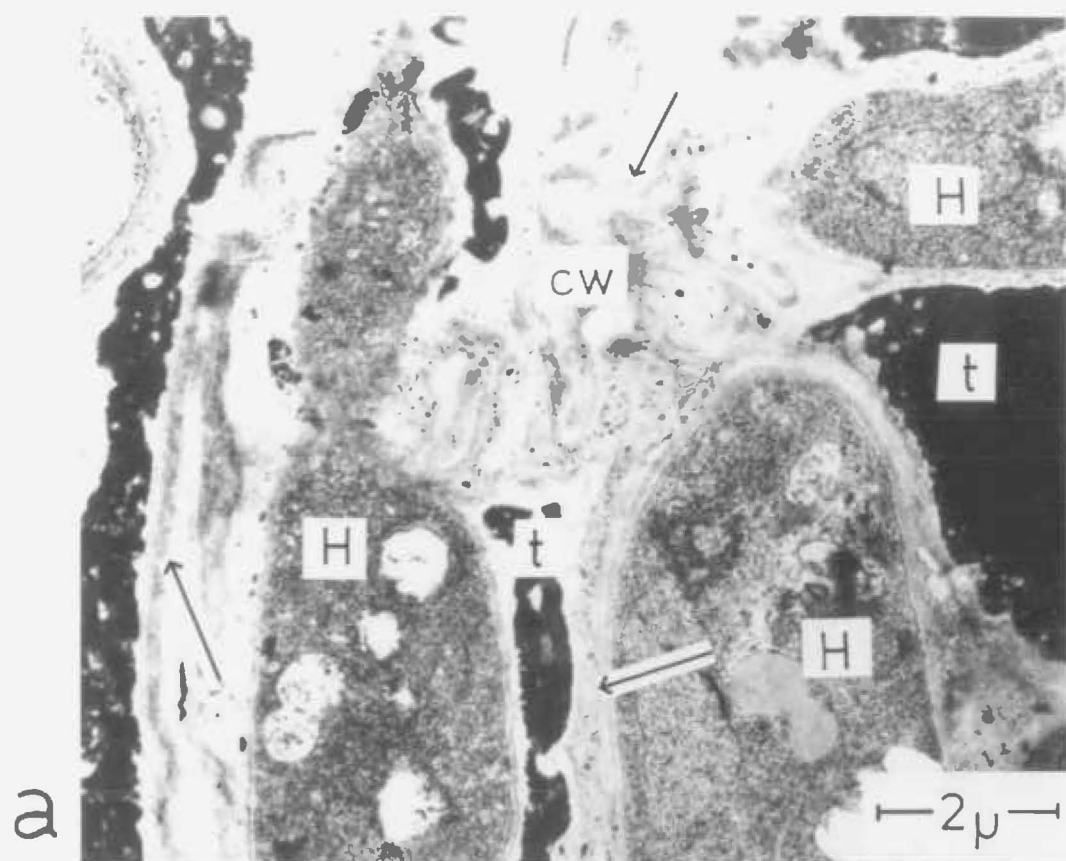


PLATE 11. COLONIZATION OF THE OUTER INTEGUMENT (T.E.M.)
 (4 days after inoculation).

Fungal hyphae colonize the outer integument of the ovule. The cells of the outer integument appear to be collapsed and degenerating, with the cell contents contracted and very electron dense. The inner integument cells, immediately surrounding the ovule, have not yet been colonized. The inner epidermis and the inner layer of the ovary wall appear still intact.

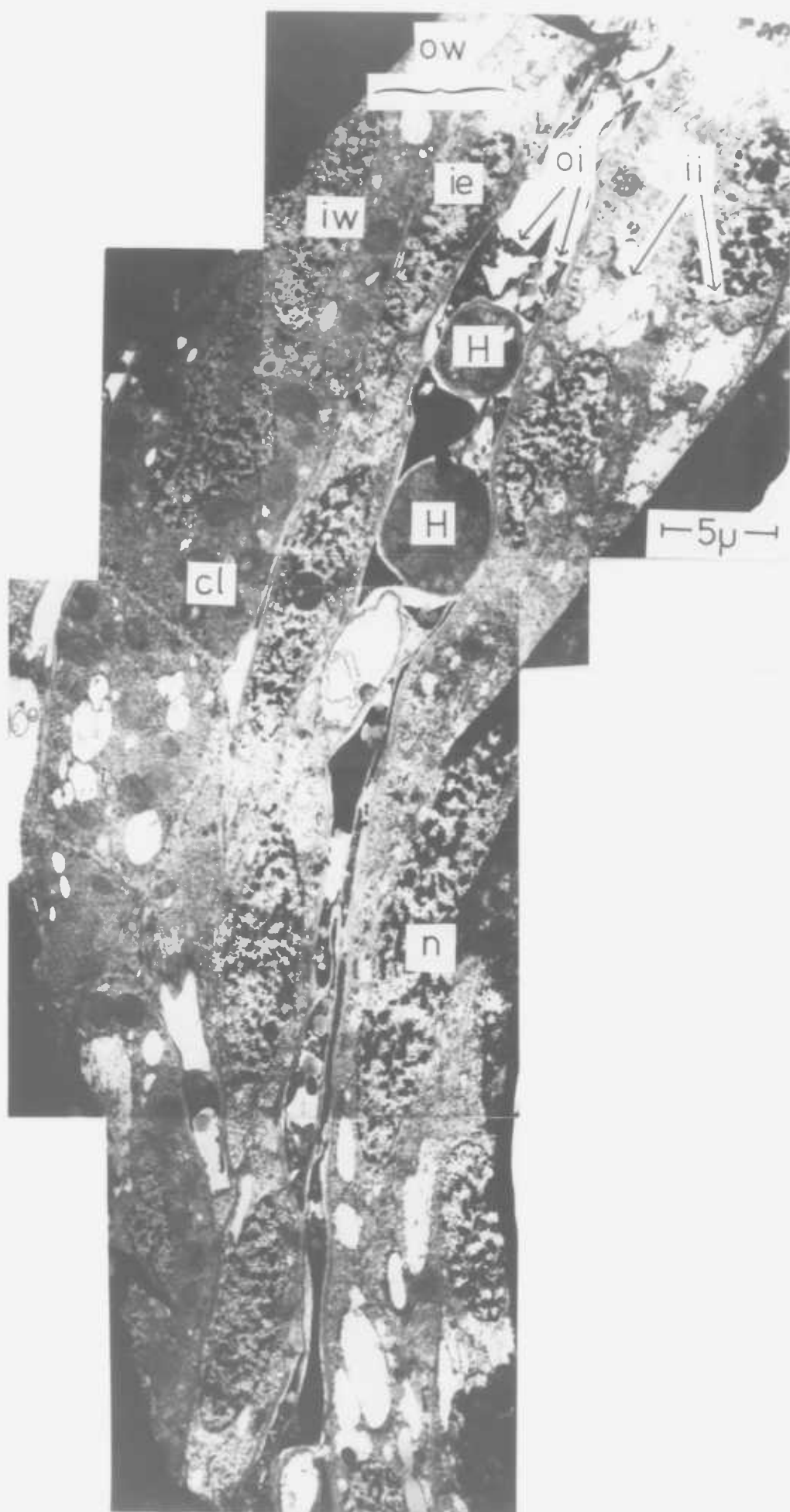


PLATE 12. COLONIZATION AROUND THE OVULE (T.E.M.)

(4 days after inoculation).

(a) Collapse and degeneration of the outer integument cells. Cells of the inner integument and the innermost layers of the ovary wall appear normal.

(b) Fungal hypha growing between the parenchyma cells of the ovary wall in close proximity to the ovule.

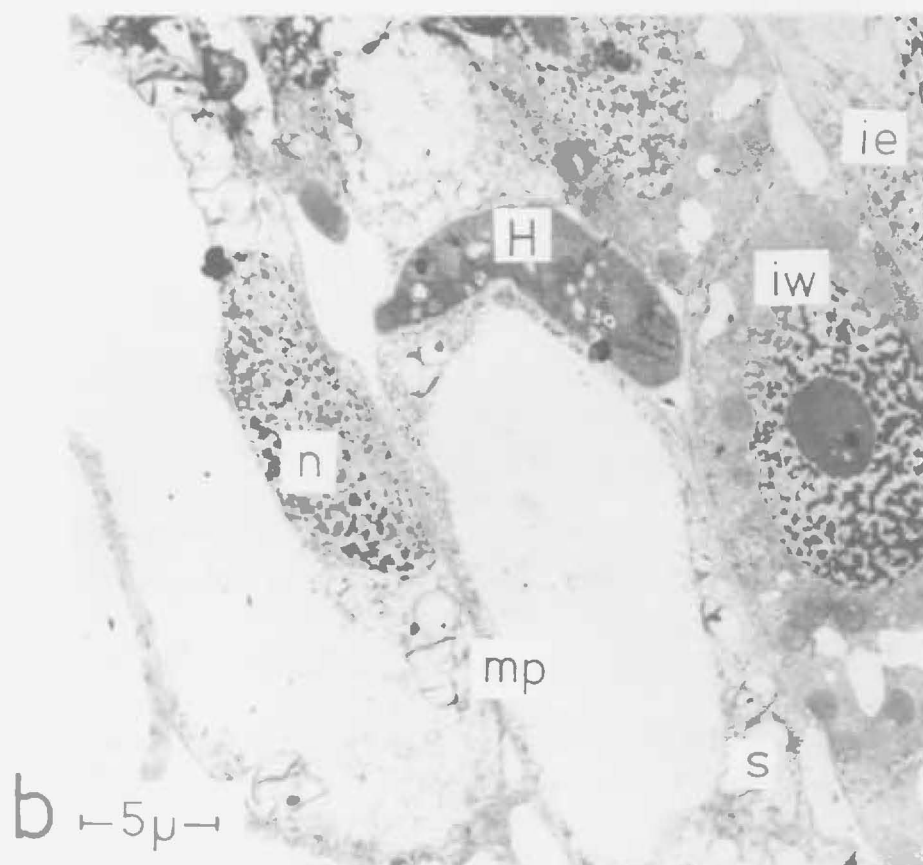
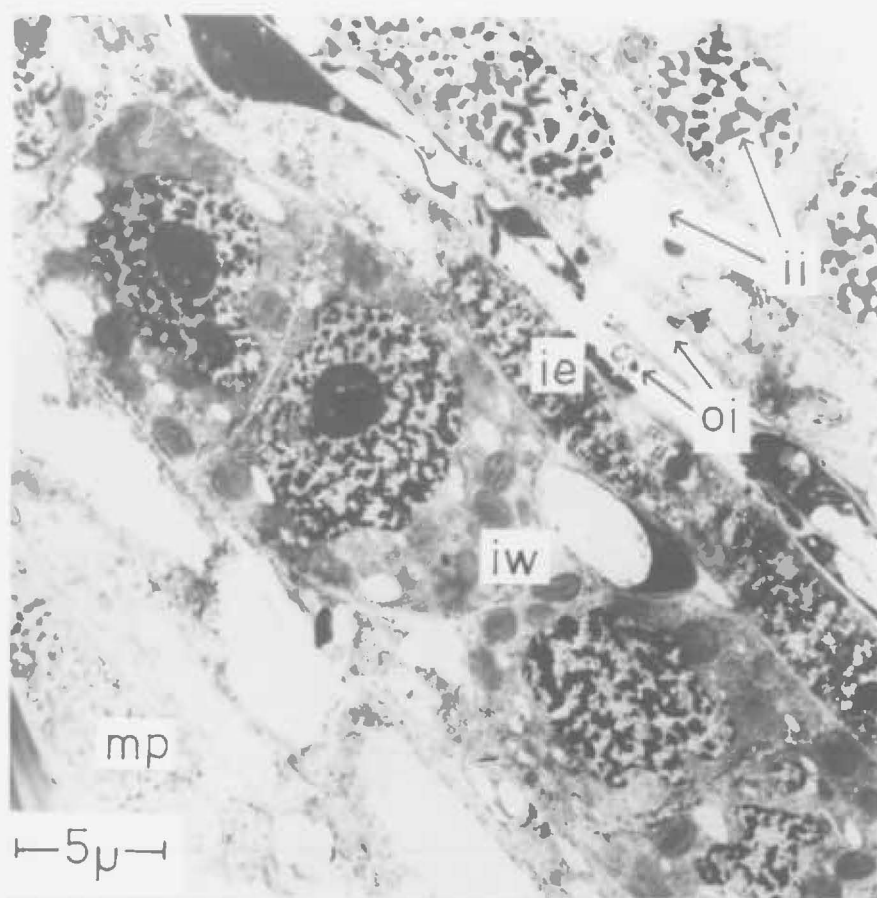


PLATE 13. EXTENSIVE COLONIZATION OF THE OVARY WALL AND OVULE.

(5 days after inoculation).

- (a) Transverse section through an infected ovary showing the pre-dominance of hyphae in and around the ovule, near the vascular bundles and in the furrow region.

- (b) Longitudinal section through the basal region of an infected ovary. There is heavy hyphal growth in and around the ovule but colonization stops at the ovary base and does not proceed into the rachilla.

- (c) Enlarged view of part of (b) showing hyphal growth separating the ovule (delimited by the nucellar epidermis) from its integuments, thus isolating the ovule. There is also extensive colonization within the centre of the ovule.

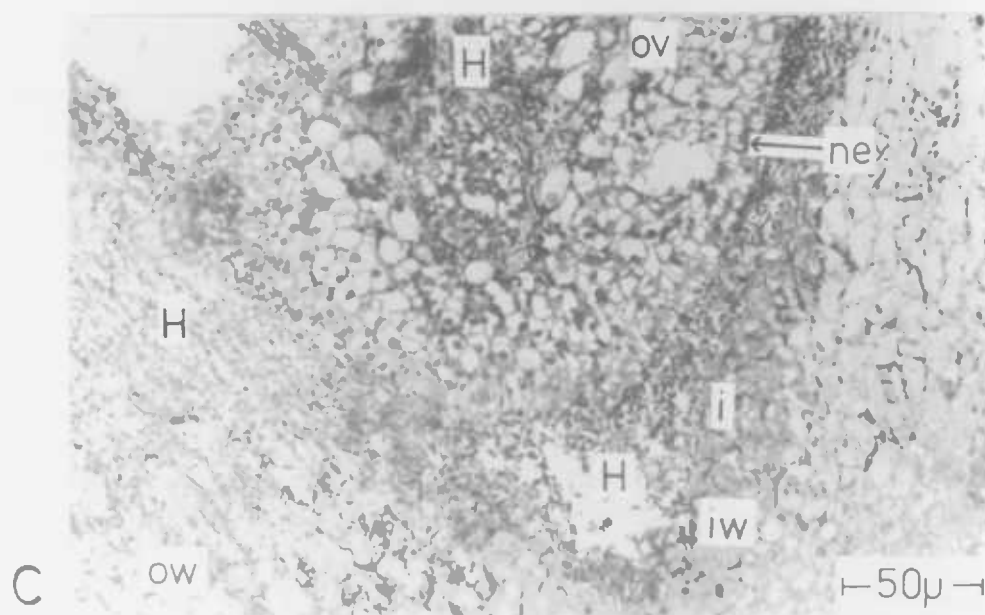
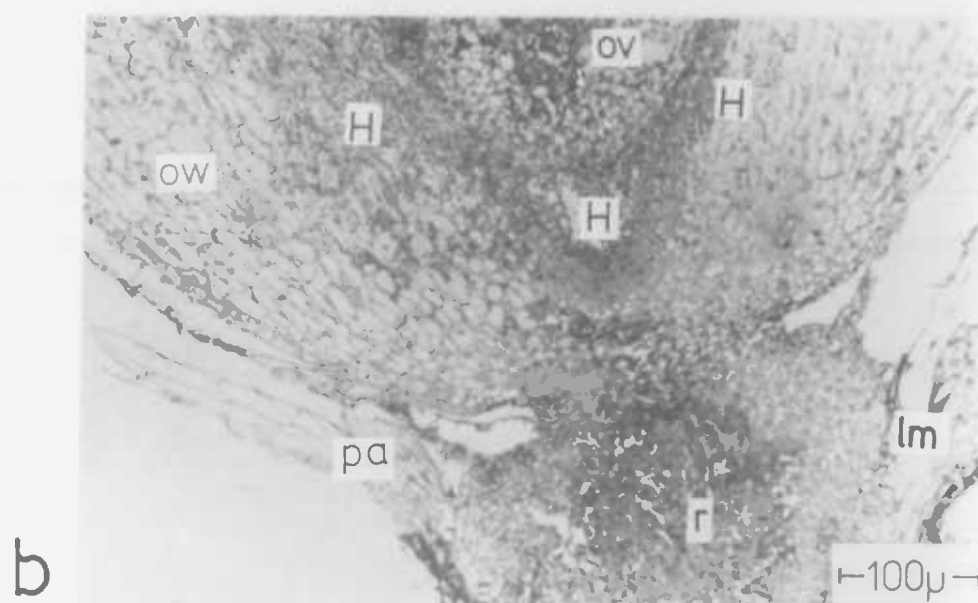
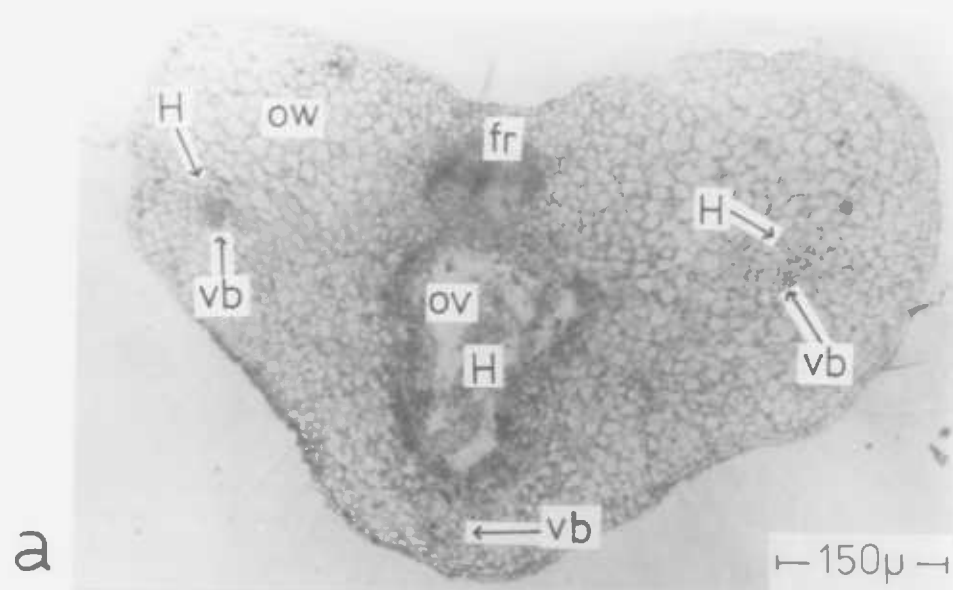


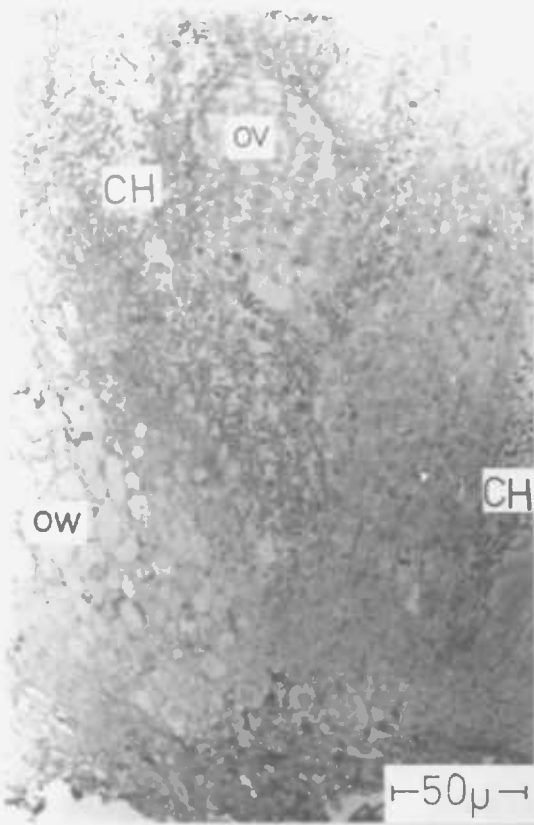
PLATE 14. EXTENSIVE COLONIZATION OF THE OVARY WALL AND OVULE.

(5 days after inoculation).

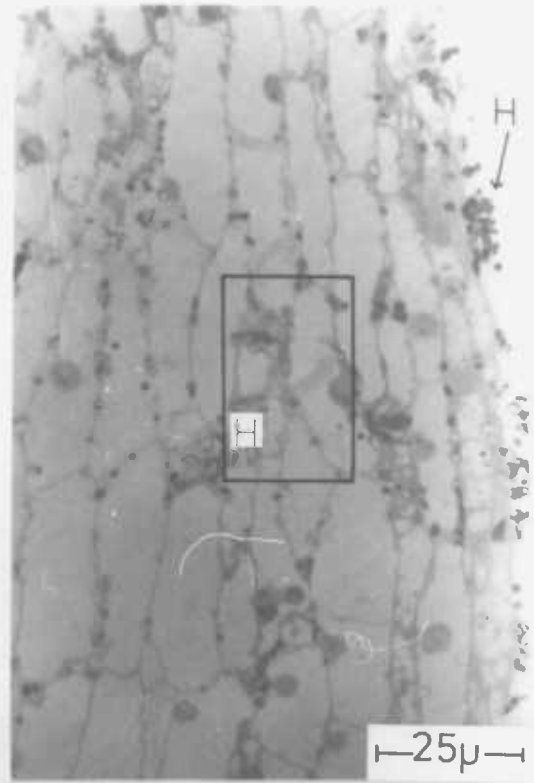
- (a) Concentration of hyphal growth in major channels in the ovary, separating large areas of ovary tissue and facilitating its total disruption. The ovule is now completely surrounded and isolated by the hyphae.

- (b) Intercellular hyphal growth in the ovary wall and on the external surface of the ovary. Area in the rectangle is shown enlarged in (d).

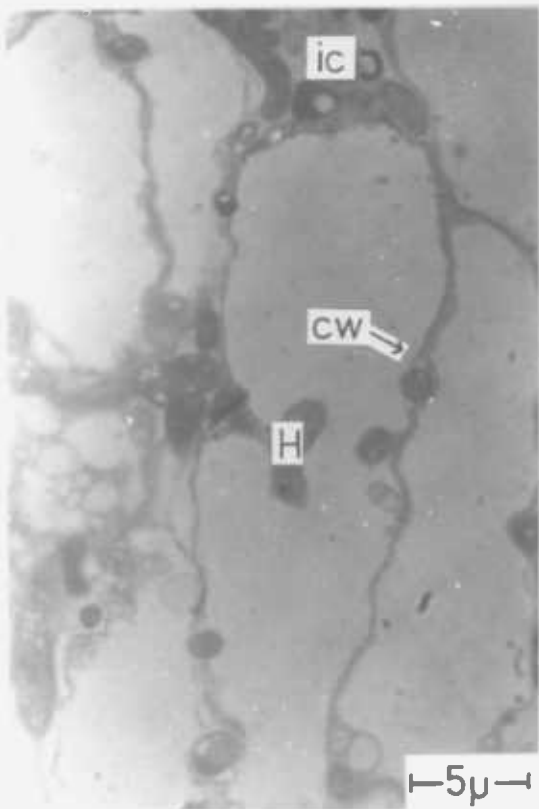
- (c) Intercellular hyphae separate the cells of the ovary wall along
&
(d) the line of the middle lamella, and proliferate in the inter-cellular spaces. The cell walls are thin and appear softened and easily invaginated.



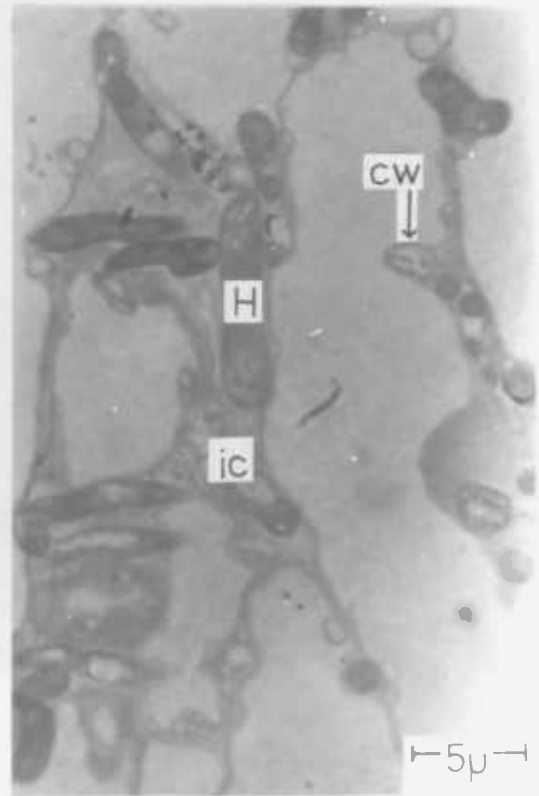
a



b



c

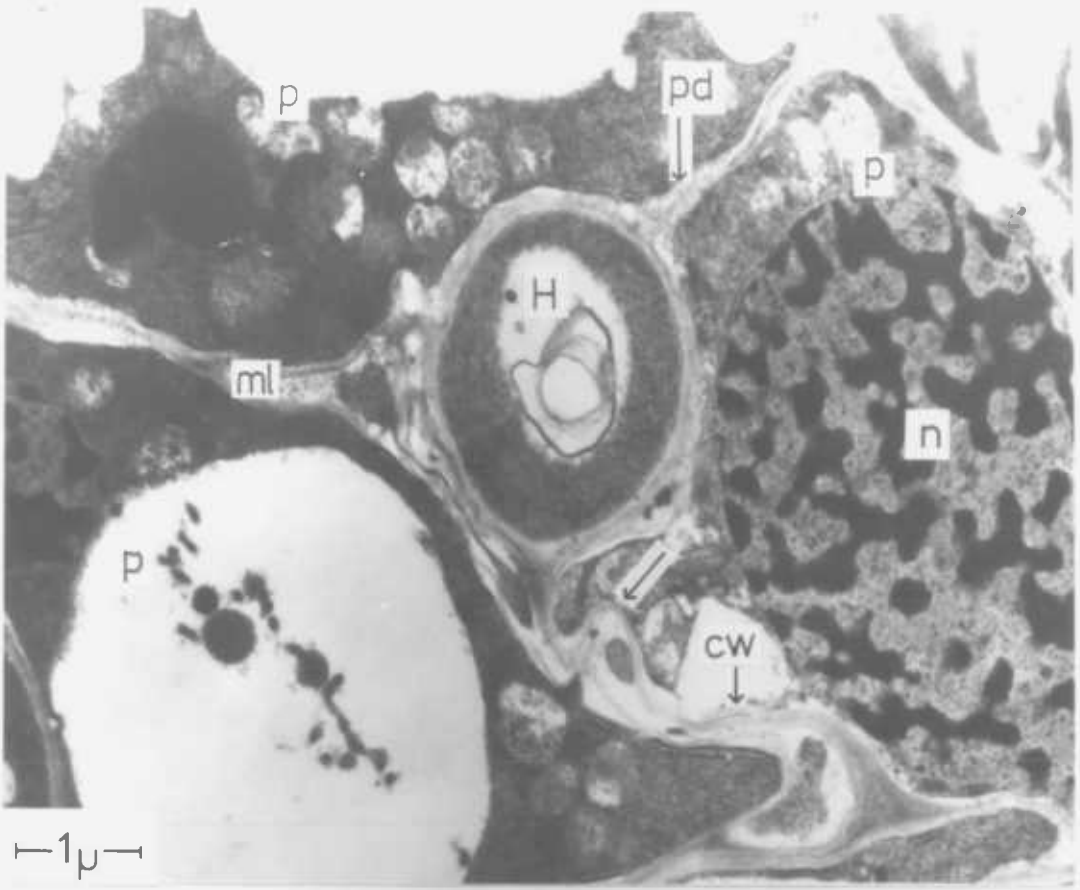


d

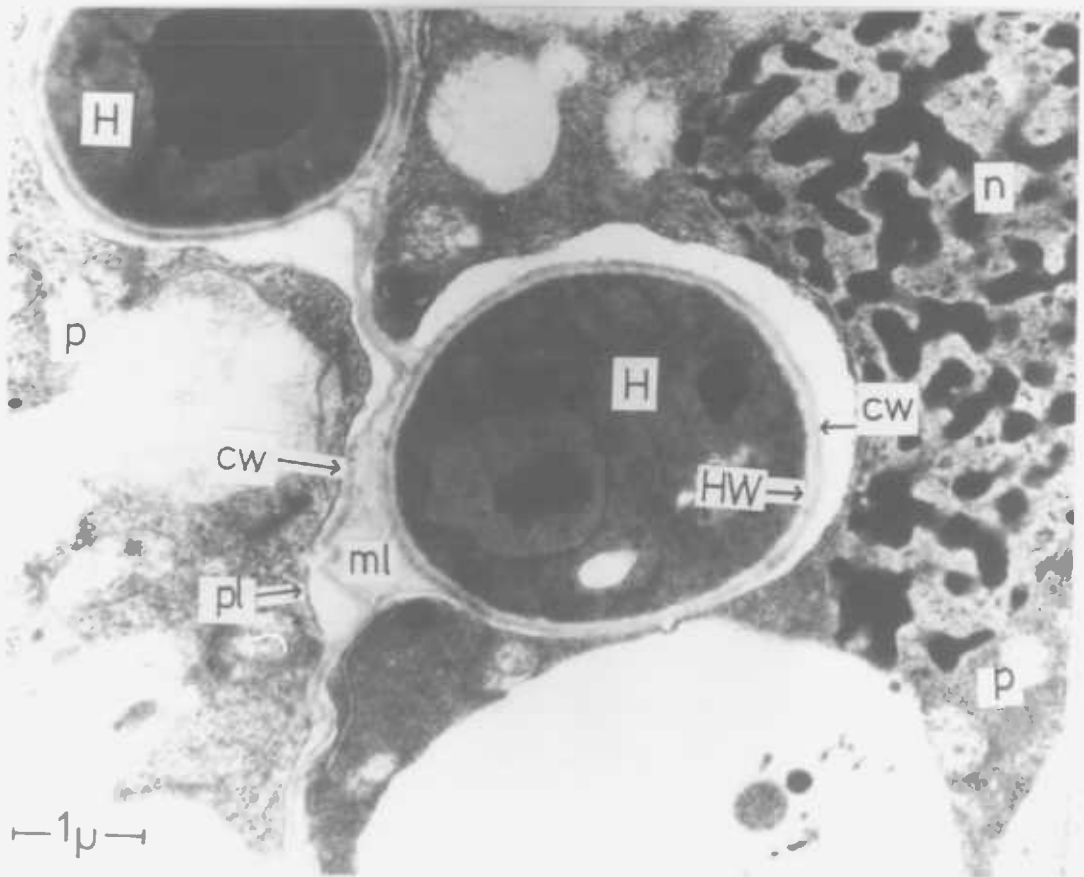
PLATE 15. COLONIZATION OF THE OVARY WALL (T.E.M.)

(5 days after inoculation).

- (a) Intercellular hyphae between 3 host cells in the ovary wall. The middle lamella has been degraded and the host cell walls softened, buckled (arrow) and eroded to a thin structure. Plasmodesmata are still visible between neighbouring cells.
- (b) The thin weak walls of the host cells are easily invaginated by the hyphae and cell disruption has begun. In the cell on the left, the plasmalemma is incomplete, has lost contact with the cell wall and the protoplast is degenerating.



a 1 μm



b 1 μm

PLATE 16. COLONIZATION OF THE OVARY WALL (T.E.M.)

(5 days after inoculation).

- (a) Invagination of the host cell wall by the invading hyphae. The cell wall has been weakened and partially degraded to a thin structure, and its layers appear to be separating. The host plasmalemma appears to be broken and the degenerating protoplast is connected to the cell wall by thin fibrils. The middle lamella has been eroded.

- (b) Detail of the eroded host cell wall bearing loose fibrils on its inner surface.

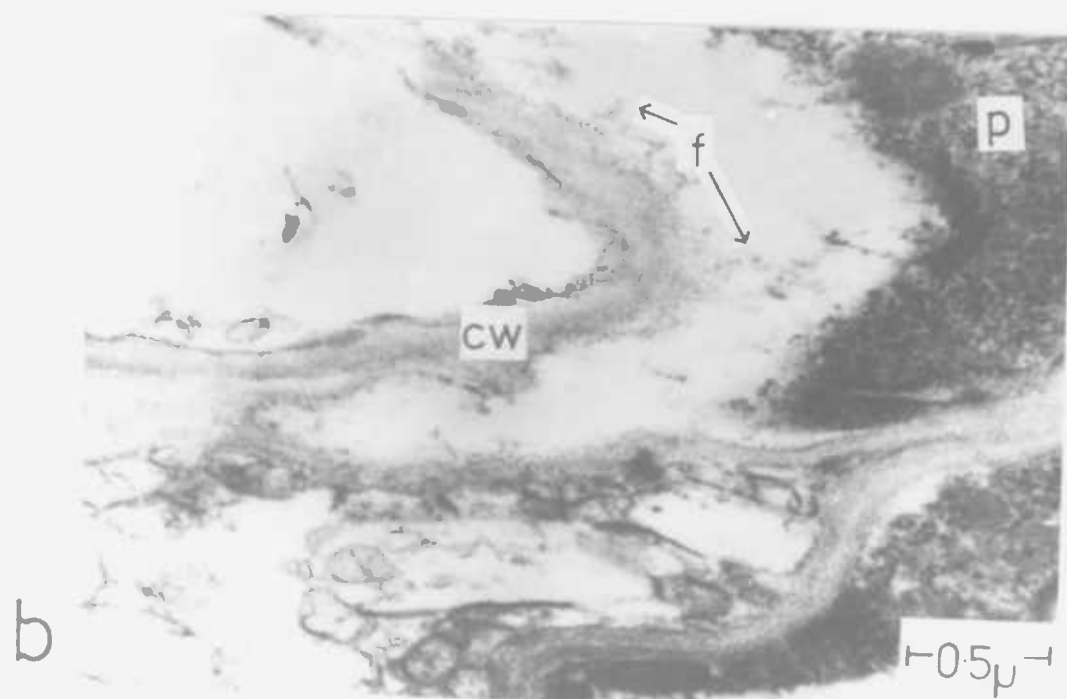
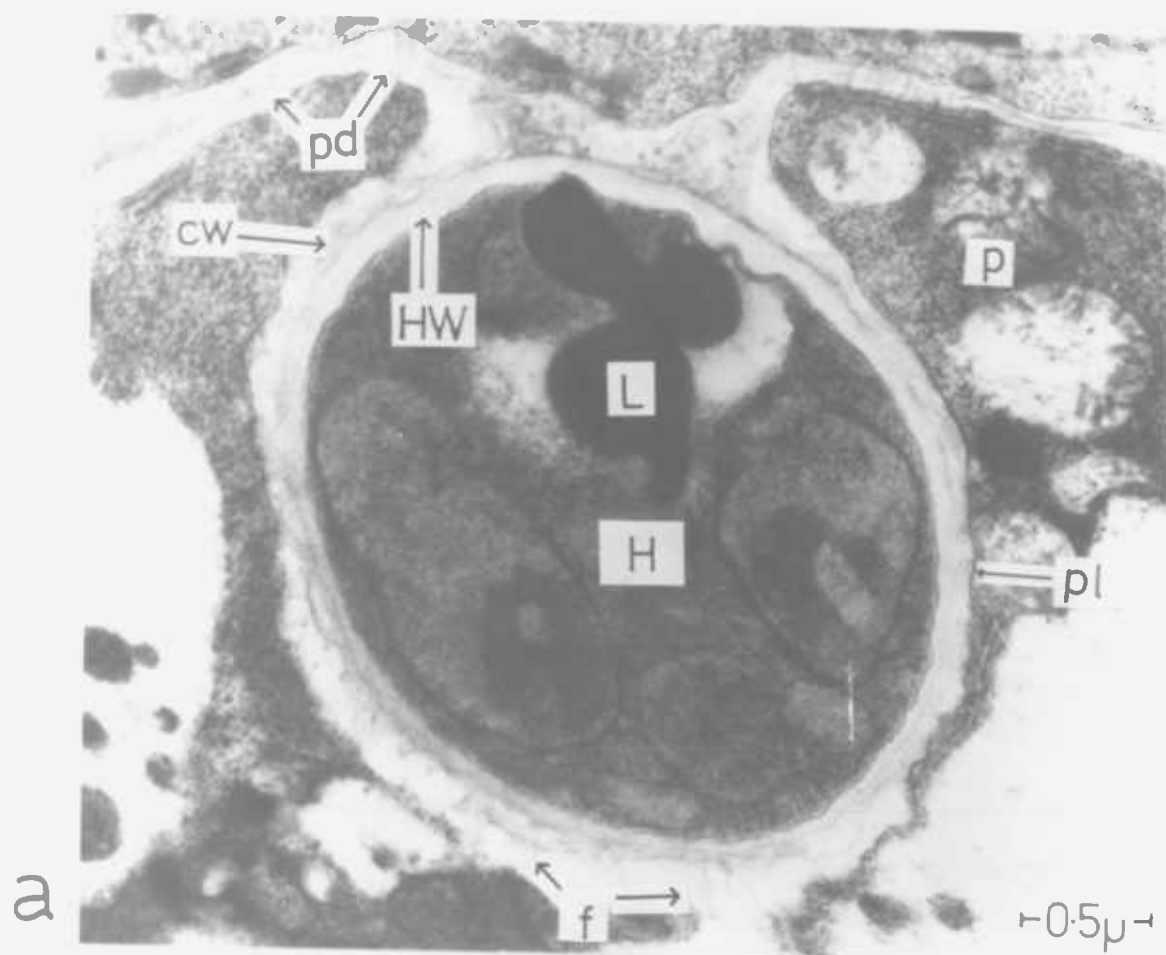
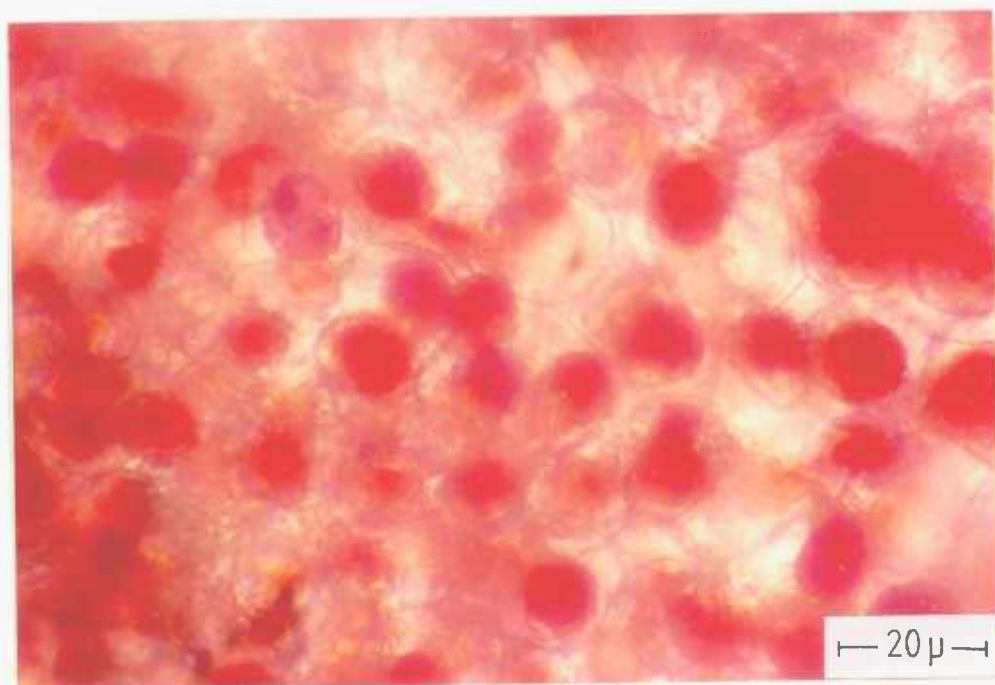


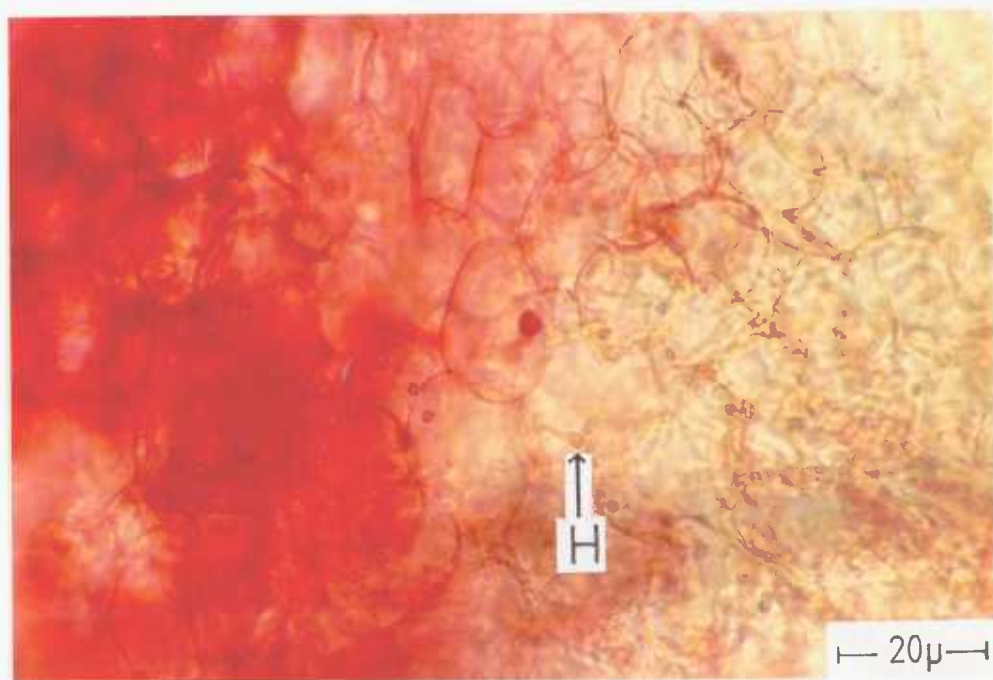
PLATE 17. NEUTRAL RED STAINING OF HEALTHY AND INFECTED TISSUE.

(a) Healthy, uninfected wheat ovary tissue, 5 days after anthesis stained with neutral red in a plasmolysing solution. The stain is retained within the vacuoles of the cells.

(b) Infected wheat ovary tissue, 5 days after inoculation, stained with neutral red in a plasmolysing solution. The stain is retained only by the uncolonized tissue on the left. The colonized tissue on the right, in which fungal hyphae are clearly visible between the cells, does not retain the stain, indicating that they are dead.



a

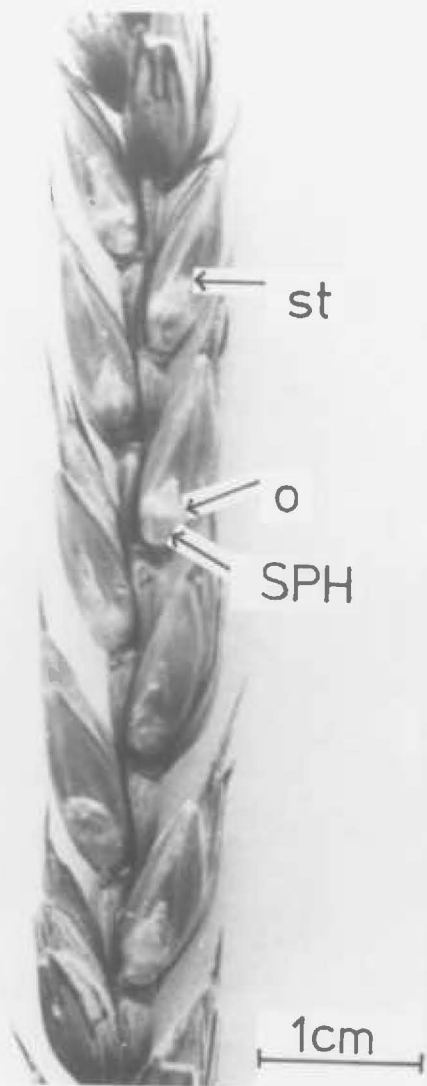


b

PLATE 18. MACROSCOPIC STAGES OF EHGOT DEVELOPMENT II.

- (a) Wheat ear six days after inoculation with glumes and lemma dissected away to reveal infected ovaries. Apex of ovaries appear intact but lower parts have been colonized and are developing into the sphacelial stage of the infection.
- (b) Wheat ear nine days after inoculation. Dissected parts show young sphacelium with the remains of the upper ovary and stigmas on its apex. Parts of the anthers still remain at this stage and are sometimes incorporated into the apex of the structure, along with the stigmas. The sphacelium is white and has a soft consistency. (The honeydew has been removed so that the view of the sphacelium is not obscured.)

a



b

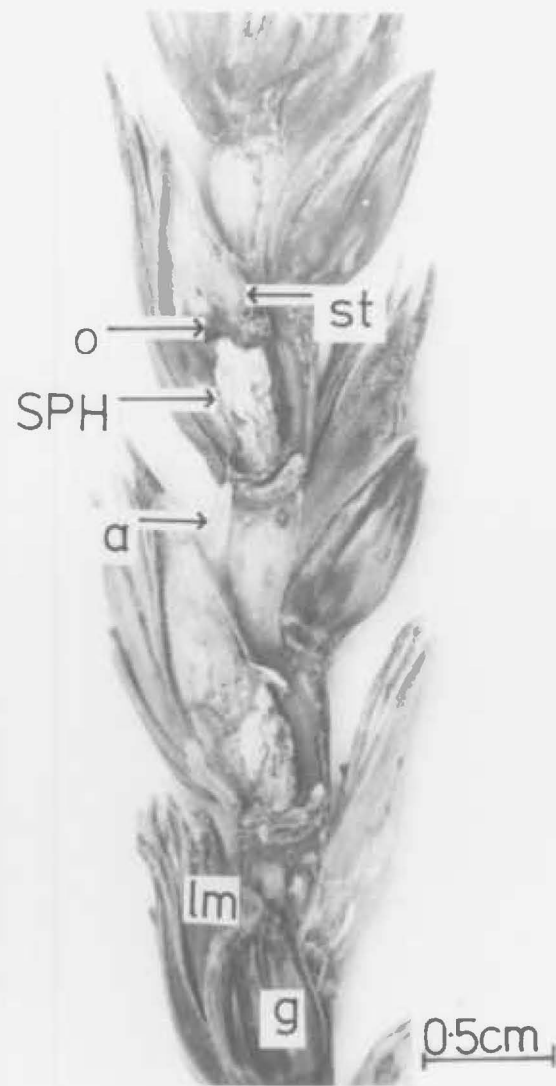


PLATE 19. EARLY SPHACELIAL STAGE.

(7 days after inoculation).

(a) Longitudinal section through sphacelium showing the replacement of the ovary by fungal mycelium of the sphacelial type. Conidia are produced on the external surface and also in internal pockets. Fungal penetration does not extend into the rachilla, where vascular tissue remains intact, but is restricted to tissues at the ovary base. The rectangle indicates area of (b).

(b) Enlarged view of part of (a) showing host-parasite interface in relation to xylem vessel. Host cells below the interface appear healthy and the border between the host and the parasite is quite distinct.

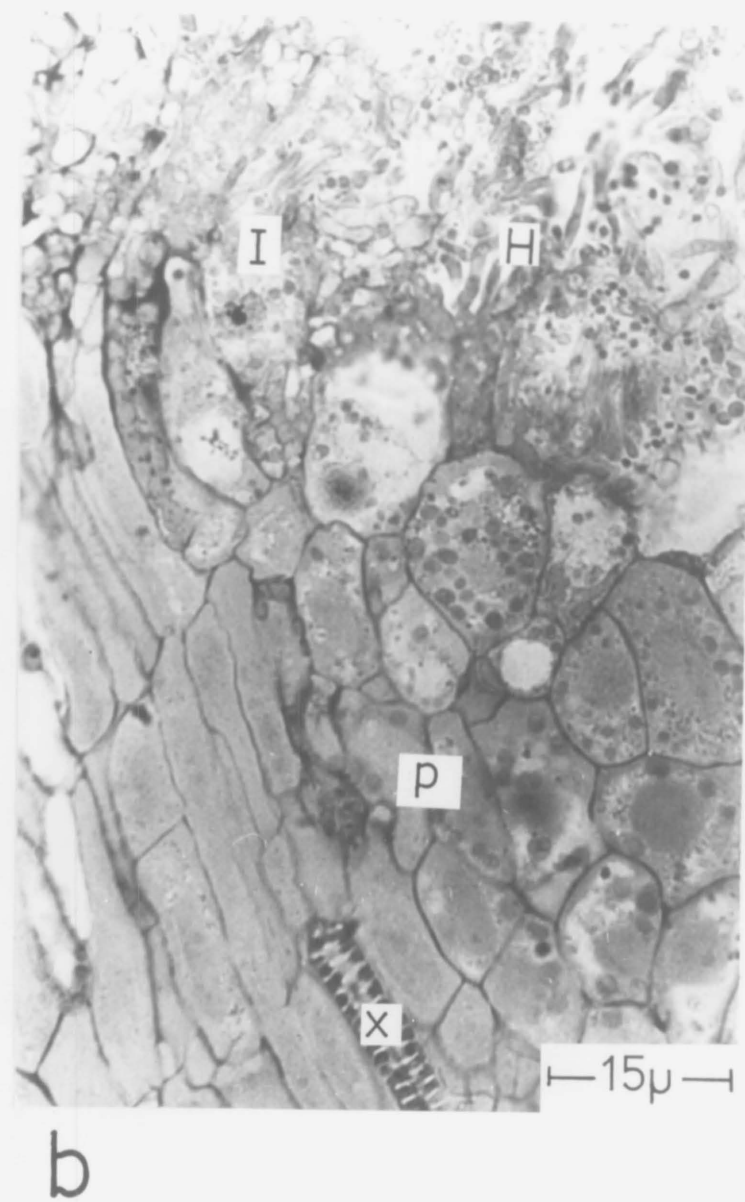
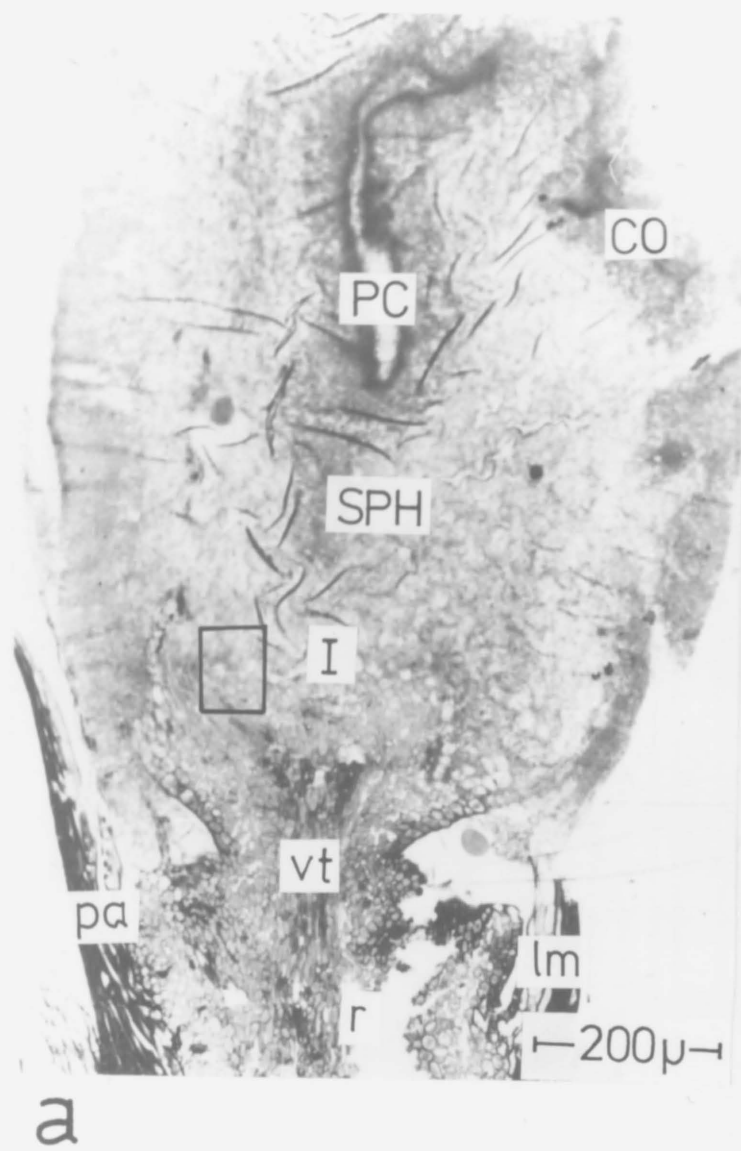


PLATE 20. MACROSCOPIC STAGES OF ERGOT DEVELOPMENT III.

Honeydew exudation from infected wheat ear. Honeydew exudes from the puncture wounds produced by the inoculating needle in the glumes as well as from the top of the florets.. Its opacity is due to thousands of conidia and its viscosity varies depending on moisture content. Its chief constituents are sugars.



PLATE 21. THE SPHACELIUM.

(10 days after inoculation).

The ovary has been completely replaced by the parasite. A few host cells remain at the apex of the sphacelium and can be recognised, by the hairs, as the upper part of the ovary. Conidia are produced externally and internally. Fungal penetration does not extend into the rachilla but appears to be restricted by a 'plate' of cells.

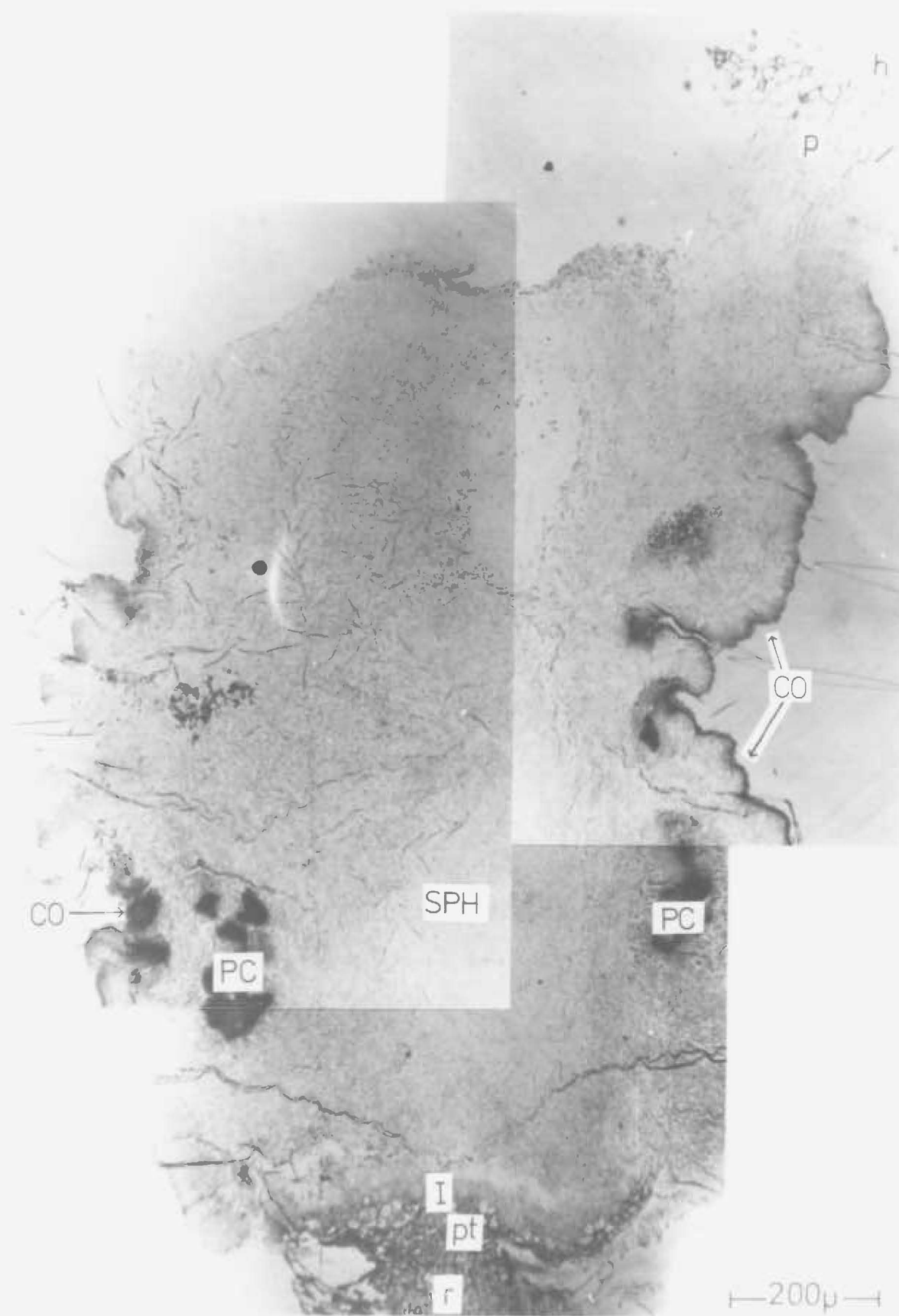


PLATE 22. THE HOST-PARASITE INTERFACE.

(10 days after inoculation).

- (a) Interface between host and parasite at the 'plate' of cells above the rachilla. At the interface, the cells are disrupted and cell walls remain in situ, forming 'cups' which become filled with hyphae. The 'core of thick-walled cells' is not visible here since it is not a median section. The core normally occurs below the 'plate' of cells in the rachilla.
- (b) Enlarged view of part of host-parasite interface showing hyphal penetration into upper layer of 'plate' cells, whose cell walls remain in situ and appear to contain hyphae. Hyphal penetration does not extend below this upper 1-3 cell thick layer of cells into the rachilla.

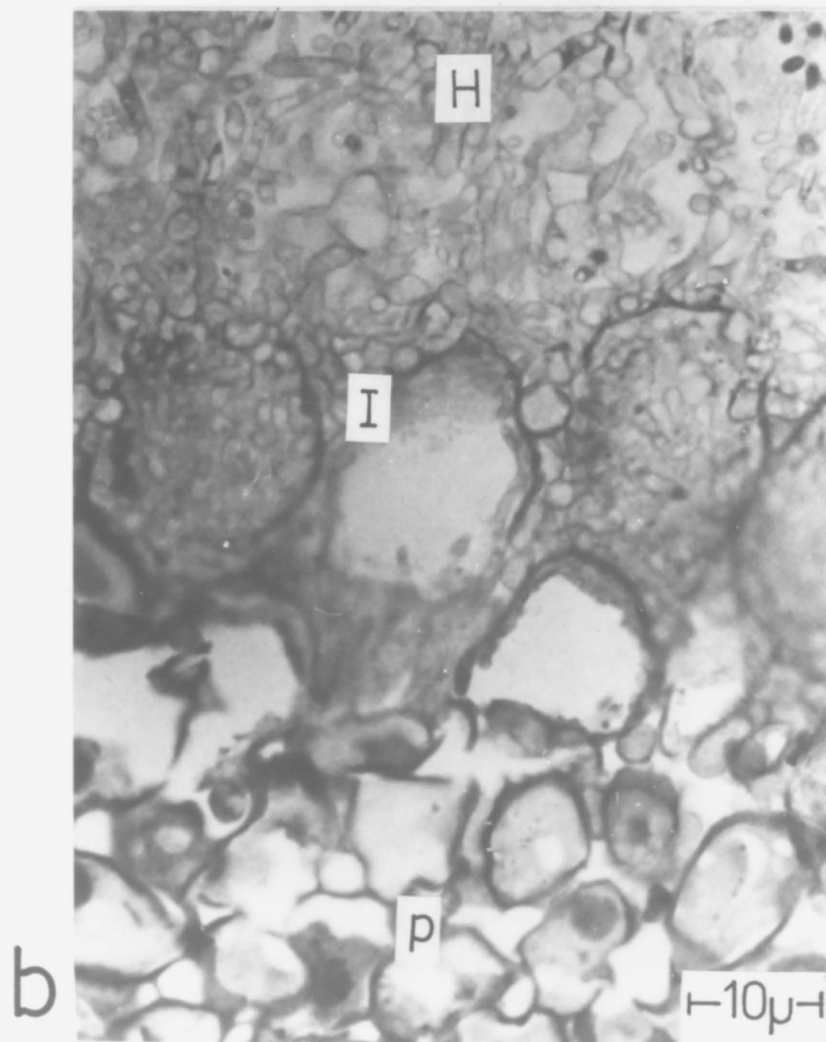
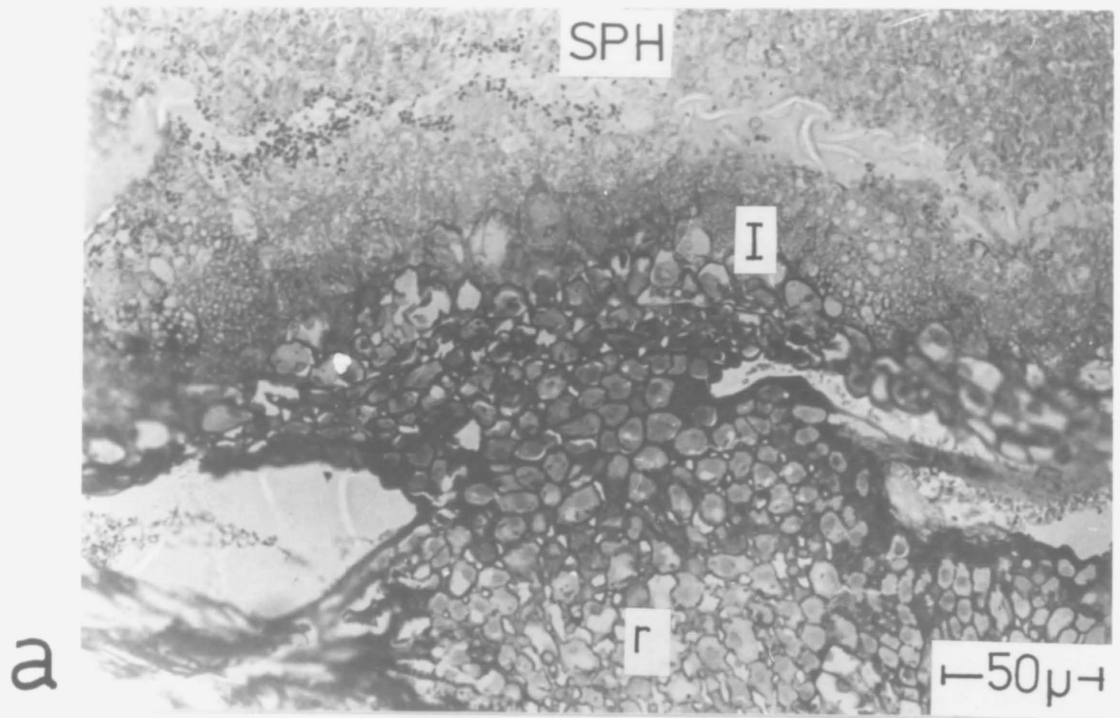


PLATE 23. HYPHAE NEAR THE BASE OF THE SPHACELIUM (T.E.M.)
(10 days after inoculation).

(a) Long sphacelial hypha with infrequent septa and occasional branches.
The liquid droplets tend to coalesce and bulge into the large
vacuoles. The rectangle shows the area in (b).

(b) Enlarged view of septum whose pore is occluded by electron dense
material which is either plugging it or passing through it into
a less dense portion of the hyphal branch.

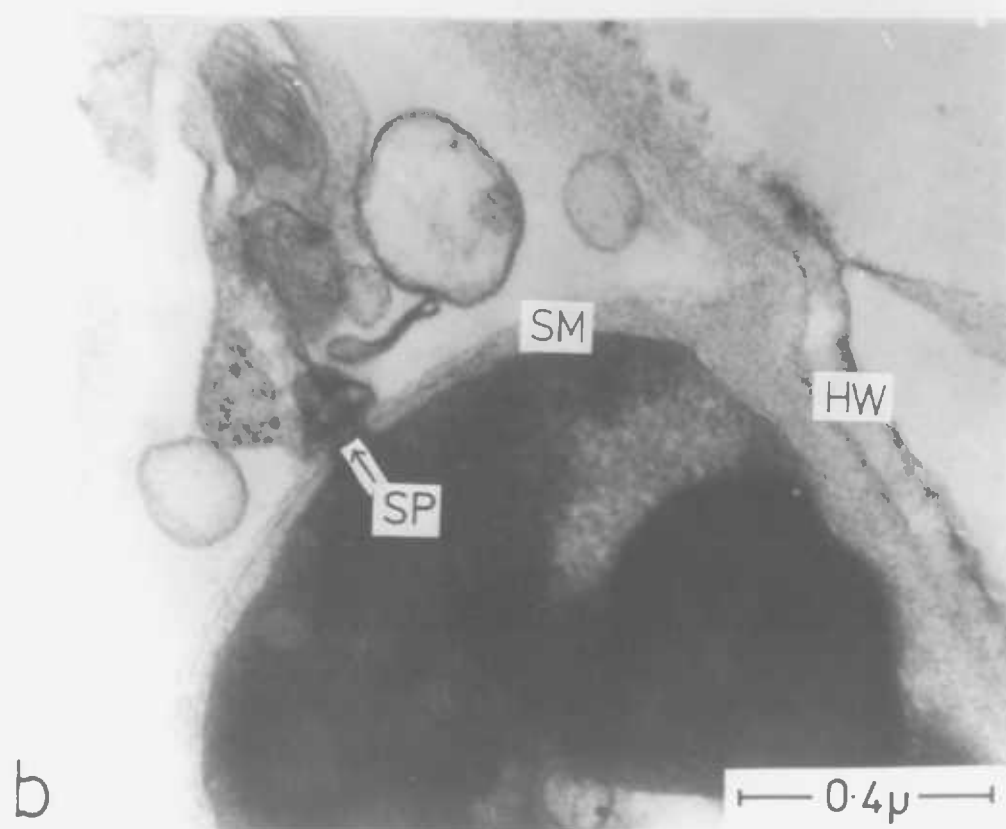
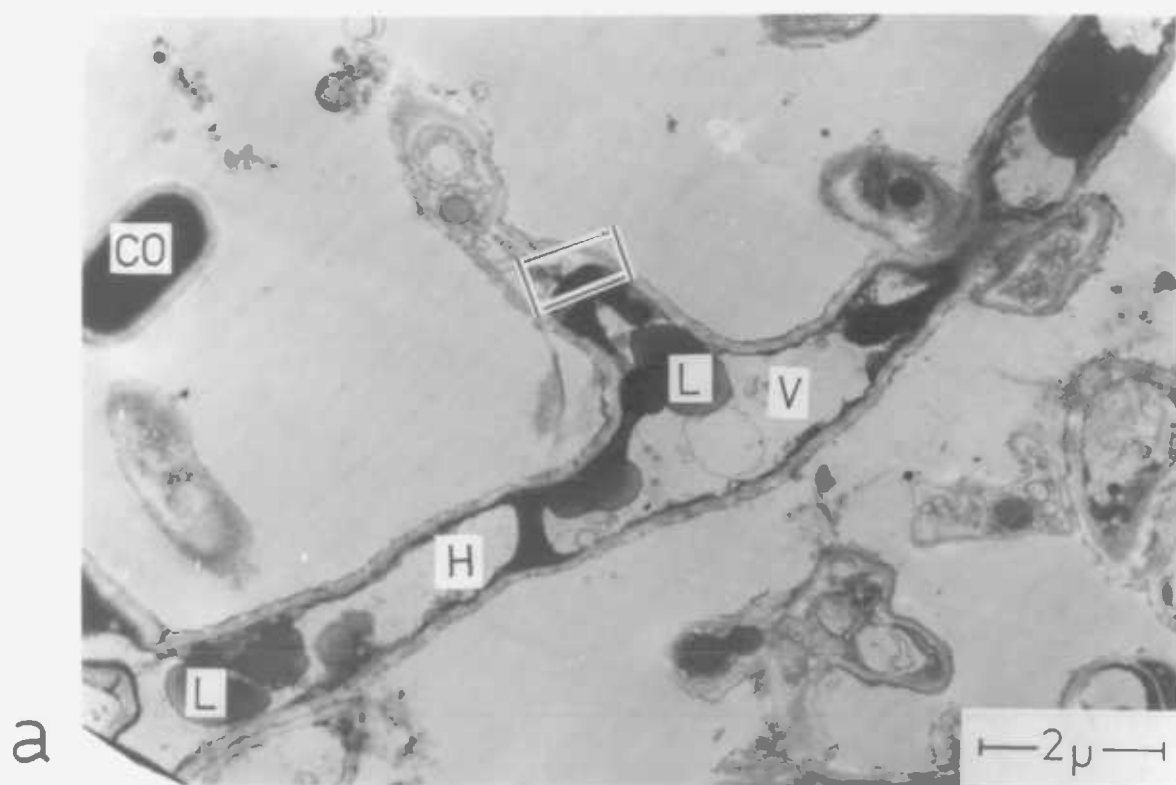
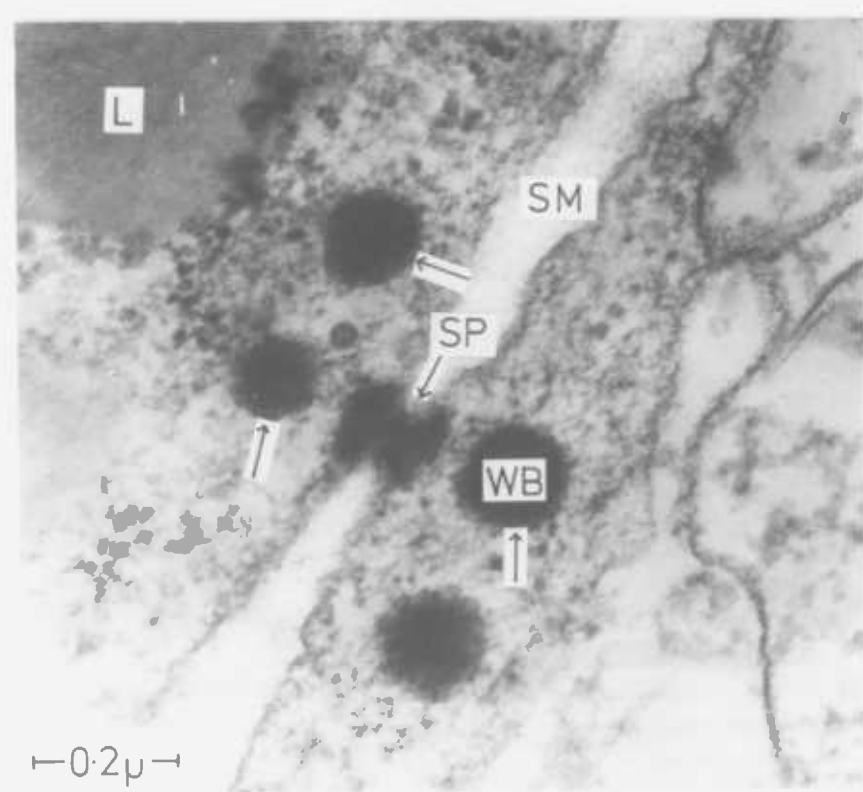


PLATE 24. WORONIN BODIES (T.E.M.)
(10 days after inoculation).

- (a) Hypha surrounded by a fine tangle of fibrils. Six Woronin Bodies are present on either side of the septum. The septum is separating a terminal cell with its dense cytoplasm from the less dense penultimate cell.
- (b) Enlarged view of a septum showing a Woronin Body within the septal pore. It may be plugging the pore, passing through it or about to degenerate. A membrane is just visible around the other Woronin Bodies (arrows).



a



b

PLATE 25. HYPHAE NEAR THE BASE OF THE SPHACELIUM (T.E.M.)

(10 days after inoculation).

- (a) The size and shape of the hyphae vary but generally they are
 &
(b) shorter, more frequently septate and more ovoid in shape than the
 sphacelial-type hypha in Plate 23. The numerous lipid droplets
 invaginate the vacuoles, and this type of morphology may indicate
 the beginnings of sclerotial cell development.

The surface of the hyphae is coated with a thin meniscus of unidentified material, which connects neighbouring hyphae. Between the coating meniscus and the hyphal wall sometimes another layer of material (possibly the same material as the meniscus) appears to be interposed (arrow). Woronin Bodies commonly occur at the septa.

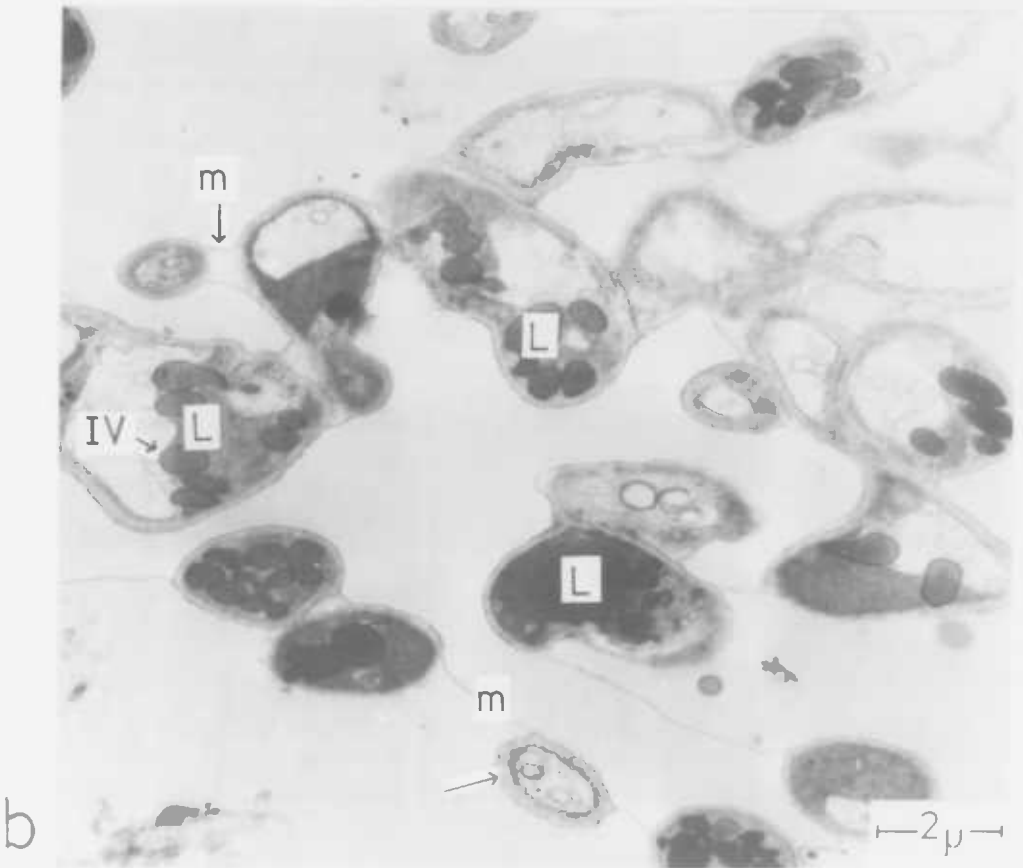
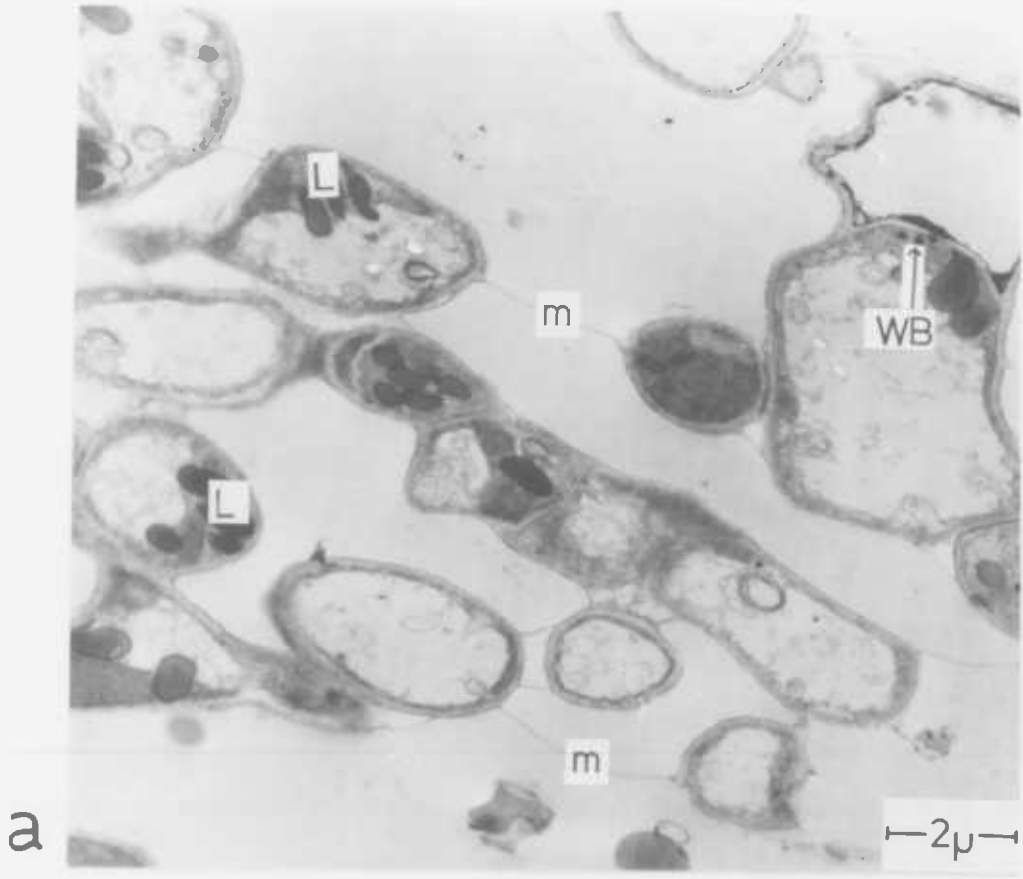


PLATE 26. BEGINNINGS OF SCLEROTIAL CELL DEVELOPMENT.

(12 days after inoculation).

Longitudinal section through sphacelium in which sclerotial cell development has begun, possibly via a transition type of cell.

The sclerotial cells stain more darkly than the sphacelial cells.

(on the left of the section) The area in the rectangle is enlarged in Plate 28(b) and shows the sclerotial cells more clearly.

Conidiation continues both internally and externally, and there is no penetration of fungal hyphae beyond the 'plate' of cells into the rachilla and the 'core of thick-walled cells'.



PLATE 27. MACROSCOPIC STAGES OF ERGOT DEVELOPMENT IV.

- (a) Wheat ear 12 days after inoculation. The sphacelia are bathed in the exuding honeydew. Small areas of dark pigmentation are just visible in the white sphacelia, and these correspond to early sclerotial cell development which are normally a purple colour.
- (b) Young sclerotium, 16 days after inoculation, developing in rye with dark sclerotial tissue at base and white sphacelial cap on top. The sphacelial cap comprises the early sphacelial mycelium as well as the remains of the ovary and stigmas.

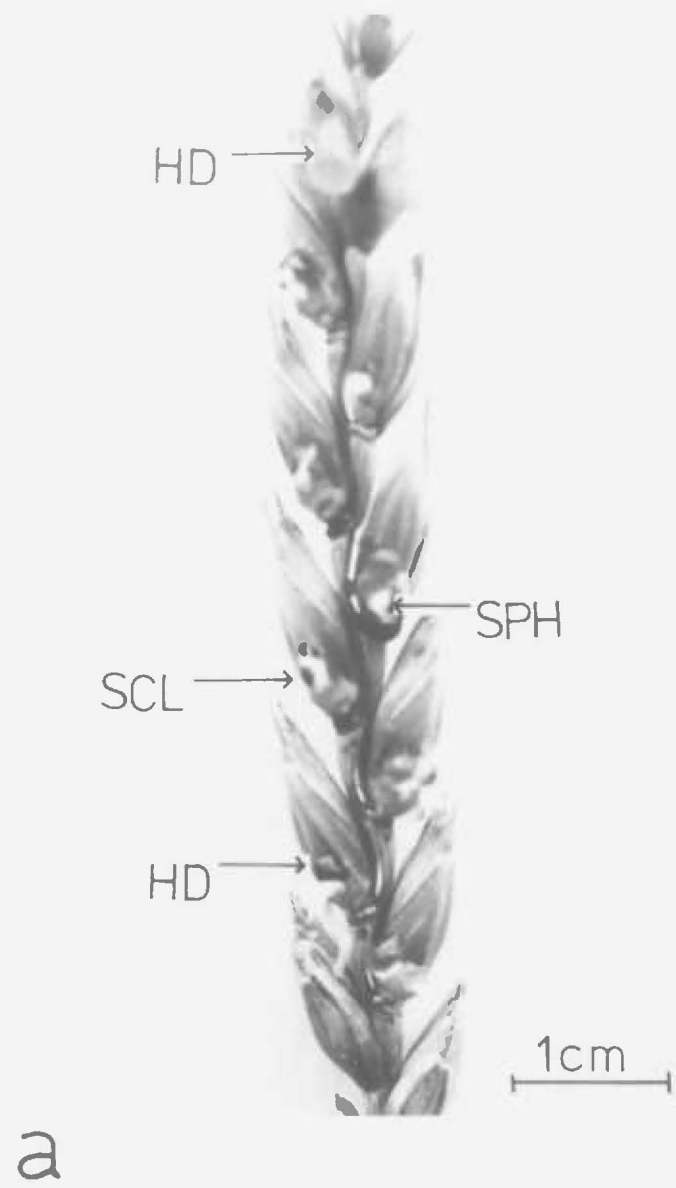


PLATE 28. BEGINNINGS OF SCLEROTIAL CELL DEVELOPMENT.

(12 days after inoculation).

- (a) Host-parasite interface showing that hyphal morphology is still sphacelial at this location. The 'plate' of cells is intact and uninvaded except for its upper 1-2 layers, thus the rachilla remains healthy.

- (b) Enlarged view of part of Plate 26 showing sclerotial cell development possibly via a transition type. Sclerotial cells stain more darkly than sphacelial cells and are shorter, more bulbous and frequently septate. In this area the first purple pigmentation, typical of sclerotial cells, was observed.

- (c) Detail of host-parasite interface showing the broken cell-walls of the upper layer of the 'plate'. Hyphae surround and invade the remnants of these cell walls and often fill them. They penetrate to the second or third layers of the 'plate' but grow no further into the rachilla.

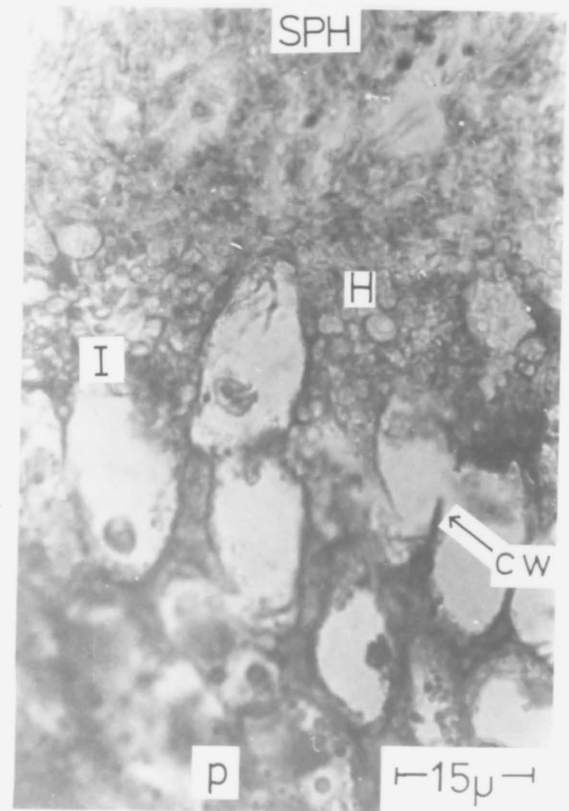
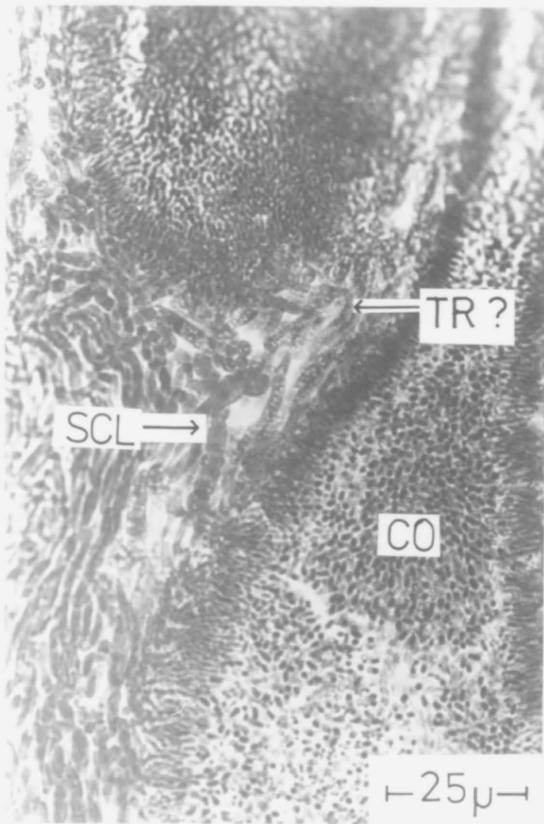
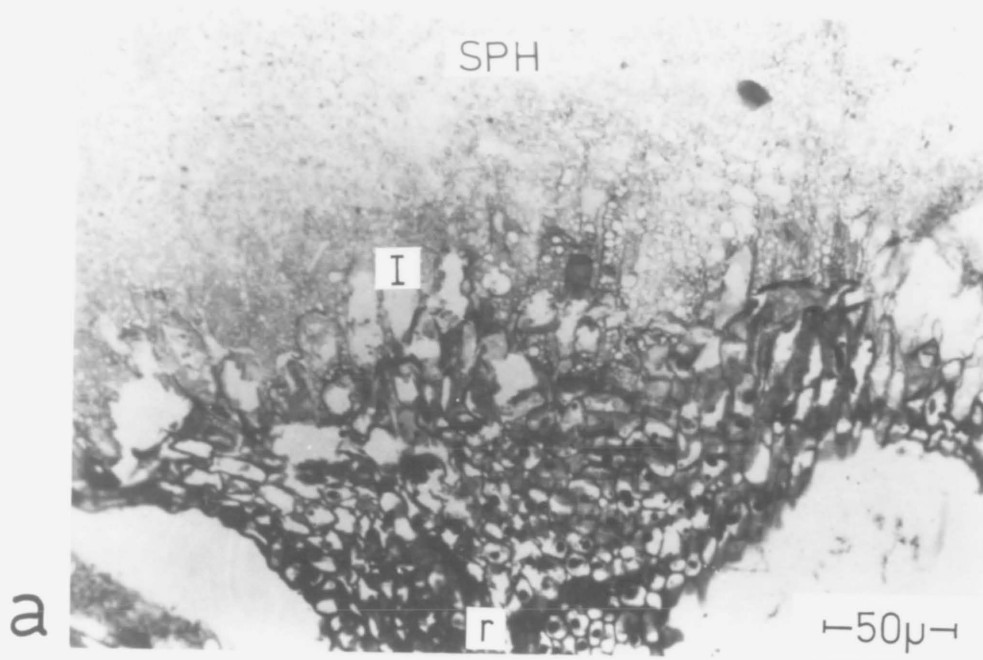


PLATE 29. LIPID CONTENT OF HYPHAE AT THE BASE OF THE YOUNG SCLEROTIUM.
(15 days after inoculation).

- (a) Fungal tissue at the base of the young sclerotium stained for
&
(b) lipid with Sudan IV and toluidine blue.

The lowermost hyphae (at the interface and just above) are devoid of lipid, have large apparently empty vacuoles, and consequently appear less bulbous than those further up in the sclerotium. They are the youngest hyphae and grow rapidly to be displaced acropetally, and develop lipid reserves within a few days. They appear to be randomly organised, unlike the lipid-containing hyphae above, which have a predominantly longitudinal close-packed arrangement. These lipid-containing hyphae are frequently septate and commonly are unbranched. The two types of hyphae are fairly sharply delimited and the junction between them possibly forms the natural breaking point of the sclerotium.

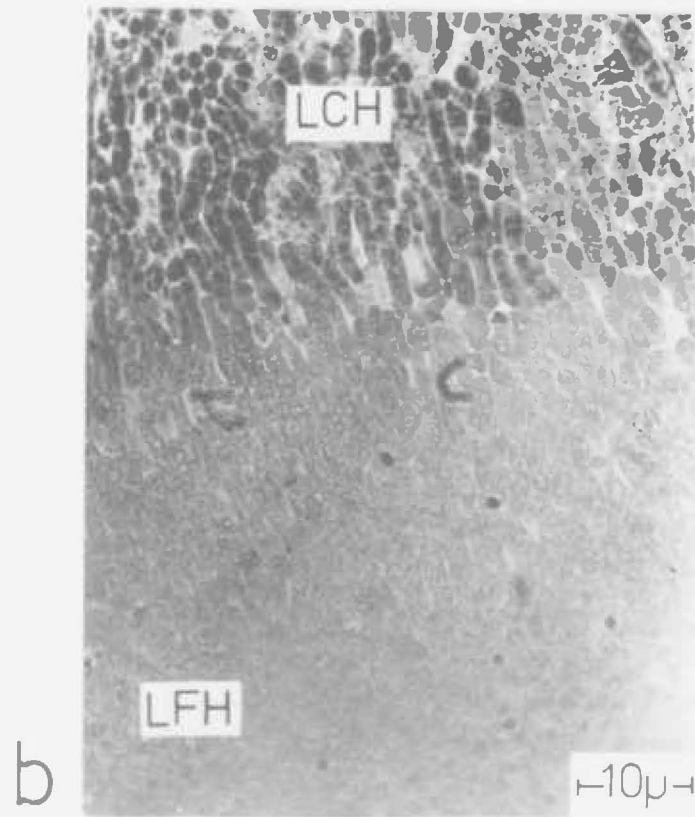
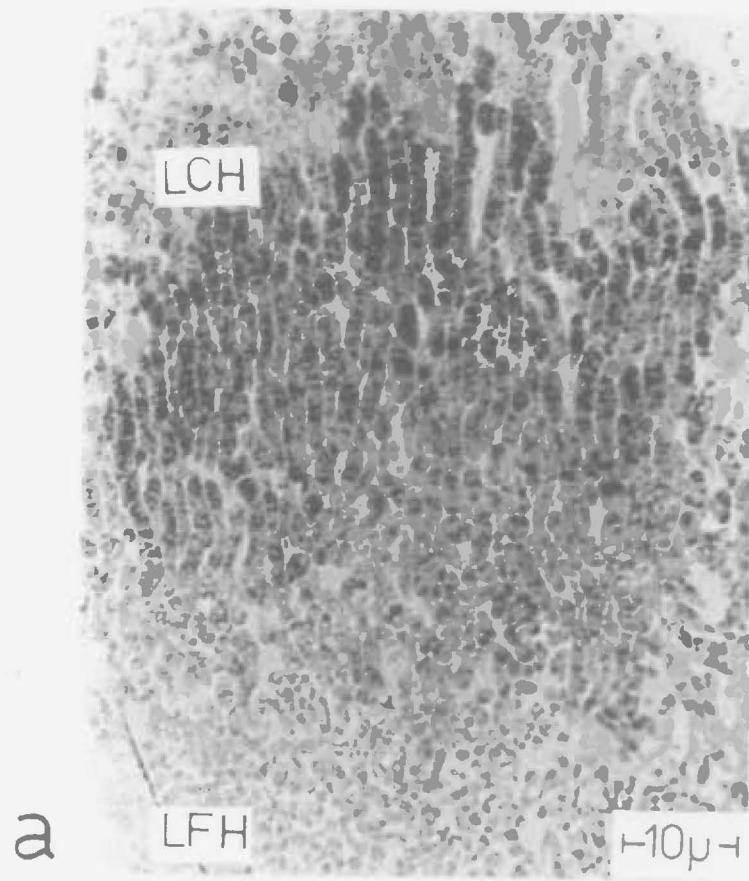
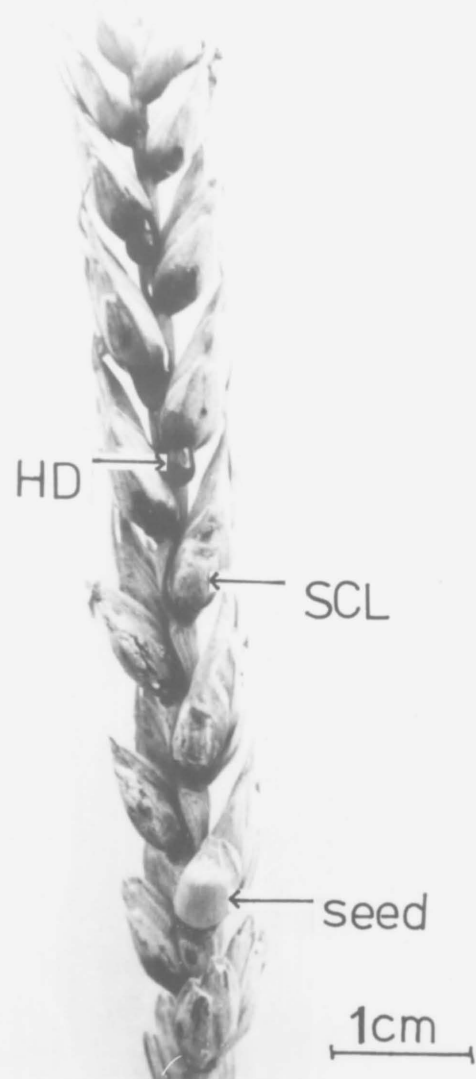
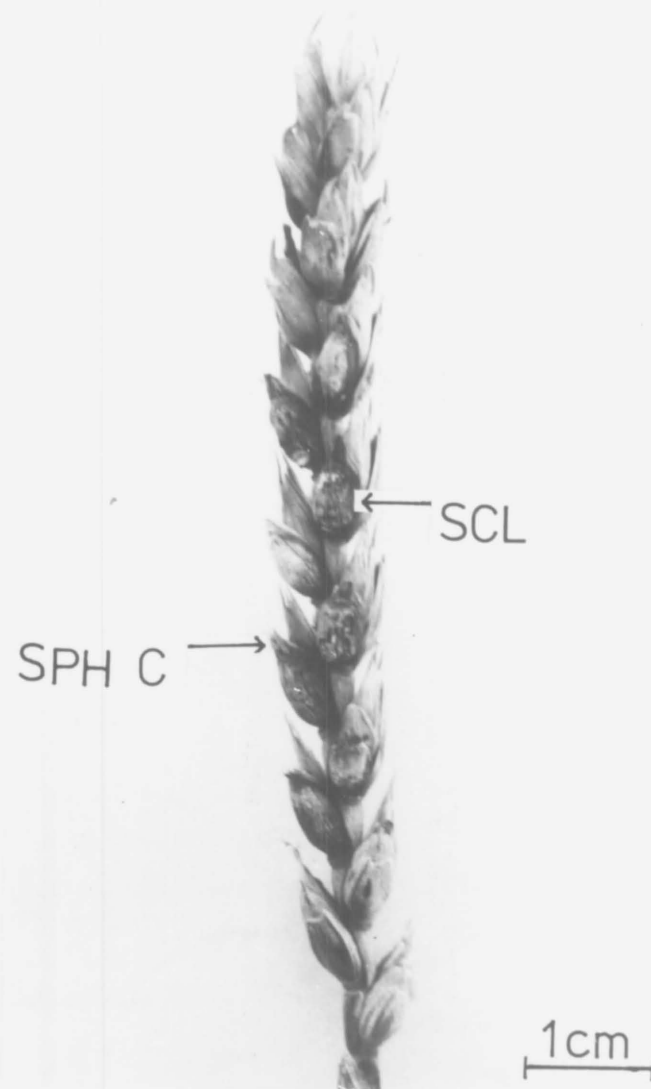


PLATE 30. MACROSCOPIC STAGES OF ERGOT DEVELOPMENT V.

- (a) Wheat ear, 18 days after inoculation, with glumes and lemmas dissected away to reveal 4 young sclerotia. Near the base of the ear is a developing seed, whose size is similar to that of the young sclerotia. Honeydew exudation is still in evidence.
- (b) Wheat ear 21 days after inoculation. The ergot sclerotia continue to grow, and still bear the remains of the sphacelial cap on their apices. Honeydew exudation has now subsided.



a



b

PLATE 31. MATURING ERGOTS.

- (a) Longitudinal section through a 21-day old ergot stained with toluidine blue. The medulla tissue of the sclerotium stains purple while the cortex remains brown. The host tissue at the base can be easily distinguished from fungal tissue because of the polychromatic nature of the staining, and is stained various shades of blue.
- (b) Wheat ear showing 4 purple 25-day old ergot sclerotia (the glumes have been dissected away). The shrivelled sphacelial caps are visible on the apices of some of the sclerotia. The shape of the maturing ergot conforms to that dictated by the glumes, and is similar to that of normal wheat seeds.



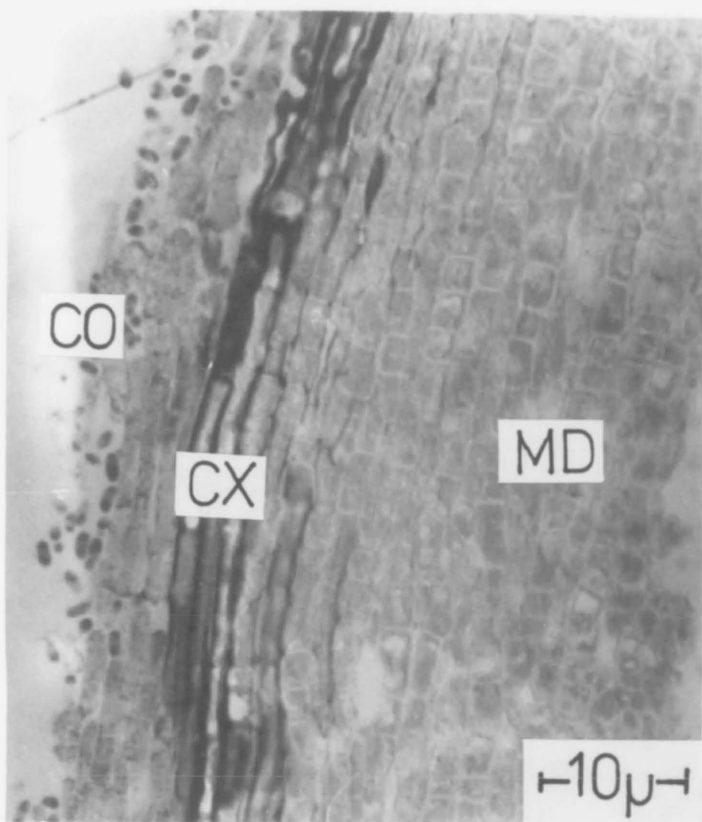
PLATE 32. SCLEROTIAL TISSUE.

(21 days after inoculation).

(a) Part of the cortex and medulla of the ergot sclerotium. Conidia occur on the external surface beneath which are found the brown pigmented hyphae of the cortex. These appear crushed and longitudinally orientated. The inner medulla tissue shown here consists of compact sclerotial hyphae arranged in a plectenchyma, although variation in hyphal types is common (see b below).

(b) Different types of sclerotial cells within the ergot. There is considerable variation in the width, length, lipid content and staining properties of the hyphae.

a



b

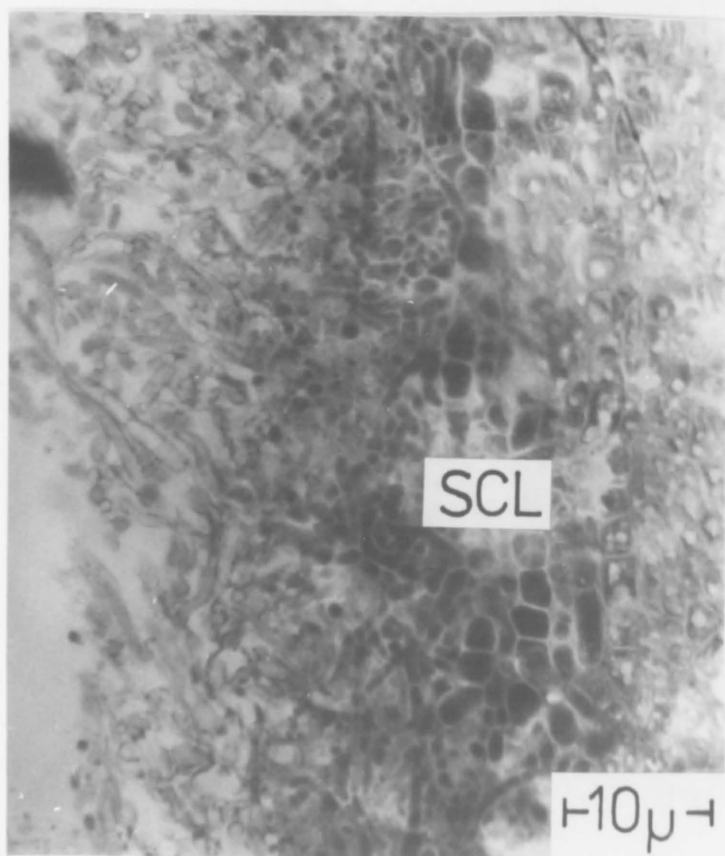


PLATE 33. SCLEROTIAL TISSUE (T.E.M.)
(21 days after inoculation).

Short, dense, septate hypha of the sclerotial type containing large lipid drops and few vacuoles. Its surface is surrounded by fibrillar material, and Woronin Bodies are associated with the septum.

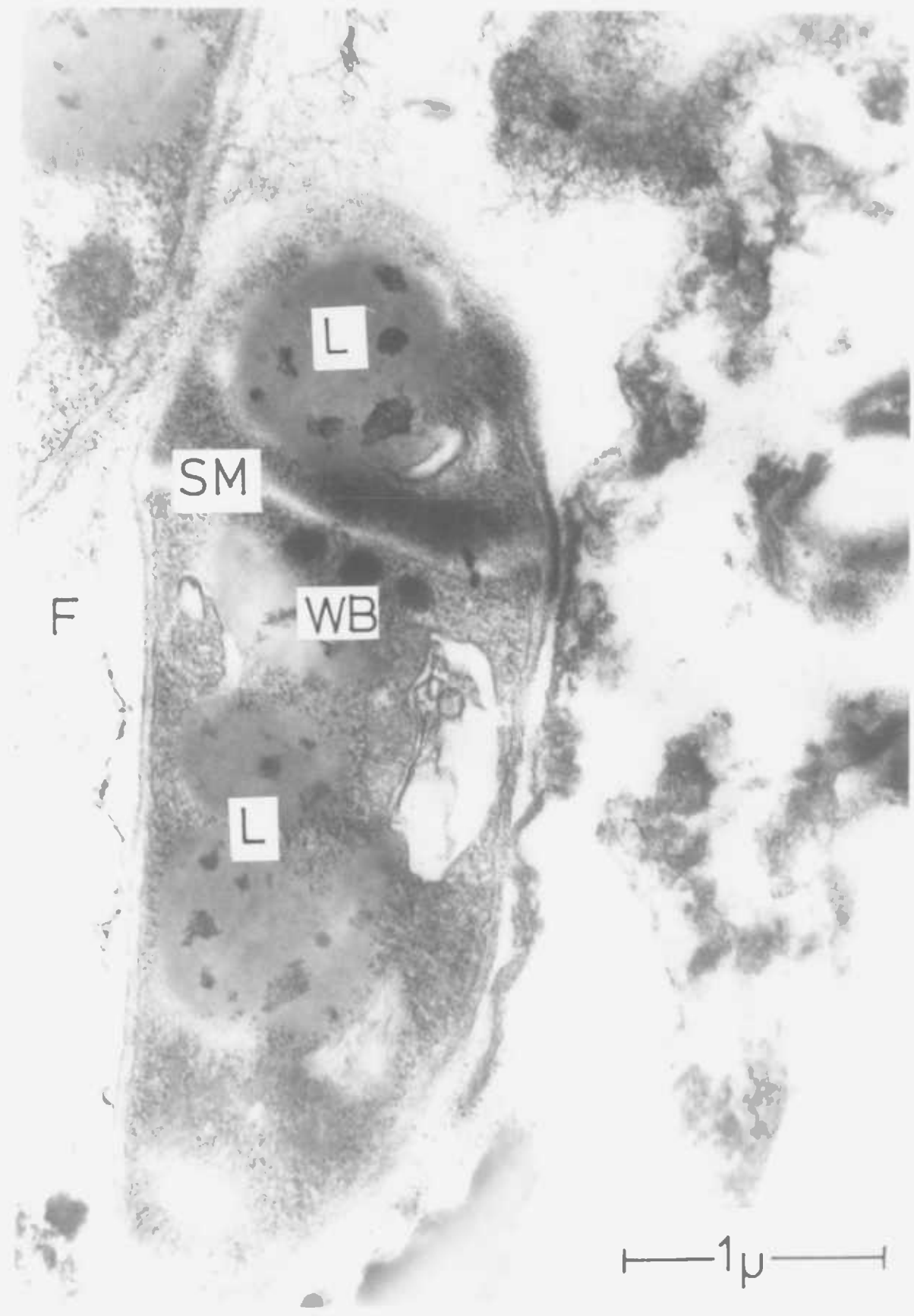


PLATE 34.

MACROSCOPIC STAGES OF ERGOT DEVELOPMENT VI.



Mature ergots in artificially infected rye.

3. 2. PECTOLYTIC ENZYMES IN PATHOGENESIS

a) Preliminary investigation of growth of C.purpurea on pectic and cellulose substrates 'in vitro', and pectolytic and cellulose enzyme production

Generally growth on the agar plates containing pectic or cellulose as substrates was slower from spore inoculations than from mycelial discs. This is to be expected since there would have been some glucose carried over in the inoculum. Growth on the three different substrates varied:- it was best on pectin, less good on sodium polypectate and poor on sodium carboxymethyl-cellulose (CMC), in both spore and mycelial disc inoculations (Table 3). When flooded with the various developers, definite opaque zones occurred around colonies grown on pectin, and indicated the activity of pectinesterase (PE) and polymethylgalacturonate lyase (PMGL). Very faint zones were obtained on sodium polypectate, showing some polygalacturonase (PG) activity, and no zones occurred on the CMC plates suggesting little cellulase activity. No attempt was made to relate the size of the zone with enzyme activity.

Table 3. Growth of C.purpurea on pectic and cellulose substrates 'in vitro'

<u>Substrate</u>	<u>Hyphal advance over 10 days (mm)</u>
Pectin (PE)*	5.2
Pectin (PMGL)	5.0
Sodium polypectate (PG)	2.2
Sodium carboxy methylcellulose (cellulase, Cx)	0.5

* (enzymes being assayed are shown in brackets)

These preliminary results demonstrate the ability of C.purpurea to utilise pectic substrates and produce pectolytic enzymes 'in vitro'. Therefore experiments were carried out to further study such enzyme production 'in vitro' and also 'in vivo'. Since growth was sparse and slow on sodium carboxymethylcellulose, cellulase activity must have been very weak and therefore this enzyme was not investigated further.

b) Growth of C.purpurea on pectic substrates 'in vitro' (liquid culture)

i) On pectin

When the fungus was grown on a series of media of increasing pectin concentration from 0.5 to 5%, growth improved with increasing pectin concentration. On 0.5% and 1% pectin, growth was poor and the mycelium was particulate while on 5% pectin, the mycelium tended to form clumps. When 0.5% sucrose was added to a similar range of pectin concentrations, best growth occurred on 0.5% and 1% pectin and the mycelium formed thick off-white clumps. Growth on 2.5% and 5% pectin was slightly more sparse than on 0.5% and 1% pectin, and the mycelium was dispersed as particles throughout the medium.

Microscopically the hyphae were long, thin and occasionally septate, i.e. of the sphacelial type.

When the culture filtrates were tested for residual pectin, those at the 2.5% and 5% pectin level, both with and without sucrose, still contained a considerable amount of pectin. However, at the 0.5% and 1% level, those with added sucrose contained very little residual pectin while those without sucrose contained markedly more. Since growth was better at these two levels when

sucrose was included in the medium, it may seem that this was due only to utilization of the sucrose. However, the residual pectin tests indicate clearly that pectin was utilized by C.purpurea, although the extent of pectin hydrolysis was not determined.

ii) On polygalacturonic acid

On this medium at pH 5.2 and pH 7.5, growth after 5 days was poor. On addition of 0.25% glucose, growth markedly improved, and after 5 days of growth on this medium, the mycelium had formed into a clump. Growth was better at pH 5.2 than pH 7.5. In flasks where no glucose was added, growth after 10 days was still poor.

An indication of growth on this medium is given in Table 4 where mycelial dry weights from cultures grown on different carbon sources are compared.

When no carbon source was supplied i.e. only the 4 salts were present and pH was adjusted to 5.5, virtually no growth (Table 4) occurred, as was expected. When asparagine (0.5%) was included, growth increased slightly, but was still minimal and yielded only 0.0033 g/100ml culture. On inclusion of pectin (3.5%) to this medium, the dry weight increased to 0.1082 g/100ml. indicating that the fungus had utilized this carbon source for growth. The fungus grew better on pectin than on polygalacturonic acid, and of the pectic media tested, growth on pectin (3.5%) + glucose (0.25%) was best. The mycelial dry weight yielded by this mixture of pectin + glucose is approximately equal to the sum of that yielded by pectin alone and glucose (0.25%) in the presence of poorly utilized polygalacturonic acid. Even so, this was still only about a quarter of the growth obtained on Type C medium, where the carbon source was 5% glucose.

Table 4. EFFECT OF DIFFERENT CARBON SOURCES ON MYCELIAL DRY WEIGHT YIELD.

<u>CARBON SOURCE</u>	<u>AGE</u> (days)	<u>DRY WEIGHT</u> (g/100ml culture)
None (salts only, pH 5.5)	5	0.0001
Asparagine, 0.5% (pH 5.5)	5	0.0033
Polygalacturonic acid (pH 5.2)	10	0.0398
Polygalacturonic acid + glucose, 0.25% (pH 5.2)	5	0.1412
Polygalacturonic acid + glucose, 0.25% (pH 7.5)	5	0.0952
Pectin, 3.5% (pH 5.5) (Medium B)	5	0.1082
Pectin, 3.5% + glucose, 0.25% (pH 5.5) (Medium B)	5	0.2631
Glucose, 5% (pH 5.2) (Type C medium)	5	1.0415
Glucose, 5% (pH 7.5) (Type C medium)	5	1.2165

Since some growth occurred on these pectic media, the fungus must be capable of utilizing them, but appears to "prefer" the methylated substrate pectin to polygalacturonic acid.

c) Production of pectolytic enzymes by *C.purpurea* 'in vitro' (liquid culture)

i) Pectolytic activity in dialysed filtrates of cultures grown on pectin Medium Type B (Table 5)

1. Pectinesterase (PE)

When cultures were grown on Pectin Medium Type B, PE activity was present in the filtrate. However, when glucose (0.25%) was included in the medium, the PE activity dropped three-fold.

2. Polygalacturonase (PG)

PG activity in the culture filtrate of glucose-supplemented medium was slightly higher (about 25%) than that in cultures where glucose had been omitted. However, whether this difference is statistically significant has not been verified since the culture filtrates from the 5 flasks were bulked on harvesting.

3. Polymethylgalacturonase (PMG)

Filtrate from cultures grown on the medium without glucose had over seven times the activity of filtrate from glucose-supplemented medium. This appears to indicate that glucose may suppress PMG synthesis or activity.

4. Polygalacturonate lyase (PGL)

No PGL activity was detected in filtrates of cultures grown on the medium containing glucose. When glucose was omitted however, some PGL activity was evident.

5. Polymethylgalacturonate lyase (PMGL)

PMGL activity was present in filtrates from cultures grown

Table 5. PRODUCTION OF PECTOLYTIC ENZYMES BY C.purpurea 'IN VITRO'.

	<u>PE</u> (mls 0.01N NaOH required for neutralization/ hr/g d.wt.mycelium)	<u>PG</u> (μmols galacturonate equivalents/ml/ mg protein/min.)	<u>PMG</u> (μmols galacturonate equivalents/ml/ mg protein/min.)	<u>PGL</u> (μmols unsaturated product/ml/mg protein/min.)	<u>PMGL</u> (μmols unsaturated product/ml/mg protein/min.)
<u>Pectin medium</u>	1.18	15.31	25.13	1.29	1.94
<u>Pectin medium + glucose (0.25%)</u>	0.36	21.4	3.3	0*	2.21

*undetectable

C. purpurea was cultured on Pectin Medium Type B (which contains 3.5% pectin) with and without glucose(0.25%).

Cultures were harvested after 5 days. Culture filtrates were then centrifuged, dialysed and assayed for pectolytic activity.

on both the glucose-supplemented medium and the standard medium, with little difference between the two.

Therefore, when grown on pectin as the main carbon source, (asparagine in the medium contributes some carbon) C.purpurea appears to be able to produce all five pectolytic enzymes. However, when glucose (0.25%) is incorporated into the medium, a different pattern of enzyme activity results. In the cases of at least two of the enzymes (PE and PMG) the inclusion of glucose causes decreased enzyme activity. The effects of glucose incorporation on the activity of the other three enzymes (PG, PGL and PMGL) is not as marked and may be minimal. No distinction was attempted between endo- and exo-enzymes.

ii) Pectolytic activity in crude filtrates of cultures grown on a non-pectic carbon source (Type C medium)

After 5 days on this medium, the mycelial growth (Table 4) was white and thick, and hyphae were typically sphacelial, i.e. long, occasionally septate with few branches and little lipid. The crude and undialysed filtrates from cultures grown at both pH 5.2 and pH 7.5 showed no detectable PE, PG or PMGL activity.

From these findings, it may appear that when grown on glucose instead of pectin as the main carbon source, C.purpurea does not produce pectolytic enzymes, i.e. the enzymes do not appear to be constitutive. While this may be the case, it is likely that the conditions of fungal growth and subsequent enzyme assay may have prevented any activity from becoming apparent. In particular, the filtrates were not dialysed, therefore the level of sugar in the filtrate could have either repressed synthesis or inhibited activity of the pectolytic enzymes.

This preliminary study therefore cannot conclude whether the

enzymes are constitutive or inducible, but this question is considered further in the Discussion.

d) Pectolytic activity of parasitic extracts of *C.purpurea*

i) Pectinesterase (PE)

All the mycelial extracts (7,12,12C,20,22,26-day old tissues) and honeydew gave positive reactions with the cup-plate assay method. When the hydroxamic acid reaction was used as the developer, a clear zone on a red background was most obvious with the 26-day old extract and slightly less so with the 20-day and honeydew extracts. With the other extracts, the zones were not as clearly defined and water gave no reaction. When the plates were developed with 10% copper acetate, positive reactions were obtained with all extracts, including 12C. Again water formed no zone. In these assays, no attempt was made to correlate the diameter of the zones with concentration of enzyme since no standard enzyme preparation was used. The assays were intended to be purely qualitative. The two developers gave slightly different results in that the 10% copper acetate produced similar zones with all the extracts, while the hydroxamic acid reagent produced zones of varying intensity, possibly differentiating between higher and lower activity. Therefore, the results indicate that pectinesterase activity exists in parasitic extracts of *C.purpurea* and also in dialysed honeydew. Since there is no intact plant tissue left in the infected floret from about 10-12 days after inoculation, any PE activity in extracts older than this must be attributed to the fungal mycelium. The results also indicate that PE activity also occurs in young seeds (sample 12C). The presence of PE in healthy plants is well known, particularly in their fruits, (see Fogarty & Ward, 1974 for review). Although it is difficult to find references to PE activity in ovary tissue in the literature,

grape seeds are known to possess it, albeit much lower than in the skin or pulp (Peynaud & Ribereau-Gayon, 1971). The 12-day control sample (12C) was a developing seed, and therefore may be expected to have some PE activity.

ii) Polygalacturonase (PG)

The following extracts were tested for PG activity and all gave positive results: 7,12,12C,22(basal part,B),26(apical part,A) days old and dialysed honeydew. The results (Table 6) indicate that the parasitic mycelial extracts possess PG activities 1.5-2 times higher than those of healthy tissue. At 7 days, when the sphacelial form of the fungus had colonized all parts of the ovary, PG activity was already high, and increased slightly at 12 days, a stage at which most of the ovary would have been destroyed. Healthy tissue at this stage had a substantially lower PG activity. The activity of the extract of the basal part (B) of the 22-day old tissue (which was sclerotial) was only two thirds that of the apical portion (A) of the 26-day sample (sphacelial cap plus some sclerotial tissue). This may possibly be explained by the exudation of honeydew. The flow of honeydew may leach some of the enzyme away from the basal hyphae leaving this area apparently low in activity relative to the apex. This is reflected in the greatly increased activity in the honeydew, and a similar situation was found in β -1-3-glucanase activity (3.3.c).

iii) Polymethylgalacturonase (PMG)

Only three mycelial extracts and honeydew were tested for PMG activity and all were found to be positive (Table 6), the activity increasing with age. Unfortunately a healthy control was not included, so the results cannot be compared with ^{un}infected tissue. However, relatively high activity is indicative of the capability of the fungus, and as with PG, activity in the honeydew is considerably greater than in the mycelial extracts. The activity of PGL and

Table 6. PG AND PMG ACTIVITY OF SOME PARASITIC MYCELIAL EXTRACTS
OF C.purpurea.

<u>EXTRACT AGE</u> (days)	<u>PG ACTIVITY</u> (μ mol galacturonate equivalents/ml/mg protein/min.)*	<u>PMG ACTIVITY</u>
7	24.1	20.3
12	25.9	52.1
12C (healthy control)	15.3	N.T. ⁺
20 (A) ^x	N.T.	62.0
22 (B) ^{xx}	21.3	N.T.
26 (A)	30.0	N.T.
Honeydew	79.7	187.6

* First order kinetics

⁺ N.T. = not tested

^x A = apical portion

^{xx} B = basal portion

PMGL in the parasitic extracts was not measured.

Therefore, parasitic C.purpurea produced at least 3 pectolytic enzymes, and enzyme production was not restricted to the initial stages of invasion, but continued into the stage where the sclerotial tissue developed into an ergot. As with PG activity, the PMG activity was greater in honeydew than in parasitic mycelia and this may be due to leaching. Although PE and PG were also found in healthy wheat tissue, and these enzymes have previously been found in other healthy plants (Peynaud & Ribereau-Gayon, 1971; Hatanaka & Ozawa, 1964), the enzymes found in the parasitic extracts could only be of fungal origin since no intact ovary tissue remains from about 10-12 days after infection.

3.3. β -GLUCANASE AND β -GLUCOSIDASE ACTIVITY IN CLAVICEPS spp.

a) Action of exo- β -1-3-glucanase from Basidiomycete QM806

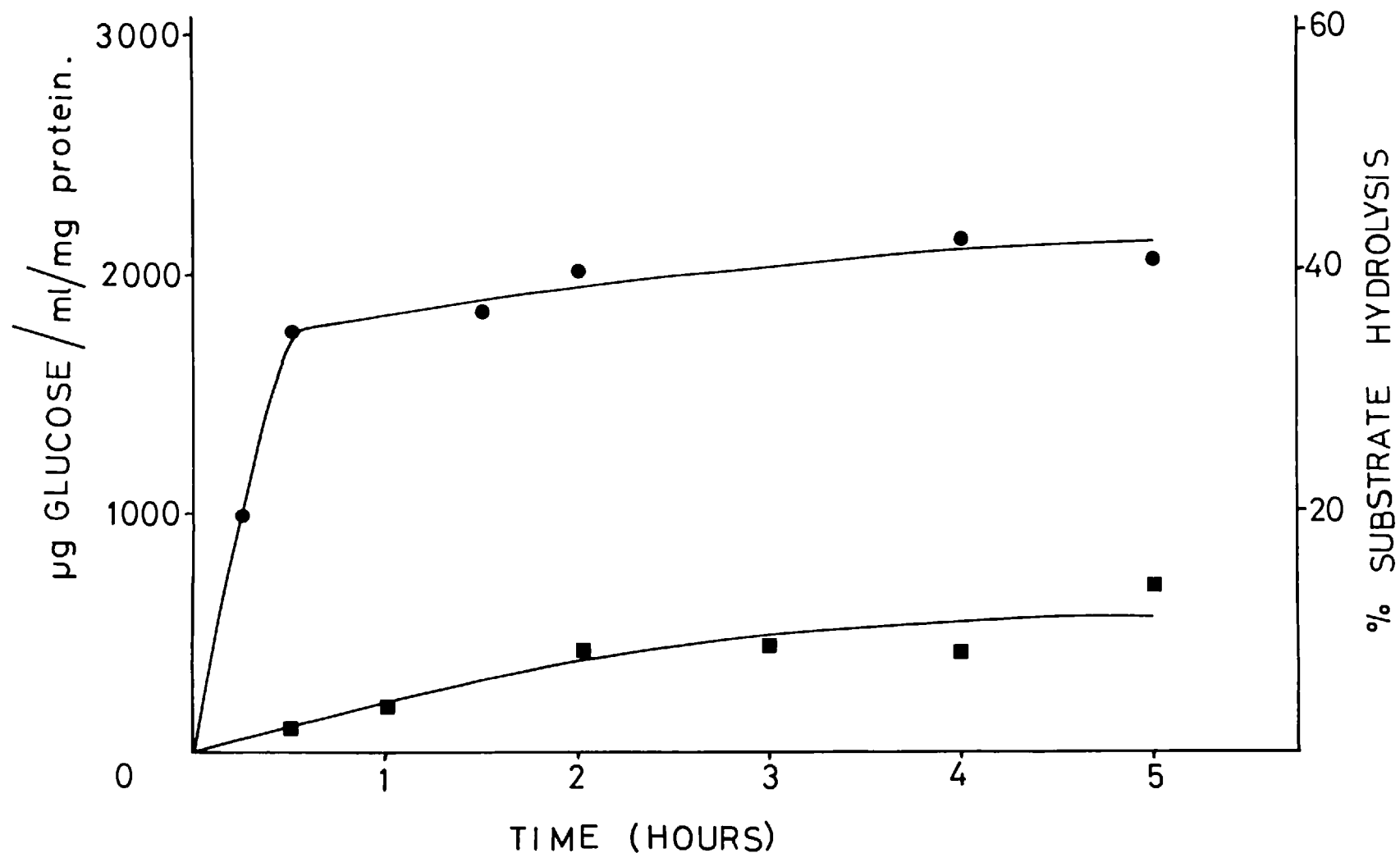
i) On glucan derived from Sphacelia sorghi

When this enzyme was incubated with S.sorghi glucan, glucose was rapidly released (Fig. 8). The initial rate of glucose production was 65.6 $\mu\text{g/ml/mg protein/min}$ (Table 7), and after 30 minutes 35% of the substrate had been hydrolysed. After 24 hrs, about 53% of the glucan was hydrolysed to glucose. This enzyme is specific for β -1-3 links and by-passes β -1-6 links so that any gentiobiose (β -1-6 linked disaccharide) formed in this manner remains unhydrolysed. Chromatography (Fig. 9) showed gentiobiose to be the only other product of hydrolysis.

ii) On laminarin

The initial rate of laminarin hydrolysis was very high: 129 $\mu\text{g glucose/ml/mg protein/min}$. (Table 7). After 30 minutes about 77% had been hydrolysed, and hydrolysis was almost complete after 1 hour. Therefore, the activity of the enzyme was about twice that when S.sorghi glucan was the substrate. The major product of laminarin hydrolysis by B.QM806 glucanase was glucose, which was detectable on the chromatogram in 10 min (Fig.10). After about 25 minutes another product was detected which was present in very small quantities up to the last sample taken at 4 hours. This other product (which was not gentiobiose) ran between sucrose and maltose on the chromatogram which suggests a disaccharide, probably laminarabiose. Nelson et al (1963) found that this enzyme hydrolyses laminarin from the non-reducing end of the chain, removing one glucose residue at a time. When there are two residues left i.e. laminarabiose ($= \beta$ -1-3 linked) hydrolysis stops, leaving this compound unhydrolysed . Gentiobiose

Fig. 8 TIME COURSE OF HYDROLYSIS OF S.sorghi GLUCAN BY HONEYDEW(■)
OF C.purpurea AND $\text{exo-}\beta\text{-1-3-GLUCANASE}$ OF BASIDIOMYCETE QM806(●)



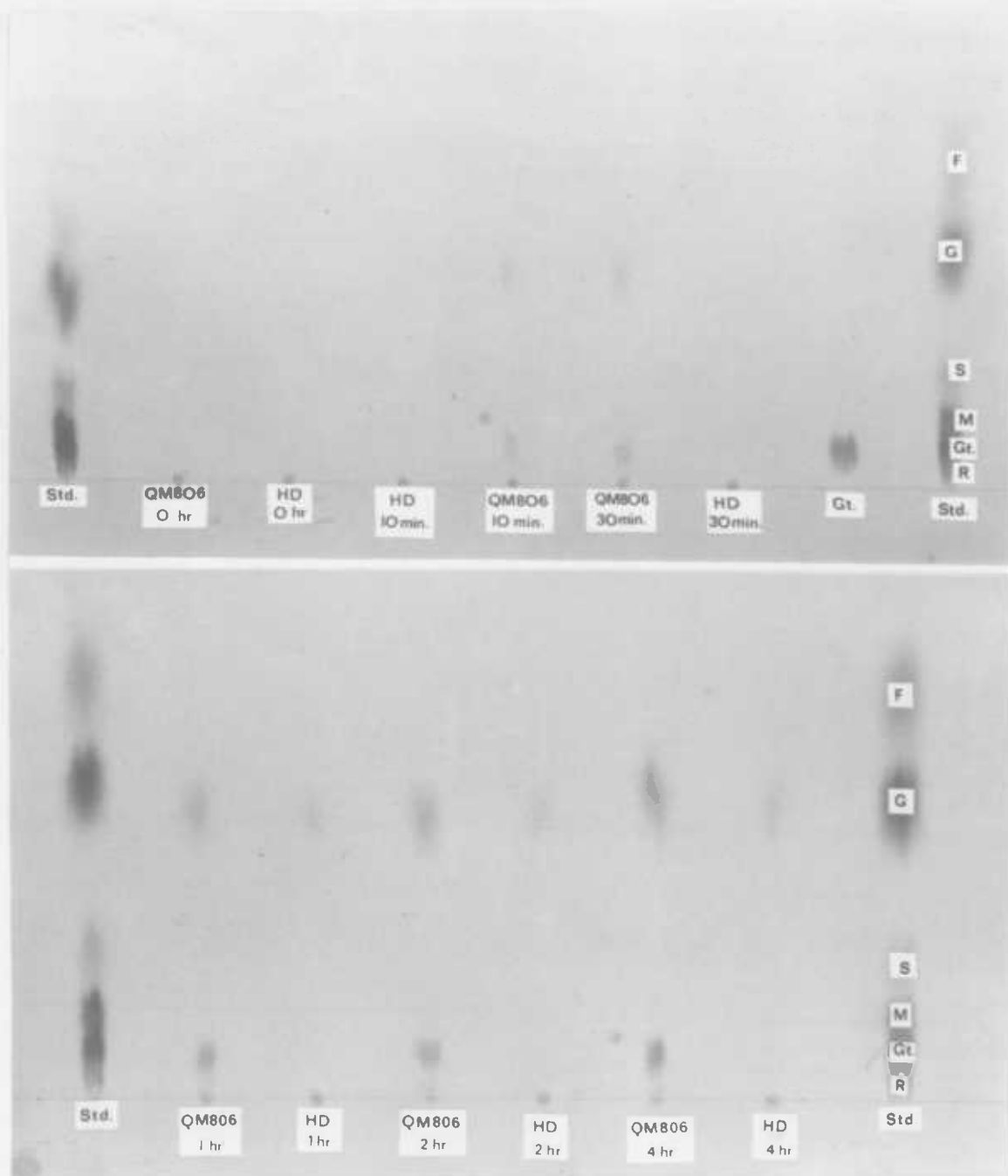


Fig. 9. Chromatography of reaction products from the incubation of *S. sorghi* glucan with a) β -1-3-glucanase from *Basidiomycete* QM806 and b) dialysed honeydew (HD) from *C. purpurea*. Samples were taken over 4 hours. Honeydew incubation produced only glucose. *B. QM806* incubation produced glucose (G) and gentiobiose (Gt).

Std = Standards: F = fructose; G = glucose; S = sucrose;
M = maltose; Gt = gentiobiose; R = raffinose.

was not detected on the chromatograms indicating that the laminarin used had, at most, few β -1-6 links.

b) Action of dialysed honeydew of *C.purpurea*

i) On glucan derived from *S.sorghi*

The only product of this hydrolysis detected by chromatography was glucose, and its initial rate of production was $2.98\mu\text{g/ml/mg}$ protein/min. This is about 4.5% of the activity of B.QM806 glucanase on the same substrate (Fig. 9 & Table 7). Honeydew contains, in addition to β -glucanase (and other enzymes), a β -glucosidase which can hydrolyse gentiobiose (see later). The action of this enzyme would have contributed to the glucose concentration in the incubate which is therefore an overestimate of the β -glucanase activity.

ii) On laminarin

Glucose alone was detected as the product of hydrolysis by paper (Fig. 10) chromatography. Any gentiobiose which might have been formed would have been hydrolysed by the β -1-6-glucosidase in the honeydew (see later). There was no sign of the disaccharide (? laminarabiose) as in the B.QM806 glucanase incubation, which suggests that either the honeydew enzyme is capable of hydrolysing laminarabiose or that its concentration was so low as to be undetectable. The rate of laminarin hydrolysis was $23.57\mu\text{g}$ glucose produced/ml/mg protein/min. which is about 17% of the activity of B.QM806 glucanase on the same substrate (Table 7). After 4 hours, just over 20% of the substrate had been hydrolysed. Therefore, when *S.sorghi* glucan is the substrate, the β -glucanase activity of *C.purpurea* honeydew is only about $\frac{1}{5}$ th of that when laminarin is the substrate.

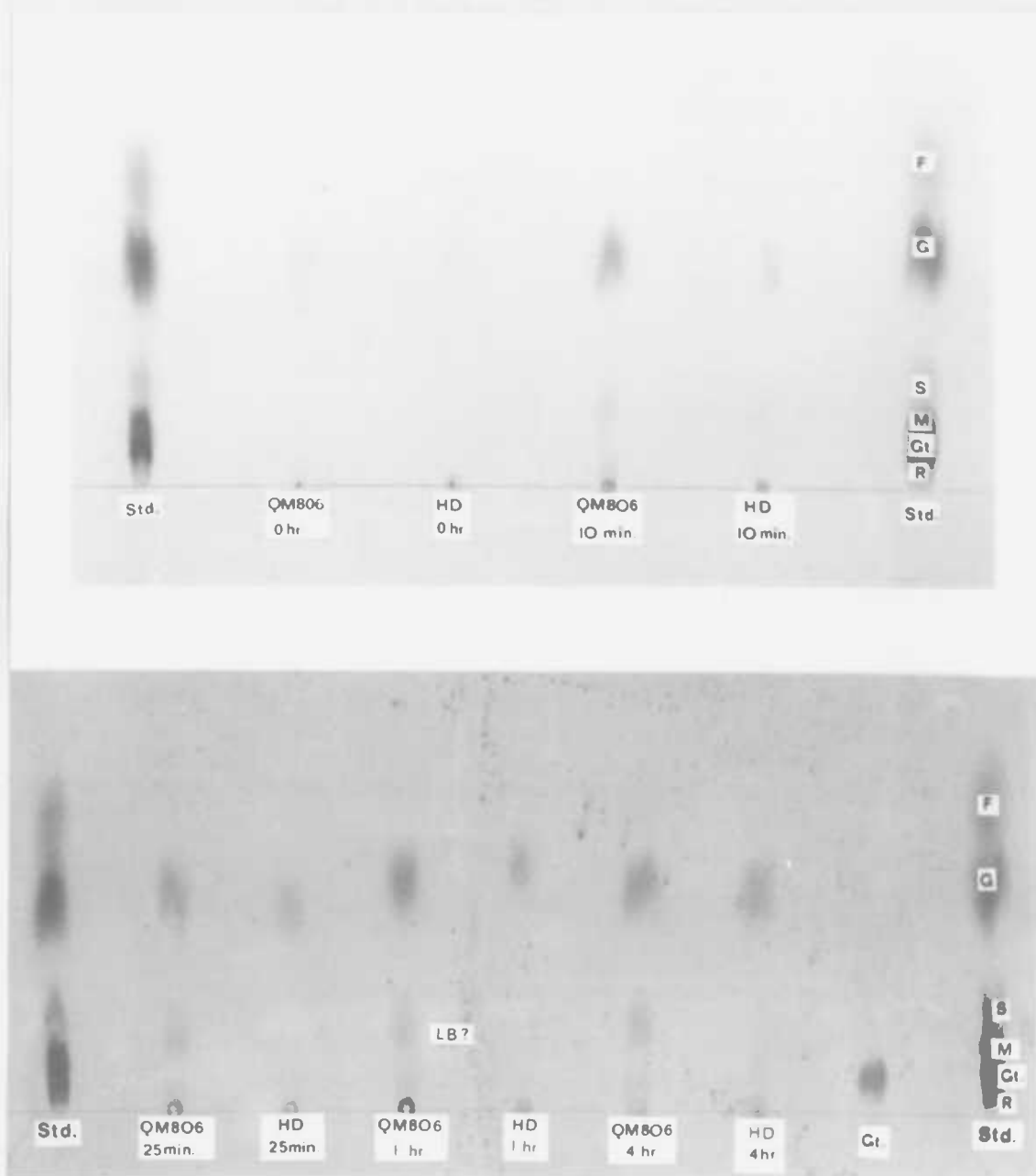


Fig. 10. Chromatography of reaction products from the incubation of laminarin with a) β -1-3-glucanase from Basidiomycete QM806 and b) dialysed honeydew (HD) from C. purpurea. Samples were taken over 4 hours. Honeydew incubation produced only glucose (G); B. QM806 incubation produced glucose + another product, probably laminarabiose (LB?).

Std = standards: F = fructose; G = glucose; S = sucrose;
M = maltose; Gt = gentiobiose; R = raffinose.

Table 7. COMPARISON OF GLUCANASE ACTIVITY* OF VARIOUS EXTRACTS
ON 2 DIFFERENT GLUCAN SUBSTRATES.

<u>EXTRACT</u>	<u>ACTIVITY* ON:</u>	
	a) <u>LAMINARIN</u>	b) <u>S.sorghi GLUCAN</u>
Exo- β -1-3-glucanase from <u>Basidio mycete QM806</u>	129.0	65.6
Dialysed honeydew from <u>C.purpurea</u>	23.57	2.98
Parasitic sphacelial extract (9 days old) from <u>C.purpurea</u>	N.T.	1.08
Dialysed honeydew from <u>C.fusiformis</u> ex Bajra	7.0	0.33
Parasitic sphacelial extract (10 days old) from <u>C.fusiformis</u> ex Bajra	2.08	0.25
Mature ergot of <u>C.fusiformis</u> ex Bajra	N.T.	0.2

* μ g glucose liberated/ml/ml protein/min. 1st order kinetics.

N.T. Not tested.

c) β -glucanase activity of extracts of parasitic mycelia of *C.purpurea*

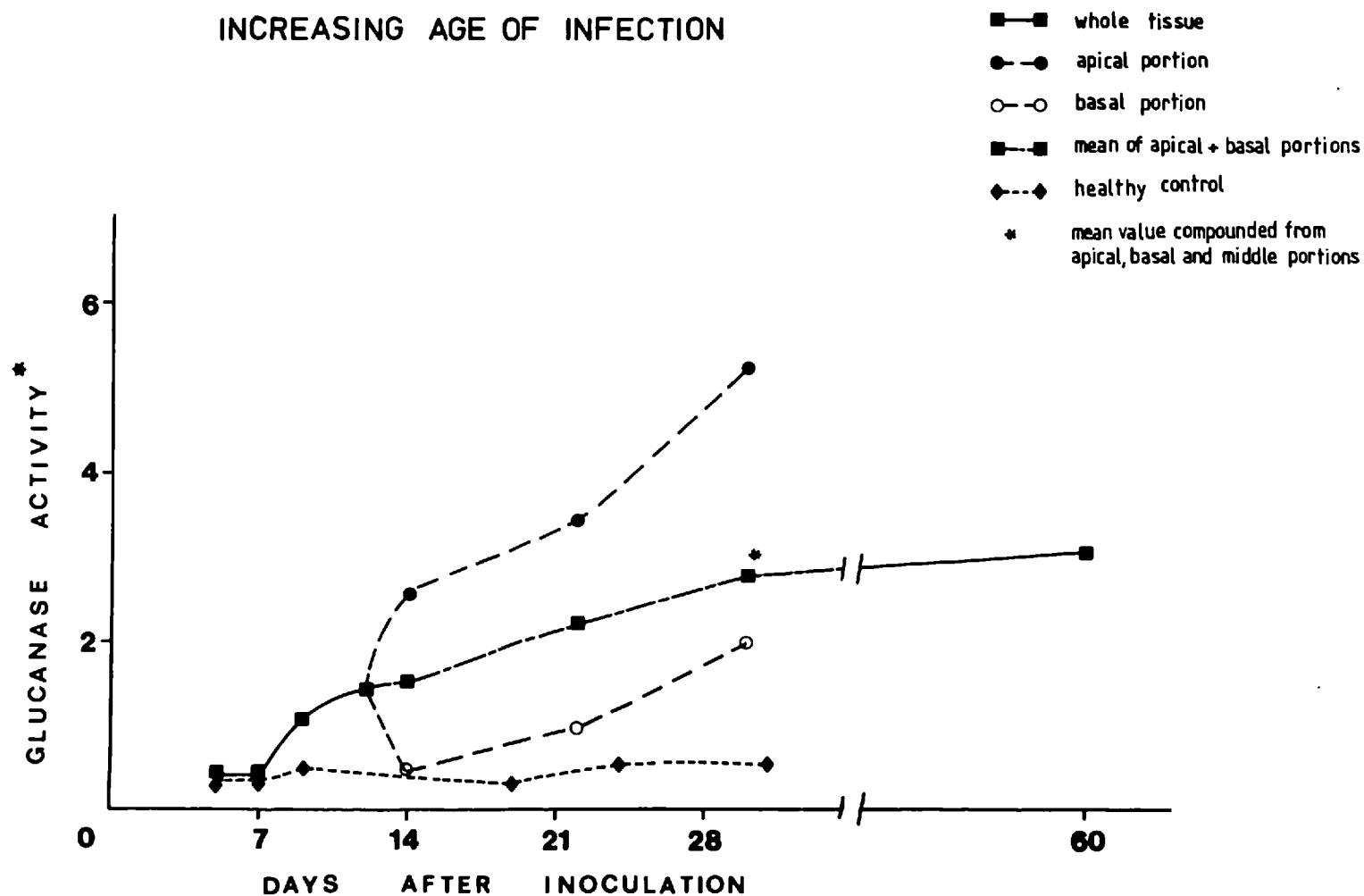
Infected wheat and rye ovary extracts sampled at various stages after inoculation showed an ability to hydrolyse *S.sorghi* glucan. Generally, up to 7 days after inoculation infected and healthy ovaries showed the same levels of β -glucanase activity, but thereafter activity in infected extracts increased while that in control extracts remained low (Fig. 11). It was noticed that when, due to climatic conditions, honeydew release began earlier than 7 days after inoculation, activity in these tissues was correspondingly higher. In the more common situation where honeydew exudation had not begun even at 7 days, activity in these tissues was still as low as in controls, and only increased coincident with honeydew release.

As the ergot sclerotia developed, apical and basal regions were analysed separately. The apical parts consistently had higher activity than the basal portions. (The sphacelial cap was included in the apical part and its glucanase activity contributed to the remaining sclerotial tissue.) The explanation for this pattern of activity could lie in the honeydew, which, when exuding freely, may leach some of the glucanase produced, especially that secreted by the basal hyphae. This is illustrated in the basal portions of 14 day samples where the activity is similar to that in control ovaries. As honeydew exudation stops, there would be less removal of glucanase from its production site. This may account for the progressive increase in activity in basal tissues with increasing age of infection. The increase in activity in the apical portion may possibly be due to the increased proportion of glucanase-secreting tissue as the hyphae are acropetally displaced throughout growth.

d) β -1-6-glucosidase activity of extracts of parasitic mycelia of *C. purpurea*

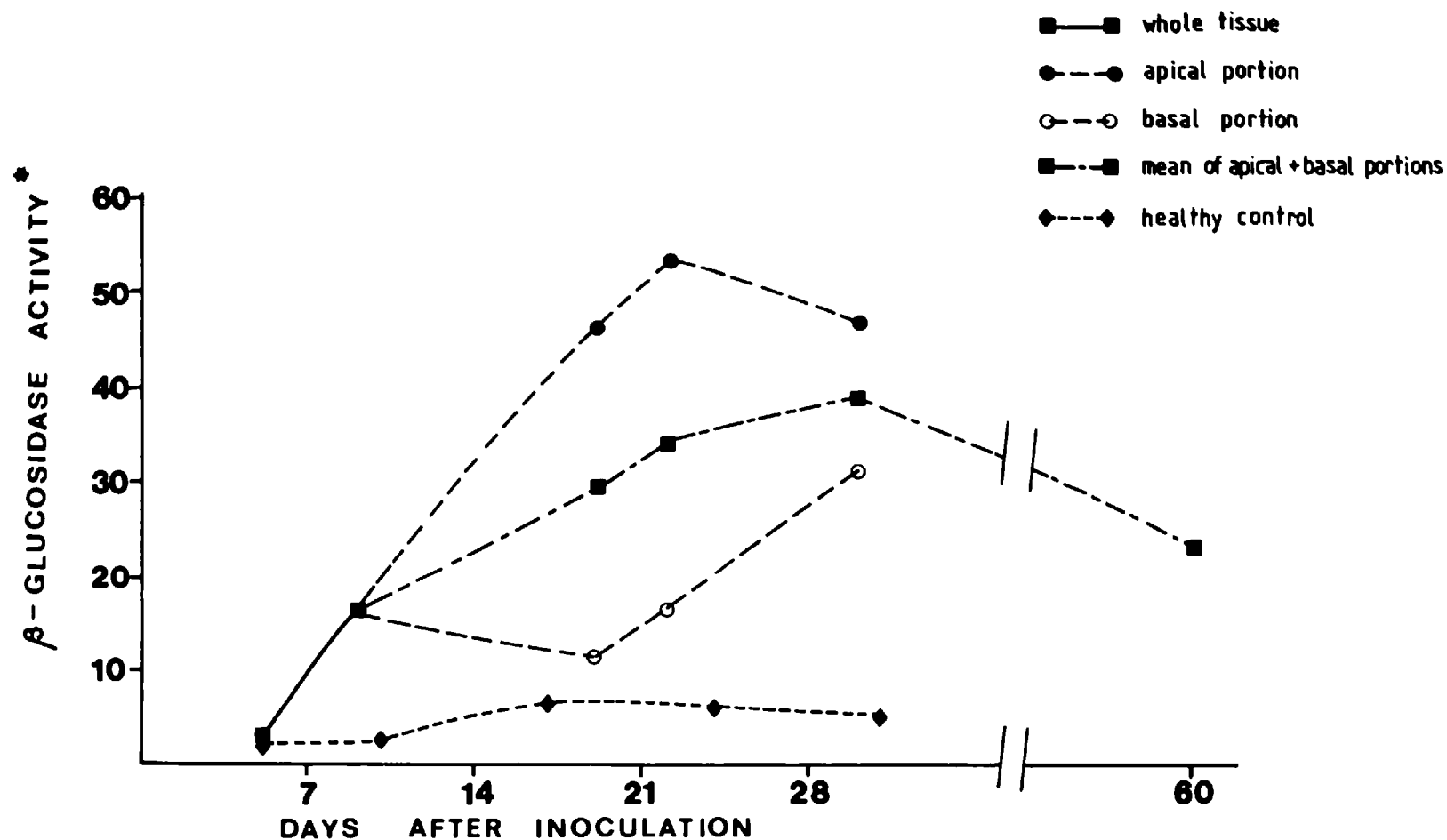
All the extracts tested were found to possess β -1-6-glucosidase activity. Fig. 12 shows the increase in β -1-6-glucosidase activity with

Fig.11 β -GLUCANASE ACTIVITY OF PARASITIC TISSUE OF C.purpurea WITH
INCREASING AGE OF INFECTION



* Activity expressed as: μg /glucose liberated/ml/mg protein/min. (First order kinetics)

Fig.12 β -GLUCOSIDASE ACTIVITY OF PARASITIC TISSUE OF C.purpurea WITH INCREASING AGE OF INFECTION



* Activity expressed as: μg glucose liberated / ml / mg protein / min. (First order kinetics)

increasing age of infection. There was a sharp rise in activity between days 5 and 9, and thereafter total activity (i.e. from the whole tissue) increased, but at a slower rate. Apical parts had higher activity than basal portions. In the apical portions of the three oldest samples (19, 22 and 30 days), the levels of activity were similar, whereas in their basal portions there was a distinct increase in activity with age: the level at 30 days is over $2\frac{1}{2}$ times that at 19 days. This rise in β -glucosidase activity with age parallels that of β -glucanase activity in similar tissues (Fig. 11) and probably also reflects the decline in honeydew exudation and associated leaching. As the ergot matured further (60 days), its β -1-6-glucosidase activity decreased slightly. The activity in honeydew ($25.7\mu\text{g}$ glucose liberated/ml/mg protein/min.) is similar to the mean value of 19 day sclerotia.

In healthy control tissues, the activity was low and fairly constant, and similar to that in 5 day old parasitic tissue, in which fungal development was relatively limited.

e) Growth and β -glucanase activity of *C.purpurea* 'in vitro'
(liquid culture)

Table 8 gives a summary of growth characteristics and β -glucanase activity in liquid culture. Throughout the growth period the level of sporulation was low and the viscosity of the culture did not change. The level of β -glucanase activity in the culture filtrates is high relative to that in parasitic extracts, and the peak occurred after six days growth. Thereafter it declined slightly to its former level, rising a little at 12 days.

Table 8

GROWTH CHARACTERISTICS AND β -GLUCANASE ACTIVITY* OF C.purpurea CULTURES.

(*activity expressed as ug glucose liberated/ml/mg protein/min.)

<u>Age (days)</u>	<u>Culture Characteristics</u>	<u>Mycelial Characteristics</u>	<u>β-glucanase activity*</u>
4	Thick white mycelial growth. Clear, fluid filtrate.	Sphacelial	4.29
6	Thick pale yellow mycelial growth. Clear, fluid filtrate.	Sphacelial + some wider hyphae having more lipid, branches and septa.	7.12
8	Thick pale brown mycelial growth. Pale brown, fluid filtrate.	Mainly sphacelial. Increase in short, wide hyphae having more lipid, branches and septa. Some extracellular lipid droplets.	4.98
10	Purple-brown mycelium and filtrate. Viscosity unchanged.	2 distinct hyphal types: a) sphacelial b) short, bulbous, frequently septate, branched hyphae containing lipid $\equiv$ sclerotial or transition to sclerotial.	4.42
12	Purple mycelium and filtrate. Viscosity unchanged.	Mainly sclerotial.	5.53

f) Growth and β -glucanase activity of *C.fusiformis* str. 139/2/1
'in vitro' (liquid culture)

Growth characteristics and β -glucanase activity of this strain are shown in Table 9 . The morphology of the mycelium appeared to be mainly sphacelial with some sclerotial differentiation. Sporulation was very low throughout growth and the culture gradually became more viscous with age. At no stage was it so viscous as to become immobile in contrast to some other strains of *C.fusiformis* (see later). This viscosity is due

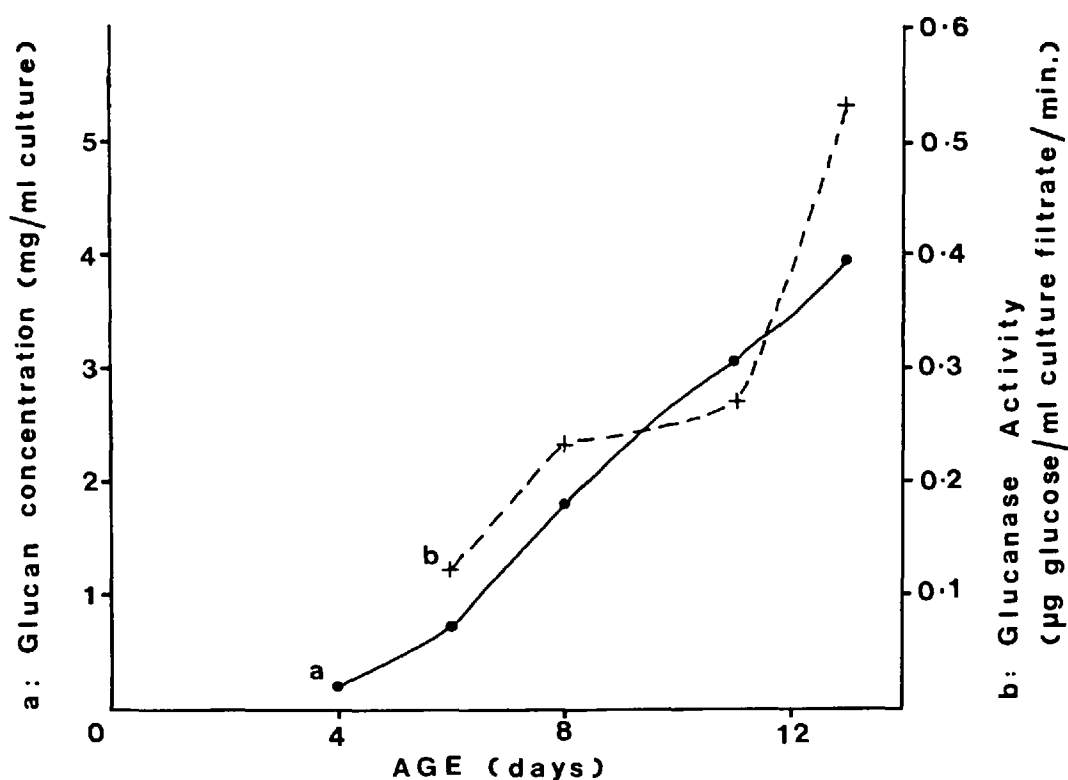


Fig.13. Glucan and β -glucanase production by *C.fusiformis*
 Str. 139/2/1 in culture.

to the production of glucan which accumulated in the culture filtrate with age (Fig.13). Simultaneously a β -glucanase was produced, whose activity per ml of culture increased with culture age (Fig.13) although its specific activity was fairly constant (Table 9). Thus the overall increase in viscosity as the culture aged is presumably due to a greater rate of glucan production than that of its breakdown.

Table 9. GROWTH CHARACTERISTICS AND β -GLUCANASE ACTIVITY* OF C.fusiformis str. 139/2/1 CULTURES

(*activity expressed as μg glucose liberated/ml/mg protein/min.)

<u>Age (days)</u>	<u>Culture Characteristics</u>	<u>Mycelial Characteristics</u>	<u>β-glucanase activity*</u>
4	White flocculent gelatinous balls of mycelium (0.5-1cm diam). Filtrate gave gelatinous ppt. with abs. alcohol indicating a polysaccharide (glucan).	Sphacelial	Not tested
6	Mycelial balls increased (1-1.5cm diam). Gelatinous ppt. present as above.	Sphacelial	2.87
8	Mixture of large and small mycelial balls tending to clump together - pale grey-brown colour. Filtrate viscosity increased slightly and ppt. present as above.	Sphacelial with occasional sclerotial hyphae.	3.29
11	Pale purple-grey mycelial clumps. Fairly viscous filtrate giving ppt. as above.	Mainly sphacelial with some sclerotial areas.	2.82
13	Purple-grey mycelial clumps. Viscous filtrate giving ppt. as above.	Mainly sphacelial with more sclerotial areas.	2.63

g) β -glucanase activity in parasitic extracts of *C.fusiformis* (ex Bajra)

After the usual purification procedures (c)ii), the dialysed extracts from both the honeydew and the 10 day old sphacelial tissue were still fairly viscous. On addition of absolute alcohol, a gelatinous precipitate was observed which was probably glucan. No such precipitate was formed by dialysed honeydew of *C.purpurea*.

The parasitic extracts hydrolysed both laminarin and *S.sorghi* glucan (Table 7). The rate of hydrolysis, however, was higher on laminarin than on *S.sorghi* glucan (21 times in honeydew). This pattern was also shown by the other enzymes tested, although the difference in rates on the 2 substrates was only 8-fold in honeydew from *C.purpurea*. Similarly, activity was higher in the honeydew than in mycelial extracts, on both substrates.

Chromatography of reaction products after 23 hours incubation of these parasitic extracts, with both *S.sorghi* glucan and laminarin, revealed that glucose was the only detectable product (Fig. 14). Since no gentiobiose was apparent as a product of hydrolysis with *S.sorghi* glucan, these extracts must also possess β -1-6-glucosidase activity.

h) Growth and glucan production by *C.fusiformis* (ex Bajra)

Growth characteristics of this isolate were observed on agar plates and in liquid culture. On agar plates of Medium T, the colonies were yellowy-white and soft for about the first week, and later appeared to be compact and developed a purple pigmentation under the surface. The hyphae were long, thin, occasionally septate and branched. Conidia were produced both in surface and liquid cultures, and were generally of two types: a) micro-conidia, average size $7.9 \times 2.7\mu$ and b) larger vacuolated fusiform conidia, averaging $15 \times 5\mu$. Production of such micro- and macro-conidia is typical of *C.fusiformis*.

In liquid culture this isolate produced a thick white growth of large

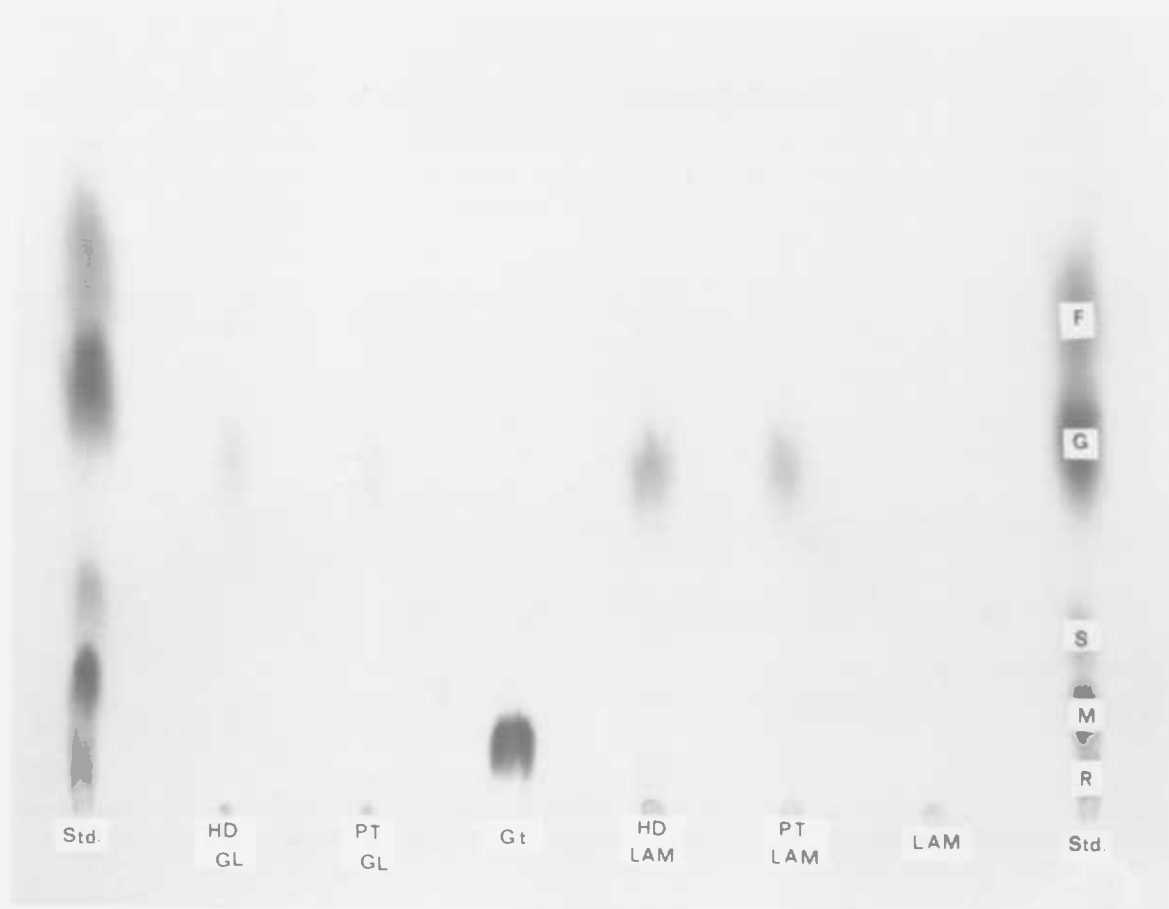


Fig. 14. CHROMATOGRAPHY OF REACTION PRODUCTS FROM 23 HOUR INCUBATION OF S. sorghi GLUCAN (GL) AND LAMINARIN (LAM) WITH DIALYSED HONEYDEW (HD) AND PARASITIC TISSUE EXTRACT (PT) OF C. fusiformis (ex. BAJRA).
GLUCOSE WAS THE ONLY REACTION PRODUCT IN ALL INCUBATIONS. LAMINARIN INCUBATED WITH BUFFER AS CONTROL (LAM) DID NOT HYDROLYSE DURING INCUBATION PERIOD.
F=FRUCTOSE, G=GLUCOSE, S=SUCROSE, M=MALTOSE, Gt=GENTIOBIOSE, R=RAFFINOSE.



Plate 35 .10-DAY OLD CULTURES OF C.purpurea
(LEFT) AND C.fusiformis (ex BAJRA) (RIGHT).

Cfusiformis culture produces foam, and gradually increases in viscosity with age until it becomes immobile, due to the production of glucan. In contrast, C.purpurea culture remains fluid throughout growth.

pellets within 5 days and spored profusely. The culture filtrate became gradually more viscous and had begun to foam by about 10 days, by which stage the mycelium was purple. The foam extended up the sides of the flasks (Plate 35) and the culture became so immobile that the flask could be inverted without its contents moving. The viscosity was not reduced even after 20 days growth. Addition of absolute alcohol to the culture resulted in a gelatinous precipitate, indicating a polysaccharide, probably glucan. β -glucanase activity in this culture must, by deduction, be very low or at least ineffective.

i) Polyacrylamide gel electrophoresis

The crude extracts from C.purpurea honeydew, and C.fusiformis (ex Bajra) honeydew and parasitic tissue were subjected to electrophoresis and compared with B.QM806. Although the extracts were impure and hence gave many polypeptide bands, some similarity can be seen.

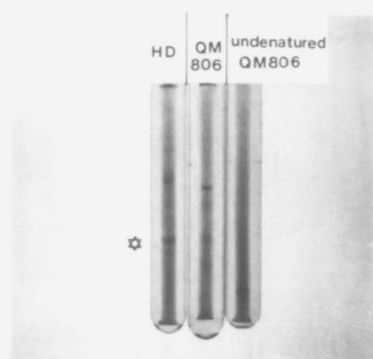
When C.purpurea honeydew and B.QM806 extracts were denatured, the position of at least 1 band (Plate 36 a) corresponded in the two extracts. (Denaturing causes disaggregation of proteins resulting in a clearer separation of bands, and can give an indication of molecular weight). Co-electrophoresis of these two extracts confirmed that at least one polypeptide band appears to be common to both (Plate 36 b).

Electrophoresis of denatured honeydew and parasitic extract from C.fusiformis (ex Bajra) suggested that two bands corresponded in these extracts (Plate 36 c). (Some of the bands in these 2 extracts may be of host origin since, during collection of the samples, anthers and other floral parts adhered to the honeydew and parasitic tissue.)

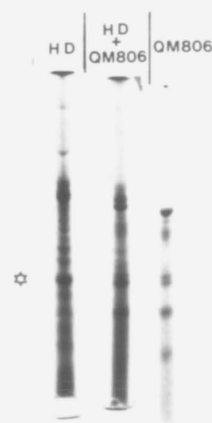
Co-electrophoresis of denatured B.QM806 and C.fusiformis parasitic tissue extracts showed (Plate 36 d) that at least one polypeptide band was common to both.

Since these gels were not all run at the same time, the exact positions

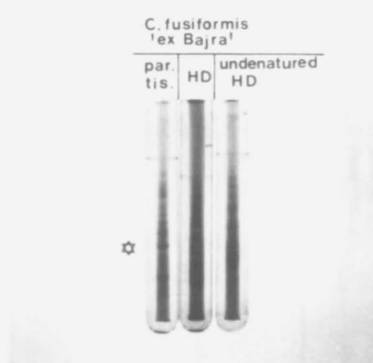
PLATE 36 POLYACRYLAMIDE GEL ELECTROPHORESIS OF PARASITIC
EXTRACTS OF C.purpurea, C.fusiformis AND
BASIDIOMYCETE QM806



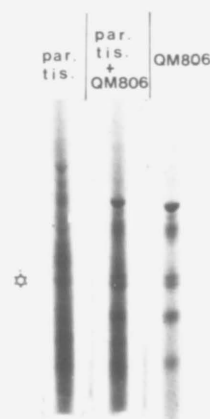
a.
DENATURED EXTRACTS OF C.purpurea
HONEYDEW (HD) AND B.QM806, WITH
UNDENATURED B.QM806 FOR COMPARISON.



b.
CO-ELECTROPHORESIS OF DENATURED
EXTRACTS OF C.purpurea HONEYDEW
(HD) AND B.QM806.



c.
DENATURED EXTRACTS OF C.fusiformis
(ex Bajra) PARASITIC TISSUE AND
HONEYDEW (HD) WITH UNDENATURED
HONEYDEW FOR COMPARISON.



d.
CO-ELECTROPHORESIS OF DENATURED
EXTRACTS OF C.fusiformis (ex Bajra)
PARASITIC TISSUE AND B.QM806.

* COMMON POLYPEPTIDE BAND

(ALL EXTRACTS WERE SHOWN TO POSSESS β -1-3-GLUCANASE ACTIVITY)

of the bands cannot be compared strictly. However, on comparing relative positions, the marked band in Plate 36a appears to be common to all four extracts. Since these extracts all possess β -glucanase activity, this band may represent the β -glucanase enzyme.

3.4 FLUORESCENCE STUDIES OF CALLOSE (β -1-3-GLUCAN) HYDROLYSIS BY *C.purpurea*

a) Fluorescence microscopy of healthy and wounded ovaries

i) Uninfected control ovaries

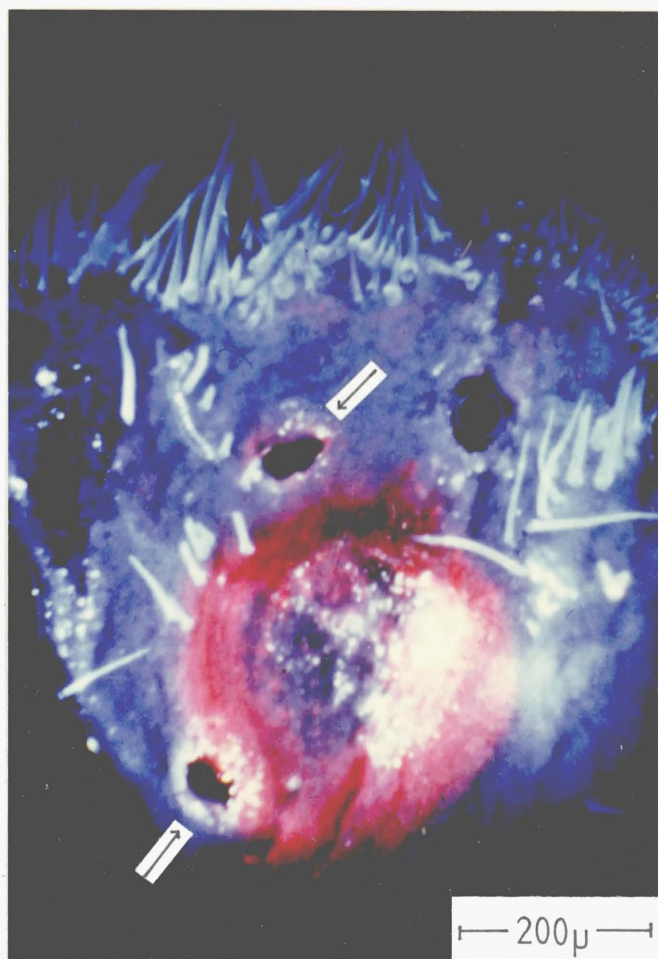
In control ovaries (i.e. healthy and not wounded) yellow fluorescence was observed only in the phloem, and occasionally in the ovule region which was probably caused by pressure damage or bruising during section preparation.

ii) Uninfected wounded ovaries

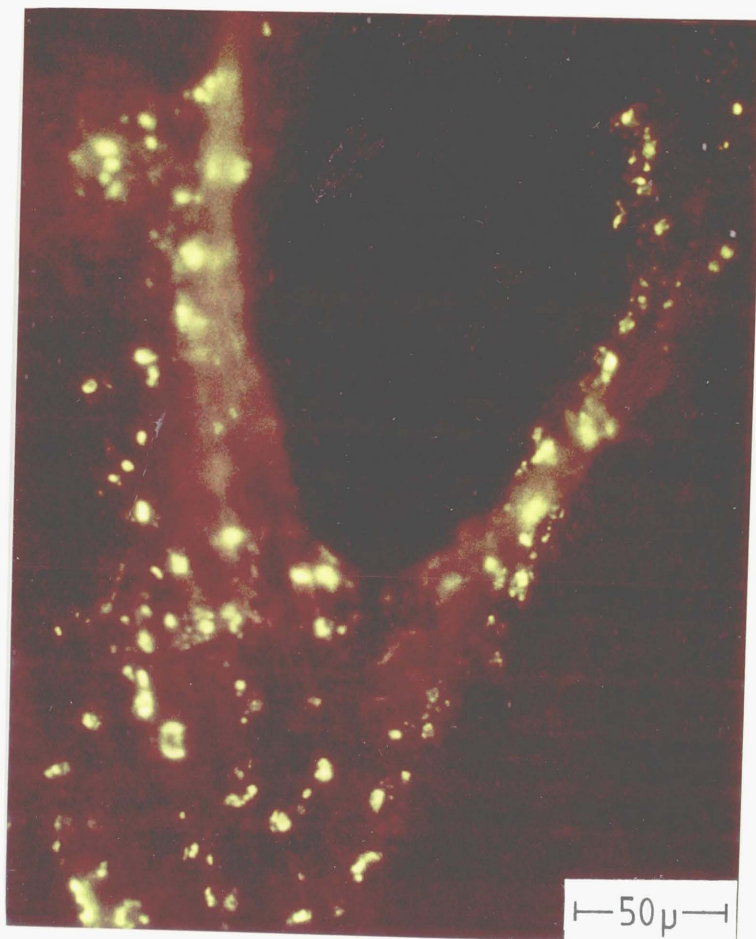
When healthy wheat and rye ovaries were wounded, incubated, sectioned and stained with aniline blue and viewed with a UV microscope, small, bright yellow fluorescent spots were observed around the wounds (Plate 37). On closer observation, these fluorescent areas (Fig 37b) were seen to be associated with the walls of cells around the wounds, although cells immediately bordering or adjacent to the wound often lacked fluorescence. This was due presumably to severe injury, leaving the still living cells with a reduced capacity to produce callose. (Engelman (1965) also noticed this phenomenon in wounded sieve tubes where ruptured cells often failed to give the wound callose reaction.) Although the usual form of the fluorescence was discrete spots or dashes, larger and more continuous areas were also sometimes observed. The ovule region also usually showed fluorescent spots, even when not lesioned. This can perhaps be explained by the fact that at anthesis, (the stage when the ovaries were sampled), the ovule cells are undergoing rapid division. Currier and Strugger (1956), have shown that active cells show the most rapid wound-callose reaction, therefore the ovule cells might be expected to detect and react to any trauma imposed on neighbouring cells. Similar yellow fluorescence was observed in the vascular traces, and was due to normal phloem callose, found in the sieve tubes.

- a) Longitudinal section through wounded wheat ovary stained with aniline blue and irradiated with UV light. Callose is deposited as a reaction around wounds in the tissue (arrows). The red area indicates chlorophyll in the ovule. Cellulose in cell walls imparts the pale blue colour to the ovary tissue and apical hairs.
- b) Enlarged view of callose deposits around a wound. The yellow colour is a more accurate photographic record of callose as seen in the microscope than in (a) above. Callose appears as discrete dots or dashes although some larger deposits also develop.

a



b



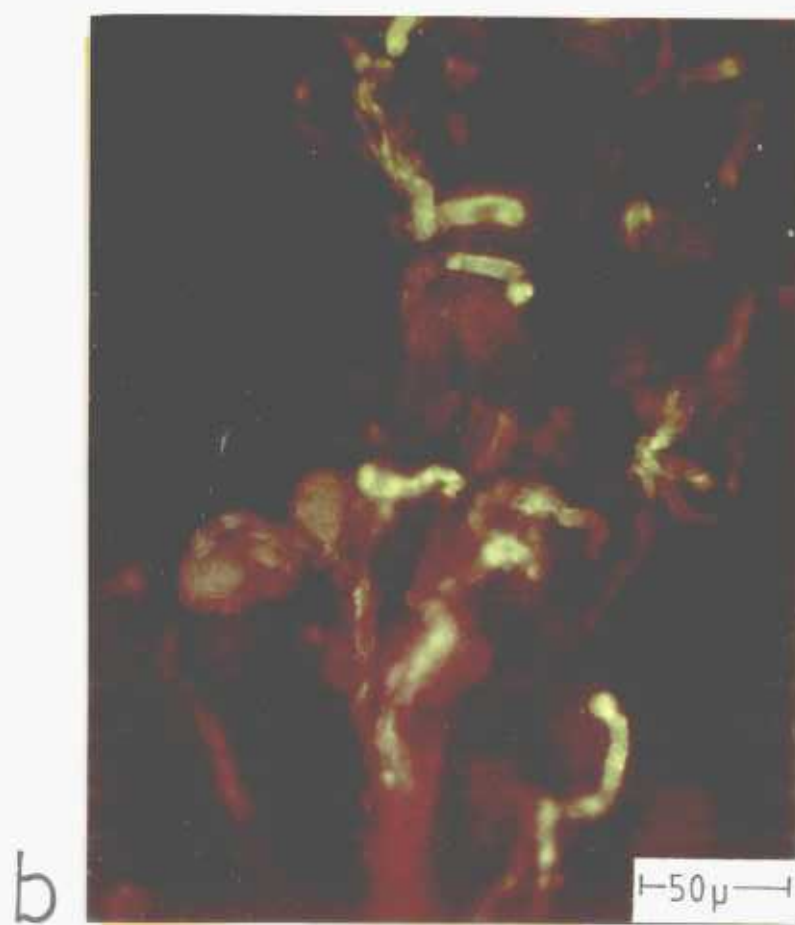
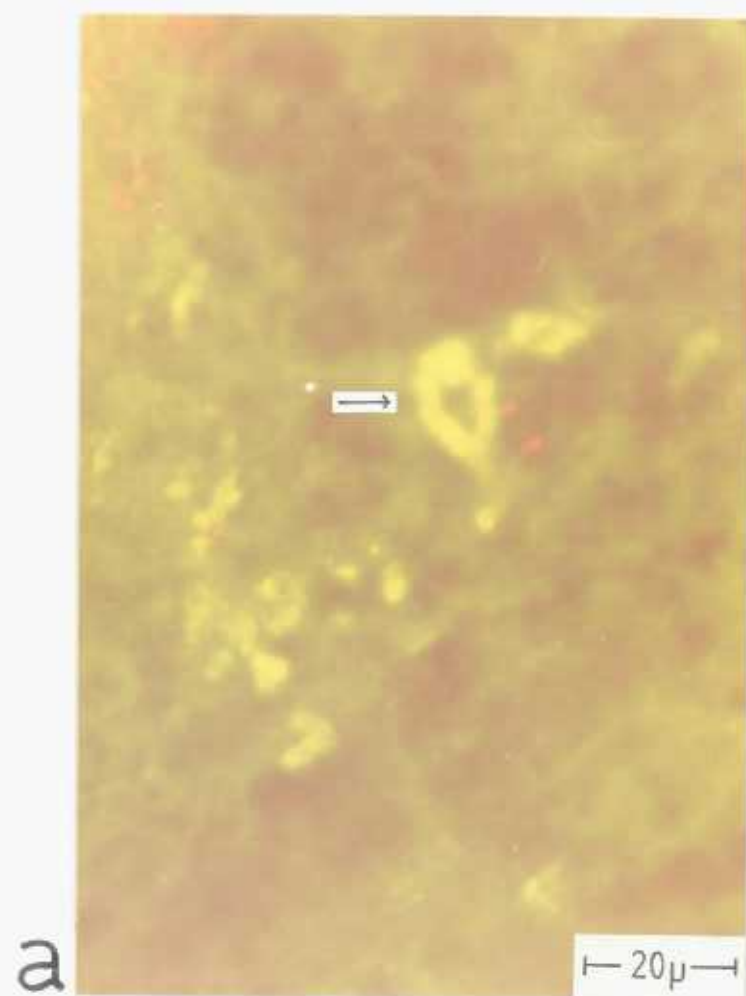
The most widespread colour after staining, both in wounded and control sections was the pale blue of the cellulose cell walls (Plate 37a). This was especially evident in the hairs at the top of the ovary. Cells containing chloroplasts, abundant in the ovule, appeared red due to the fluorescence of their chlorophyll on exposure to UV. The integuments of the ovule sometimes stained a dull orange. The bright yellow fluorescence of wound and phloem callose could be easily distinguished from these background shades. Neither callose nor aniline blue is autofluorescent (Eschrich and Currier, 1964) but when in mutual contact, produce this characteristic yellow fluorescence (Plate 37b).

iii) Infected control ovaries (i.e. not wounded)

When ovaries at early stages of infection were sectioned and stained with aniline blue and viewed with UV light, small tubular-shaped yellow fluorescent structures were visible throughout most of the area of the sections (Plate 38a). At 5 days these fluorescent forms could be seen in the ovary wall and around the ovule, but not within it. This pattern corresponded to that of hyphal invasion and it was concluded that these fluorescent structures were hyphae because of their location and size. They could be clearly distinguished between and around ovary cells, and their fluorescence could be due to the β -glucans in their walls (Bartnicki-Garcia, 1968). The pattern of fluorescence of the hyphae was not uniform: some did not fluoresce at all, others appeared yellow, while the rest fluoresced bright yellow. The distribution of β -glucans or the stain's accessibility to the β -glucans in various parts of the hyphal walls could be partly responsible for this non-uniformity of fluorescence.

When spores, germ-tubes and hyphae of saprophytically grown C.purpurea were stained with aniline blue and observed under UV, their walls fluoresced in a manner different from those of parasitic

- a) Infected ovary tissue stained with aniline blue and irradiated with UV light. The yellow fluorescent tubular structures (arrow) are probably hyphae; β -glucans in the walls are responsible for the fluorescence.
- b) Pollen tubes growing in rye stigma stained and irradiated as above. Callose is deposited in the walls and as plugs in the pollen tubes.



hyphae. Ungerminated spores fluoresced at one or both ends. Germ tubes and hyphae gave variable results but usually fluoresced brightly at their tips and septa.

At slightly later stages of infection i.e. 7 and 10 days, fluorescing hyphae were observed to have penetrated the ovule and colonised every part of the ovary. At 10 days there were very few plant cells remaining, and the fungus had begun to form pockets of sporulation which did not fluoresce. There was no indication of wound callose deposition in the plant cells at any of these stages. Older tissue was not examined. When infected ovaries (5 and 10 days old) were incubated in B.QM806 β -glucanase, the hyphal fluorescence diminished, suggesting that the glucan responsible for the fluorescence had been at least partly degraded.

iv) Infected wounded ovaries

When 5 day old infected ovaries were injured mechanically with a thin wire and stained for callose, some wound callose was observed in some of the plant cells around the wound. This callose appeared as small discrete fluorescent spots in the cell walls, and occurred a small distance from the wound border as well as in intact cells at the edge of the wound. The hyphae fluoresced as in the infected control ovaries described above.

When 6 day old infected ovaries were injured in a similar manner, callose fluorescence around the wound was not as marked as in the 5 day old material. Fewer cells reacted by producing callose, and the total amount of fluorescence in the walls of cells which did react appeared to be less than in wounds of 5 day old infected ovaries and of healthy wounded ovaries. This suggests a reduction in the cells' ability to produce wound callose. This could be explained by the fact that colonization by the fungus increases rapidly (see 3.1.) at this stage, separating the plant cells and leaving them damaged,

dying or dead. Since callose deposition is an active metabolic process requiring synthesising enzymes, such cells would have an impaired capacity to produce wound callose in response to mechanical injury. Non-uniform fluorescence of fungal hyphae was again observed.

In mechanically injured ovaries 7-8 days after inoculation, even fewer plant cells could be distinguished as coherent tissue and wound callose was not evident. In older samples, the ovary was completely replaced by fungal mycelium, and therefore were not tested for mechanically induced wound callose.

b) Hydrolysis of wound callose

Since the above tests demonstrated the cells' ability to produce callose after mechanical wounding, studies concerning the degradation of callose and hence a reduction in its fluorescence were carried out.

i) Visual assessment of fluorescence intensity.

1) Effects of B.QM806/3- β -glucanase

This enzyme was tested at 4 different temperatures for varying periods, at concentrations of 1 and 5 mg/ml as shown in Table 10. The results can be compared with those from control sections, incubated in water.

Incubation with this enzyme resulted in little or no fluorescence when conditions were within the range indicated. Neither wound nor phloem callose fluorescence was detected. In some sections, although there was no fluorescence, a faint dull yellow was sometimes observed both in phloem tissue and around wounds, and was considered to indicate partial callose breakdown. It was found to be quite useful for locating wound areas and vascular traces in sections which appeared otherwise featureless. Control sections generally gave good fluorescence on incubation up to 45°C (except at 23°C for 20 hrs) and both phloem and wound callose was seen to fluoresce. Above this temperature, the duration of

Table 10. Effect of B.QM806 β -glucanase and water on callose fluorescence of wounded ovary sections incubated under various conditions.

B.QM806 β -glucanase treated:

<u>Temperature</u> (°c)	<u>Concentration</u> (mg/ml)	<u>Duration of</u> <u>incubation (hrs)</u>	<u>Fluorescence*</u>
23	1	1	+
23	1	2	+
23	1	19	0
23	1	20	0
30	1	1	+
30	1	2	0
30	1	3	0
45	1	1	0
45	5	1	0
45	5	2	0
50	5	3	0

Control: (water)

23	1	+++++
23	2	+++++
23	20	++
30	1	+++++
30	2	+++++
30	3	++++
45	1	++++
45	2	++++
50	1	++++
50	3	++

*Estimated on an arbitrary scale:

0 = no fluorescence

+ = very weak +++++ very strong

incubation became critical, and at 50°C, incubation for 3 hours resulted in noticeably reduced fluorescence. It was decided that in future experiments, especially those which were to involve quantitative measurement of fluorescence, the 'safest' conditions of incubation were 45°C for 1-2 hrs, when there should be little risk of controls showing diminished or variable fluorescence. The reason for this loss of fluorescence at 50°C when incubated for 3 or more hours is obscure, but a similar reduction was observed in sections incubated at a lower temperature, 23°C, for 20 hours. Since these sections were stained after incubation, degradation or removal of aniline blue could not be responsible. It is possible that prolonged incubation in water at room temperature, or for a shorter period at a higher temperature could alter the structure of callose, its binding capacity to the stain or cause partial degradation. These may in turn affect its fluorescence.

Even with the standardization of procedure and conditions, variation in fluorescence intensity sometimes occurred in sections which necessitated the use of many replicates to give statistical validity to the results.

2) Effects of parasitic tissue extracts of *C.purpurea*

Having established the ability of B.QM806 β -glucanase to degrade wound and phloem callose, extracts from parasitic mycelia of *C.purpurea* of differing ages were tested. Wounded ovary sections were incubated in dialysed honeydew or selected parasitic mycelial extracts as shown in Table 11. The parasitic extracts used here have been shown to possess relatively high glucanase activities, (see 3.3.). In all cases fluorescence around the wounds and in phloem tissue appeared to be weaker than in controls (Table 11).

It would seem therefore, that parasitic tissue extracts have the ability to degrade not only wound callose, but also phloem callose. However, subjective comparison of the brightness of different micro-

Table 11.

Effect of parasitic extracts of C.purpurea on callose fluorescence of wounded ovary sections

<u>Extract</u>	<u>Temperature</u> (°C)	<u>Protein Concentration</u> (mg/ml)	<u>Duration of incubation</u> (hrs)	<u>Fluorescence*</u>
Dialysed honeydew	23	1.3	2	+++
	45	2.5	1	+
	45	2.5	2	+
Apical part of 14-day old parasitic tissue	45	1.2	3	++
Apical part of 22-day old parasitic tissue	45	1.4	2	++
Apical part of 30-day old parasitic tissue	45	1.5	2	+
Control	23	-	2	+++++
	45	-	2	++++

* Estimated on an arbitrary scale: + = very weak

+++++ = strong

scopical preparations is notoriously difficult to assess (Nairn, 1969). It is difficult for the human eye to discern small differences in light intensity especially if the two light sources are viewed consecutively (and not placed side by side). Inevitably this leads to errors. The present method therefore, is recognised as being inexact and inadequate to wholly justify the above conclusion.

In addition, since sections were illuminated by transmitted light, the thickness of the section greatly influenced the intensity of fluorescence observed: i.e. intensity is reduced with thicker sections. This problem does not arise with incident or epi-illumination (see Appendix II). Since up to this stage observations had been made using transmitted light, the observed fluorescence was subject to some error due to small variations in section thickness. Because the visual assessment of fluorescence used in the present study had so many shortcomings, a quantitative method utilising the advantages of epi-illumination (see Appendix II) was adopted to provide numerical evidence of reduction in fluorescence when sections were incubated in C.purpurea extracts.

ii) Microspectrofluorimetric assessment of fluorescence intensity

Measurements of fluorescence emitted from the wound areas after treatment with various exo- β -1-3-glucanases are shown in Table 12 which includes results from both methods of measurement (i.e. whole wounds and long axes of wounds). The value for whole wound fluorescence show a significant difference between control sections and those incubated in either dialysed honeydew or the exo- β -1-3-glucanase from Basidiomycete QM806. The difference in fluorescence between control sections and those incubated in the parasitic Claviceps extract was not significant. This indicates by this method of measurement, that both B.QM806 glucanase and dialysed honeydew hydrolysed callose to a marked extent and that the Claviceps extract apparently did not.

The results of any experiment are as good as the method used

Table 12. Effect of Exo- β -1-3-glucanases from various sources on the fluorescence of wound callose, measured by two different methods.

<u>Fluorescence</u> (photons/sq. μ /sec.)				
<u>Area measured</u>	<u>Water</u>	<u>B.QM806 β-glucanase</u>	<u>Dialysed</u>	<u>Claviceps</u>
	<u>Control</u>		<u>Honeydew</u>	<u>Extract</u>
1) Whole wound	0.446	0.255	0.271	0.372
	(± 0.033)	(± 0.053)	(± 0.053)	(± 0.042)
'P' value*	-	0.01	0.01	N.S.
2) Long axes of wounds	19.66	5.57	6.54	7.84
	(± 1.96)	(± 2.78)	(± 2.78)	(± 1.96)
'P' value	-	0.002	0.002	0.01

*Significance of difference between control and treatment

N.S. = Not significant

to obtain them, and, the present measurement of fluorescence from whole wound areas leads to several inaccuracies. The size of the wounds and distribution of fluorescent spots around the wounds were such that relatively large areas of black background had to be incorporated into the area measured (Fig. 7). This background included non-fluorescing tissue as well as the actual hole in the section, and the relative proportions of background to fluorescing tissue varied between sections. This clearly reduces the fluorescence intensity on a per area basis. Another significant factor was the inclusion of non-callose fluorescing material e.g. cellulose of hairs, which appeared brighter than normal cell walls, chlorophyll especially in the ovule, dust etc.

By measuring fluorescence using the second method, the above factors were largely eliminated, resulting in more reliable estimates. This method ensured that only callose fluorescence was measured, and the amount of non-fluorescing background was considerably reduced. This increase in selectivity and specificity is reflected in the results (Table 12), which show that all three β -1-3-glucanase sources hydrolysed wound callose to a significant degree as compared with controls. Sections incubated in B.QM806 β -glucanase showed no residual fluorescence that could be attributed to callose. In all treated sections, it was noticed that phloem callose fluorescence was also greatly reduced, although quantitative measurements were not obtained. This supports the observations from the previous tests in which reduction of fluorescence was assessed visually, and a greater range of Claviceps extracts was used.

Statistical analysis of these results show that the readings from controls are significantly different from B.QM806 and dialysed honeydew incubations at the $P=0.002$ level, and from Claviceps extract at the $P=0.01$ level. When the three treatments were compared with each other for differences, B.QM806 and honeydew readings were found not to be significantly different from each other, neither were honeydew and Claviceps extract,

but the difference between B.QM806 and Claviceps extract incubations was significant at the $P=0.02$ level. This is perhaps to be expected since Basidiomycete QM806 produces a very active β -1-3-glucanase and the extract used had a protein concentration of around 60%. The protein concentration of the Claviceps extract was only about 29%, and that of honeydew about 50%. Had the tests been performed using extracts of equal protein concentration, the Claviceps extract might well have reduced the fluorescence to a more statistically significant value.

In fluorescence microscopy, one of the major problems is fading of fluorescence on exposure of the section to UV light. The rate of fading is influenced by many factors, and is particularly significant in fluorescence photomicrography, where exposure times can be very long. The fluorescence of the present sections did fade during photomicrography and this topic is discussed later (Appendix III). In microspectrofluorimetry, the exposure times are generally shorter than in fluorescence photomicrography, so that measurements of fluorescence can often be taken before fading occurs. The duration of exposure is therefore an important consideration in this context, especially if duplicate readings of fluorescence are to be taken. In the present study, sections were exposed for 10 seconds and the accumulated fluorescence was recorded. When this was repeated almost immediately on the same area, the readings indicated that no fading had occurred as a result of the previous exposure.

c) Hydrolysis of pollen tube callose

The results presented so far have indicated that both wound and phloem callose of cereals can be degraded by various parasitic extracts of C.purpurea and B.QM806 β -glucanase.

Tests were carried out to investigate whether callose from pollen tubes is subject to similar degradation. Daffodil (Narcissus spp.) pollen was used in addition to rye and wheat pollen.

i) Germination of pollen

Daffodil pollen germinated very easily and quickly (within 1 hour) in both Rosen's and Loo and Hwang's media, either liquid or solid. However, the appearance of the pollen tubes was different; in Rosen's medium, they were long and fairly straight whereas in Loo and Hwang's medium they were shorter, often coiled with swellings along their length. On incubation in a mixture of these media, the tubes appeared less stunted and coiled, and were of intermediate length. When stained with aniline blue and viewed under UV light, pale yellow fluorescence was observed in the pollen tube walls, and brighter fluorescent structures occurred at irregular intervals within the tubes, often appearing as plugs. The pollen grains stained a dull blue.

Neither rye nor wheat pollen grains germinated in either medium, nor when incubated in stigma extract (stigmas at anthesis ground in 0.01M acetate buffer pH 5.2, and centrifuged). Often the grains were ruptured; the cytoplasm appeared to have been released through a break in the wall. It is well known that grass pollens are particularly difficult to grow in vitro (Rosen, 1968) and tests on wheat pollen have not yielded satisfactory results (Goss, 1968). Loo and Hwang (1944) however, claimed success with their medium, but results were disappointing when their medium was used in the present study. Varying the temperature, sucrose concentration or light conditions had no effect on germination. Only when pollen grains were placed in contact with the stigmas did germination and tube growth occur. After staining, when viewed with UV light, the tube walls fluoresced bright yellow (Plate 38b) some along the whole length, others at intervals. Most of them fluoresced at the pollen grain pore, where the germ tube emerged. Fluorescence of the plugs was also obvious (Plate 38b).

ii) Effects of β -glucanases

The fluorescence in daffodil pollen tubes was assessed visually

and appeared to diminish in intensity or be removed after treatment with either B.QM806 β -glucanase or freeze-dried extract of 14-day old parasitic ergot tissue. Incubation in crude undialysed extract of the same tissue resulted in fluorescence similar to that of controls, where plugs, parts of walls and pollen grain pores fluoresced bright yellow.

When rye pollen tubes, growing on excised stigmas, were incubated in the various β -glucanases at 37°C and 50°C, fluorescence was absent in those incubated in B.QM806 β -glucanase at both temperatures. The 14-day old parasitic ergot tissue extract appeared to remove most of the wall and plug fluorescence at both temperatures, but some often remained at the pollen grain pore. Undialysed honeydew caused inconsistent reduction in fluorescence and controls fluoresced as above.

It seems therefore, that B.QM806 β -glucanase and partially purified parasitic ergot tissue extract can degrade pollen tube callose of rye, and also of a plant (Narcissus sps.) taxonomically unrelated to rye. This conclusion is drawn from visual assessment of fluorescence and is therefore subject to the same criticism as that discussed (p.220) for wound callose. However, in many cases, the fluorescence in the pollen tubes had been so completely removed that the activity of the enzyme was obvious. The results suggest that both B.QM806 β -glucanase and partially purified parasitic ergot extracts are capable of degrading all three types of rye callose: wound, phloem and pollen. It is not known whether the three callose types have identical structures and how specific the enzymes are for β -glucans with slight variations in structure. It has been suggested (Currier, 1957) that callose might be "a heterogeneous substance exhibiting source-dependent compositional differences". When wound and pollen callose of rye were irradiated at 400nm in the microspectrofluorimeter, and the fluorescence emission spectrum was scanned, the peak wavelength for wound callose fluorescence was at 540nm whereas

that for rye pollen tube callose was 496nm. This difference may perhaps be caused by structural variations in these two β -1-3-glucans, possibly in the degree of type of branching.

In summary, therefore, although the 3 calloses may vary slightly from one another, the fungal enzyme appears able to hydrolyse them all. The significance of this in relation to infection (where degradation of pollen-tube callose may be important) and further colonization by the parasite (where phloem and wound callose degradation may subsequently become more important) is discussed later.

4. DISCUSSION

4.1. MICROSCOPY OF INFECTION AND ERGOT DEVELOPMENT

When the host plant is inoculated artificially, the infection process and development of C.purpurea on rye is virtually identical to that on wheat. In the field, wheat flowers do not always open at anthesis, and fungal spores probably have more difficulty infecting wheat than rye whose flowers open widely. It may be, therefore, that the range of natural primary infection sites is different for the two cereals and that consequently the infection processes vary. However, in the present studies, the colonization sequence described for the fungus was common to both cereals. In artificial inoculation, the inoculation procedure may also affect the primary infection site and may partly account for inconsistencies in the literature on this subject i.e., spraying or painting a spore suspension on anthesing florets is likely to lead to a different distribution of spores on the receiving ovaries than injecting a spore suspension to fill the floral cavity. The former method may be expected to apply spores preferentially to the stigma while the latter could distribute inoculum on all ovary surfaces.

The present account shows that hyphae are capable of penetrating via the ovary surface or through the stigma. Several workers (Kuhn, 1863; Kirchhoff, 1929; and Campbell, 1958) have discounted stigma infection as the primary site although they allow that stigmas could become infected, but only after the base of the ovary has been colonized. Engelke (1902) and Milovidov (1954) maintain that the stigma is the primary site. In a recent study of ergot infection in dallisgrass (the causal organism is C.paspali), Luttrell (1977) showed that infection occurred through the stigma and style. Engelke (1902) likened infection by C.purpurea to pollen germination and pollen tube

growth through the transmitting tissue to the ovule. This is supported by the present study and is clearly shown by electron microscopy (Plate 7-10). The transmitting tissue, evolved to facilitate the passage of pollen tubes, is utilized by the fungal hyphae in a similar manner. Having reached the apex of the ovule, the hyphae take the path of least resistance and colonize the outer integument fairly extensively before penetrating the ovule. In ergot of pearl millet (Pennisetum americanum) caused by C.fusiformis, Thakur & Williams (1978) showed that the major infection path is through the stigmas, since once the stigmas withered, little infection occurred. The flowering habit is an important factor in this since pearl millet is highly out-crossing and extrudes its stigmas 2-5 days before the floret opens. The stigmas, therefore, are usually the only part of the pistil exposed to spores. Once pollination occurs, the likelihood of infection is severely reduced since the stigmas rapidly wither. In rye however, the stigmas and anthers normally emerge simultaneously. Therefore, the stigmas are not usually exposed for such a long period before they are pollinated. In such cases the chance of ovary wall infection probably equals that of stigma infection. Since wheat is usually self-pollinated, the flowers often open only slightly or not at all, thus the stigmas are the most likely parts of the pistil in which infection can occur.

However, according to Kuhn (1863), the ovary base is the major infection site, in which region infection becomes extensive while the rest of the ovary, including the ovule, is still healthy. Kirchhoff (1929) agreed and suggested that spore germination occurs on the stigma where long germ tubes develop and grow over the external surface of the ovary for 1-2 days before penetrating at the base, which he regarded as the most sensitive site. Campbell (1958) found no evidence of spore germination on the stigma nor long germ tubes on the ovary surface, and suggested that a limited mycelium is produced which penetrates

the ovary at the base.

The evidence provided in the present account does not negate the mechanisms proposed by the other authors and in certain conditions one method may be more dominant. However, it is difficult to envisage the stigma as being unreceptive to the spores, since it is a very delicate and easily penetrable structure. In addition, stigma infection is characteristic of several ovarian pathogens e.g. Smuts (Weihsing, 1956; Fischer & Holton, 1957; Luttrell, 1976), and it seems likely that stigma infection by Claviceps spp. would occur fairly commonly in natural conditions.

There are several possible explanations for the contradictions in the literature regarding this stage. First, as has already been mentioned, the various authors used different inoculation procedures and often used different cereals. Campbell (1958) for instance, sprayed barley with a spore suspension using an atomiser, while Milovidov (1954) studied rye, having dipped the ears into a spore suspension. In the present work, wheat was used to a greater extent than rye, and ears were inoculated by injecting a spore suspension into the floral cavity. Milovidov (1956) found that although the internal anatomy of sclerotia from ten different grasses was indistinguishable from that of rye ergots, the transition from sphacelial to sclerotial growth occurred faster in the grasses. This could suggest that comparison of the development process, especially the timing of events, between different cereals may not be strictly valid.

Secondly, environmental conditions influence the development rate. Previous authors have often compared their findings with those of other authors without allowing for substantial differences in the environments of the host. I have noticed that in glasshouse grown cereals, the colonization rate of the ovary may be faster than in cereals grown outside in plots, and it is easy to miss certain

stages and be unaware that they have in fact occurred. Campbell (1958) states that "different stages of the infection phenomenon follow one another very rapidly" and that "unless observations are initiated within one day after infection and continued daily thereafter the various events may well be missed or misinterpreted." Therefore most accurate results may be obtained when the development is at a sufficiently slow rate to allow full observation of the various stages, and outdoor cultivation of the cereal provides these conditions. Hence the different stages that Campbell describes are supported by the present work although his time-scale is shorter by about 2 days with regard to onset of honeydew exudation. In both Campbell's and the present account, penetration into the ovule was first noted about 5 days after inoculation. According to Campbell, sporulation and honeydew exudation also began at this stage, whereas in the present description sporulation began 5-6 days after inoculation and honeydew exudation did not occur until about the 7th day. These differences could be due partly to the fact that Campbell studied glasshouse grown plants while the present work deals with plants grown outdoors.

A third factor in the discrepancies between authors is that some of the earlier work was performed over a century ago e.g. Tulasne, 1853. Although the standard of some of the early work is very high, there has since been considerable sophistication in instruments and technique, and it is now possible to prepare and view better sections with better microscopes and interpret accordingly. In the previous studies (Kuhn, 1863; Engelke, 1902; Kirchhoff, 1929; Milovidov, 1954; Campbell, 1958), where micrographs have been published of supposedly intracellular hyphae, the micrographs are often of poor quality and it is difficult to ascertain whether the mycelium simply appears intracellular because of the thickness of the section and abundance of hyphae around and over the cells, or whether they are truly intra-

cellular. Carew & Schwarting (1959) working on rye callus, state that growth is mainly intercellular with occasional cell penetration and intracellular growth. Campbell (1958) maintains that at about 5 days after inoculation of barley florets, the hyphae become intracellular, and Milovidov (1954) also noticed intracellular hyphae. The micrographs presented by Carew & Schwarting, Campbell and Milovidov are not, however, a convincing basis for this conclusion, which is also not supported by the present investigation. Luttrell's work (1976,1977) supports the present study and he states that the ergot fungus is intercellular in habit. However, this argument hinges on the interpretation of the term 'intracellular'. In its simplest form, intracellular means inside the cell i.e. the cell wall must be broken for the hyphae to invade. But should this term still apply if the cell is dying or dead when the wall is finally broken? The present study shows that in the early stages of colonization, the hyphae are intercellular, and erode the middle lamella and soften the cell walls as they grow, presumably enzymatically. There is no evidence of localised cell wall degradation as with intracellular parasites. By 5 days after inoculation, the host cell walls are considerably invaginated and eroded to a thin structure (Plate 14-16). The protoplast at this stage is also beginning to degenerate and soon dies (Plate 17). Therefore there may be a short period when the hyphae breach the cell wall and the host cell is still living, albeit degenerating. Invasion of such senescing tissue could be interpreted as intracellular penetration. Hyphae were often observed within the confines of host cell walls, particularly the upper layer of the 'plate' of cells at the ovary base (Plate 21,22). Careful examination however, showed that the cell contents had already degenerated and disappeared, and the lumen of the cells had become filled with fungal hyphae. Therefore the present study supports the view that C.purpurea is an intercellular

parasite which causes widespread enzymatic erosion of the middle lamella and general weakening of the host wall, such that the cell soon dies and moribund cells may then be colonized. An intracellular growth habit would be incompatible with the rapid destruction and replacement of the ovary which is essential for ergot development.

During the present ultrastructural studies, the occurrence of Woronin bodies was discovered in the hyphae, which organelles have not been previously reported in C.purpurea. Woronin bodies were first discovered in 1886 by Woronin in cells of Ascobolus pulcherrimus, and since then have been found only in certain Ascomycetes and Deuteromycetes (Buller, 1933). Their origin is uncertain but they are usually found associated with the septa, and often near or in the septal pore. Two possible functions have been suggested for Woronin bodies:

- 1) By plugging the septal pore they regulate the cytoplasmic flow in healthy hyphae. If the hyphae are injured, septal plugging prevents leakage of the contents, and in the event of cell death, it separates the living cells from the dead. (Reichle & Alexander, 1965; McKeen, 1971; Wergin, 1973; Brenner & Carroll, 1968; Bracker, 1967)
- 2) Woronin bodies may be sites of enzymatic activity. They resemble microbody inclusions (Maxwell et al, 1977) and their development within and extension from microbodies has been observed in Fusarium oxysporum f. sp. lycopersici (Wergin, 1973). Since some microbodies have enzymatic activity, it is thought possible that the same applies to Woronin bodies. For example, catalase activity has been shown in a particle, found in Aspergillus tamarii, which has very similar characteristics to Woronin bodies (Graves et al, 1976).

In C.purpurea, Woronin bodies were often found in equal numbers on either side of a septum. When they were found in the septal pore, adjacent cells usually had different densities. In such cases they may have been plugging the pore, or, as observed in Ascodesmis sphaerospora (Brenner & Carroll, 1968) were in the process of degradation, especially if the cells were old. Their origin, development and function in C.purpurea, however, are as yet unexplored.

One point on which all previous workers in C.purpurea pathogenesis agree is the lack of fungal penetration into the rachilla. Fungal penetration does not proceed into the rachilla beyond a 'plate' of cells at the base of the ovary where the complex of vascular tissue is situated. The core of thick-walled cells and its ring of transfer cells and sieve tubes remain intact (Plate 26) and presumably continue to function in supplying the ovary base. The lower portions of the 3 ovarian vascular traces were often observed in the 'plate' of cells, and sometimes continued into the fungal tissue for a short way. The reason for the absence of rachilla penetration has not been fully ascertained. It is possible that the upper layers of the 'plate' cells may inhibit further fungal invasion by, for example, host cell wall modification and production of phenolic compounds. It is equally possible, however, that since the ergot is effectively 'sitting' on a continuous nutrient supply, there is no necessity for further penetration. Although the ovary "serves as little more than a portal of entry" (Luttrell, 1977) in both C.purpurea and C.paspali infections, the essential factor in infection is the establishment of a compatible relationship with the host tissue in the rachilla. Further ergot development depends on the nutrient supply from this region, through the maintenance of this compatible relationship. However, whether the 3 ovarian traces alone have the capacity to

supply the rapidly developing ergot is questionable. Large quantities of honeydew often exude for 1-2 weeks, and, since the ovarian traces are small, and designed to supply the ovary only until shortly after it has been fertilised, it is doubtful that they are the only channels for nutrient flow to such a large sink as the ergot. An alternative is that the contents of the vascular tissue around the core continually flood into the plate of cells at the host-parasite interface. The parasite, therefore, would receive nutrients from the whole surface area of the plate of cells, rather than from 3 distinct points of the vascular traces. This is perhaps more compatible with the observation of large volumes of honeydew exudation. If this were the case, the parasite would need to initiate and maintain the flooding by vascular contents, and prevent the possible sealing of the tissue by a wound-healing type reaction. Since β -glucanase activity of the parasite has been shown in the present study, this could provide a mechanism to fulfil the above requirements, as further discussed later.

At the time of transition from sphacelial to sclerotial growth form, several anatomical and biochemical changes occur in the hyphae. (1) The first visual indication was a pale purple pigmentation on the surface of the sphacelium, in the lower half (Plate 26). This occurred at about 12 days after inoculation, and the fungal hyphae stained darker, were wider and more bulbous, and appeared to have more frequent septa than the surrounding sphacelial hyphae (Plate 28b). (2) There were earlier microscopic indications that differentiation was imminent i.e. at 10 days electron microscopy revealed some changes in growth form. The hyphae were shorter, more ovoid in shape and had more frequent branches and septa than those observed at earlier stages (Plate 25). A major feature was their increased lipid content; lipid occurred as small droplets which coalesced to form large deposits and bulged into the vacuole, in-

vaginating the tonoplast. (3) Earlier work (Corbett et al, 1974) showed that the transition from sphacelial to sclerotial growth form was associated with major metabolic changes (i.e. lipid content and dry weight increased while total protein, RNA, malate and polyol content decreased. Carbohydrate levels remained fairly constant and total amino acid content declined slightly.) Nisbet (1975) showed that the respiration rate fell and there was a switch from aerobic respiration to fatty acid oxidation during mycelial differentiation. These changes indicate a lower metabolic activity in the sclerotial mycelium. (4) Sclerotial development having commenced within the lower sphacelium, further sclerotial proliferation occurred from the base, with continuous acropetal displacement of older tissue. Within the compact, barrel-like cells of the sclerotial plectenchyma, there are hyphae showing variations in branching, cell dimensions and septation, as observed previously (Milovidov, 1954; Campbell, 1958).

Therefore, the microscopical evidence suggests:

- a) that the sphacelial mycelium has the capacity to differentiate into a sclerotial form, and usually this begins at one or two locations in the lower half of the sphacelium,
- b) that once this transition has been initiated, further fungal growth at the base results mainly in sclerotial hyphae instead of the sphacelial type.

However, the youngest absorbing hyphae at the base (or host/parasite interface) are not themselves sclerotial. They are distinct in that they contain very little lipid and have large vacuoles. They resemble sphacelial hyphae in their low lipid content and ability at least at the periphery, to form conidia. They appear, however, to be shorter and wider, with noticeably larger vacuoles than typical sphacelial hyphae. Therefore their morphological classification is open to question. During sclerotial proliferation, a small amount of conidiation was obser-

ved at the base, in the region of these lipid-free hyphae, up to at least 21 days after inoculation. The conidia were produced near the external surface of this zone, with no internal pockets of conidiation. The reason for this is not clear, but aeration may be involved since it has been shown to be a factor in sporulation in other fungi (Morton, 1961). The sclerotial hyphae above this zone did not appear to produce conidia. This zone of lipid-free hyphae forms the first contact between the developing sclerotium and the nutrients from the host. It extends distally for about 150 μ and above it the hyphae become sclerotialised.

The processes involved in sclerotial morphogenesis are poorly understood even though a considerable amount of work has been done on sclerotia-forming fungi (Willettts, 1972; Smith & Berry, 1974) but the results are often difficult to interpret. Also, the 3 stages of sclerotium growth i.e. initiation, development and maturation (Townsend, 1952) can be affected differently by a single factor. Sclerotial formation in C.purpurea is perhaps all the more puzzling since such structures are produced only in parasitic conditions. In culture, sclerotial cells are produced, which have several characteristics similar to those in the ergot sclerotia (e.g. lipid content, production of alkaloid (Mantle, 1975; Nisbet, 1975) but have not yet been shown to be able to initiate the sexual stage). In most other sclerotia-forming fungi e.g. Verticillium, Sclerotinia, Sclerotium spp., the sclerotia are usually produced in saprophytic conditions, and hence are more amenable to study (Cooke, 1970; Chet & Henis, 1968; Brandt & Reese, 1964).

It has been shown in C.purpurea, that ergot growth occurs by proximal addition of material, which is forced distally as growth proceeds (Nisbet, 1975). Therefore, the region at the base must have a relatively high rate of nutrient uptake, metabolic activity (Dickerson et al, 1976) and hyphal growth. (One way to test this could be by autoradiography following feeding of the ear with labelled sugars.) Since hyphal elongation occurs at the apex of the hypha (Grove et al, 1970; Bartnicki-Garcia, 1973),

there is presumably a high proportion of hyphal tips in this basal region. Hyphal growth also occurs by branching, which is one of the main prerequisites for sclerotial cell initiation. Therefore, the bulk of the increase in fungal material must occur in the basal region, whether by apical growth or sub-apical branching and septation. These two types of growth might be spatially and temporally separated within the basal region:

- a) the lipid-free hyphae, morphologically not sclerotial, may grow by hyphal tip extension,
- b) the zone of hyphae immediately distal to a) (and which is now distinguished from a) by the deposition of lipid) may grow primarily by branching and septation, which processes are intrinsic to sclerotialization and may continue for some time as the tissue ages.

In this way hyphal tip growth could account for the increase in ergot length, while branching and septation determine the compact plectenchymatic nature of the sclerotium.

An important aspect of branch and septum formation is the change in wall plasticity (Willettts, 1972). Such changes presumably involve the activation/production of wall-lysing and synthesising enzymes at the site of branch or septum initiation (Rosenberger, 1978). The production of lysing enzymes is affected by the nature of the nutrient source (Rosenberger, 1978). In addition, the availability of sulphur might affect branching and septation since S-bands are important in maintaining the structure and activity of proteins - in this case particularly wall proteins (Brachet, 1964; Nickerson & Falcone, 1959; Chet et al, 1966; Chet & Henis, 1968).

It is clear that the nutrients which the sclerotium receives play a major part in sclerotial morphogenesis. In parasitically growing C.purpurea, exactly what triggers the transition from sphacelial to sclerotial is not known. However, in several isolates, such a transition

'in vitro' has been initiated by certain amino acids (Nisbet, 1975; Mantle & Nisbet, 1976). In particular asparagine/aspartic acid and glutamine/glutamic acid are important, where the amide supports sphacelial growth while the acid supports sclerotial growth. In the parasitic state, the fungus receives a complex mixture of amino acids, some of which are more readily utilizable than others. The changes in the relative proportions of the amino acids may be involved in the switch mechanism from sphacelial to sclerotial growth (Nisbet, 1975).

During ergot development, the ergots themselves can be dislodged relatively easily from the floral cavity, unlike healthy ovaries and seeds. Sclerotia break at their base, leaving behind some fungal tissue, which appeared to correspond to the zone of lipid-free absorbing hyphae. The difference in mechanical properties between the lipid-free and lipid-filled hyphae appears to be responsible for this line of weakness. It may however serve a useful purpose at ergot maturity, when easy dislocation from the ear would be advantageous in ergot dissemination and help to ensure its overwintering in the field.

4. 2. PECTOLYTIC ENZYMES IN PATHOGENESIS

a) 'In Vitro'

The aim of the present study was to demonstrate whether C.purpurea has the ability to produce pectolytic enzymes 'in vitro' and 'in vivo'. Since this is the first report of pectolytic enzyme production by this fungus, either 'in vitro' or 'in vivo', many questions remain to be resolved. Fundamental information on optimum cultural conditions such as pH value, temperature, buffer system, requirement for certain ions (e.g. calcium for certain lyases) and age of culture at maximum enzyme production is unavailable. Optimum conditions for enzyme extraction (from culture filtrate and infected tissue) and purification had not been established and so, in the present studies, enzyme preparations were necessarily relatively crude. However, workers investigating other host-parasite interactions have used similarly crude material and have obtained positive results (Cole & Wood, 1961; Talboys & Busch, 1970; El Goorani et al, 1976; Kurowski & Dunleavy, 1976). The enzyme assays used here have been previously used by many workers, but again the optimal conditions for assay of C.purpurea enzymes were not known. Since there is so much variation in pectolytic enzyme activity from different pathogens, it is difficult to extrapolate a set of conditions from one organism to another. Therefore, although this fungus was shown to produce pectolytic enzymes, it is likely that measurements of enzyme activity are inaccurate, probably under-estimated and certainly incomplete.

Since C.purpurea grows 'in vitro' on pectin and polygalacturonic acid as main carbon sources, (asparagine in the medium also contributes some carbon) it can evidently degrade and utilize it. However, pectin (3.5%) and polygalacturonic acid (2.5%) as the

main carbon sources supported less growth (about half and one third respectively) than when glucose (0.25%) was included in the medium (Table 4). Glucose (5%) supplied as the main carbon source yielded the highest mycelial dry weights. This is not unexpected since glucose is a most readily utilizable substrate, and other workers have noted that pectic substances supplied alone often supported insignificant growth (Ayers et al, 1969; Papavizas & Ayers, 1964). Correspondingly, the pattern of pectolytic enzyme production varies depending on the substrate supplied. This phenomenon is well known in a variety of microorganisms (Waggoner & Dimond, 1955; Hancock et al, 1964; Mussell & Strouse, 1971; Talboys & Busch, 1970). When C.purpurea was grown on pectin as the main carbon source, all five pectolytic enzymes (PE, PG, PMG, PGL and PMGL) were produced (Table 5). However, when glucose was incorporated into the medium, the activity of PE and PMG was depressed. The cause of this is probably catabolite repression, since some pectolytic enzymes are known to be subject to this type of regulation (Bateman, 1976). In the case of PGL, no activity was detected when glucose was included in the culture medium. Although this may also indicate catabolite repression, the activity in the glucose-free medium was also low (1.29 μ moles product/ml/mg protein/min.), therefore the zero reading may represent an undetectable amount but not significantly different from the above value. In a similar way the slight increase in PMGL activity observed when glucose was included may only be within the normal variation expected between replicate flasks of glucose-free medium. PG activity also appeared to increase when the fungus was grown on pectin + glucose medium. Therefore at least the activities of two enzymes, PG and PMGL, are apparently not diminished by the addition of 0.25% glucose. It may be that these two enzymes are not subject

to catabolite repression when the glucose (repressor) concentration is only 0.25%. Other workers have found that not all pectolytic enzymes of pathogenic fungi are subject to catabolite repression (Ayers et al, 1969; Mussell & Strouse, 1972; Bateman et al, 1973), and so far these enzymes have been found to be constitutive. Hancock et al (1970) found that glucose repressed pectate lyase synthesis in Hypomyces solani when its concentration exceeded 0.025% and similarly for galactose and galacturonic acid at 0.1%. Possibly PG and PMGL of C.purpurea are constitutive enzymes which are subject to catabolite repression at glucose concentrations higher than 0.25%. Similarly PE, PGL and PMG could be constitutive but repressed at much lower glucose concentrations. This however is speculation since there is little evidence to demonstrate it in the present study. A different pattern of pectolytic enzyme activity may have resulted if the filtrate samples had been taken after a longer period of growth (e.g. 12 days instead of 5). By 12 days, a substantial amount of glucose may have been utilized, thereby releasing enzyme synthesis from catabolite repression and allowing increased production of such enzymes as PE, PGL and PMG.

In order to investigate whether enzymes are constitutive or inductive, many workers have grown the organisms on a non-pectic carbon source and measured any resulting pectolytic enzyme activity. When this was done in the present study, and the glucose concentration was 5%, no PE, PG or PMGL activity was detected (PGL and PMG activities were not tested). While this may suggest that PE, PG and PMGL are only inducible, other explanations are equally possible. It may be that these enzymes are in fact constitutive but were under catabolite repression. This has been shown in other organisms (Moran & Starr, 1969; Hsu & Vaughn, 1969). Cooper & Wood (1975) pointed out that in much of the work on induced and

constitutive enzyme synthesis, the effects of catabolite repression were either ignored or insufficiently allowed for, and therefore results were difficult to interpret. They suggested supplying potential inducers at rates sufficiently low as to preclude catabolite repression. When this was done, enzymes previously thought to be inducible were shown to be constitutive but subject to catabolite repression in circumstances where the catabolites were present slightly in excess of the requirements for growth (Cooper & Wood, 1975; Cooper, Rankin & Wood, 1978). In the present study, glucose was supplied at 5% in the medium (Type C), which concentration could readily repress synthesis of pectolytic enzymes. Further studies using different levels of glucose or a poor carbon source, and purer enzyme preparations would elucidate whether these enzymes are inducible or constitutive, and the extent of catabolite repression. Therefore, from the evidence presented in this 'in vitro' study, a conclusion cannot be drawn as to the inducible or constitutive nature of pectolytic enzymes of C.purpurea.

b) Pectolytic Enzymes in Ergot Parasitism

In the present study, three lines of evidence suggest that pectolytic enzymes may be secreted during parasitism of the cereal ovary by Claviceps purpurea, and possibly play a role in pathogenesis. First, histological evidence from light and electron microscopy shows that there is considerable degradation of the middle lamella. Since this fungus has an intercellular growth habit within host ovaries, one might expect an effective method of penetration to have evolved. However, this need not involve wall degrading enzymes since mechanical pressure (by hyphal growth or osmotic force) might be sufficient to allow fungal passage between cells. Indeed, until scanning and transmission electron microscopy became

widely used, direct penetration of walls by pathogens was thought to be primarily a mechanical process (Gaumann, 1955; Goodman et al, 1967). Fungi have been shown to be capable of exerting sufficient force to indent a film of cured epoxy resin (Mercer et al, 1971), but there is little evidence to show that such force is required or exerted during cell wall penetration. What has been demonstrated many times is that hyphae can penetrate cell walls through small holes formed apparently by solubilization of part of the wall. Also cell wall softening and invagination has been shown with clear signs of alterations in appearance, staining properties and texture of the wall (Mercer et al, 1971; Bracker & Littlefield, 1973; McKeen, 1974). This kind of evidence has led to the general view that enzymatic activity plays an important role in cell wall penetration, with mechanical pressure taking second place. According to Albersheim et al (1969), cell wall degrading enzymes are universally produced by plant pathogens. Their relative importance in pathogenesis, however, must be assessed in each individual host-parasite combination since their mere presence need not indicate a significant function. In the present microscopical study there is evidence that in the initial stages of colonization, the middle lamella in the vicinity of the intercellular hyphae is less electron dense and takes on a loosely fibrillar arrangement, which distinguishes it from the normal appearance of the middle lamella (Plate 3b, 6c). This erosion appears to continue so that at a slightly later stage the cell wall itself becomes softened and appears to invaginate easily (Plate 10a, 15, 16). There is no sign of fracturing or cracking of the wall which buckles and seems pliable, and yields to the fungal hyphae without much apparent resistance. There is no evidence of direct penetration through the cell wall into the cell lumen, as with intracellular pathogens,

since C.purpurea confines itself to the intercellular areas of living tissue. In some pathogens e.g. Erysiphe spp. and Colletotrichum spp., the action of wall degrading enzymes appears to be highly localised and only the material in direct contact with the hyphal tip is degraded. This is especially so however in intracellular fungi where only a small perforation in the wall is required for fungal entry otherwise the cell would die rapidly. In C.purpurea, the enzymatic activity appears to be diffuse, and results in softening of larger areas of wall without actually breaching it, in a manner similar to the early stages of haustorial invagination (Kajiwara, 1971; Wheeler, 1975). These properties are consistent with those caused by pectolytic enzymes and therefore, from microscopical evidence, it seems that these are involved in colonization by C.purpurea.

The second piece of evidence of pectolytic enzyme involvement in parasitism is the neutral red test performed on sections of infected tissue (Plate 17). This demonstrated, by their inability to retain this stain, that host cells surrounded by hyphae were not viable. Cells adjacent to these, not yet extensively invaded, retained the stain, showing, by Tribe's criteria (1955) that they were still functioning. Since cell injury by pectolytic enzymes is characterized by loss of ability of cells to retain neutral red stain, the above observed phenomenon implies that pectolytic enzymes may play a part in cell separation and death. In the present study, conductivity measurements were not taken and therefore the neutral red test may have underestimated the extent of damage to the cells. The extent of cell death in the immediate vicinity of hyphae has not been determined in the present study. Cole (1956) has shown that apple cell death occurred in advance of Sclerotinia laxa hyphae which suggested a diffusion of a toxic substance a

short distance ahead of the hyphae. However, in the present microscopical study, several hyphae were noticed, during the early stages of infection, to be very near or even adjacent to cells undergoing division. This seems to imply that the presence of the hyphae at that stage did not obviously interfere with the normal cell processes. It may be that at such an early stage (3 days) the sparseness of hyphae in the tissue was such that the concentration of 'toxic substances' was insufficient to disrupt functioning of the cells. Alternatively their action may be slow, and their effects may not have manifested themselves so rapidly in such actively dividing tissue as the ovary. Therefore in the initial stages at least, the factor(s) causing cell death, which may be pectolytic enzymes, does not appear to affect cells far in advance of the hyphae. Whether this situation changes as colonization proceeds has not been elucidated.

Thirdly, biochemical assays of pectolytic enzymes in diseased tissue have shown that at least three such enzymes were present in infected tissue. Several workers, studying other plant pathogens, have shown by iso-electric focusing and other methods, that most of the increase in pectolytic enzyme content of infected tissues is due to the pathogen (Byrde et al, 1973). However, there are reports of increases in host pectolytic enzymes following infection e.g. polygalacturonase (Barash & Khazzam, 1970) and pectinesterase (Bateman, 1963). In the present studies the pectolytic enzymes were detected at stages when very little intact host tissue remained, which implies a fungal origin for these enzymes. The three enzymes were: pectinesterase, polygalacturonase and polymethylgalacturonase. The presence of the transeliminative enzymes was not investigated. However, the 'in vitro' studies of pectolytic enzyme production by C.purpurea showed that transelim-

inases were produced on a pectic medium. It is recognised that 'in vitro' enzyme production cannot be correlated with 'in vivo' activity (Bateman, 1976) because of the great differences in these two environments. The effects of single components within these environments can have profound influence over enzyme activity. But since C.purpurea has at least the ability to produce transeliminases 'in vitro', these enzymes may also be produced 'in vivo'. As previously mentioned, two types of pectolytic enzymes, namely polygalacturonases and pectic transeliminases of the endo-type, have been shown to cause tissue maceration and cell death. The presence of at least one of these enzymes in infected tissue could implicate it in some aspect of pathogenesis.

c) Possible Control Mechanisms of Pectolytic Enzyme Activity

The evidence presented thus far appears to suggest that pectolytic enzymes may be involved in the parasitism of cereals by C.purpurea. The regulation of the control system of pectolytic enzyme production may be significant in this parasitic relationship, as has been shown for other host-parasite combinations (Horton & Keen, 1966 ; Keen & Horton, 1966; Weinhold & Bowman, 1974; Biehner et al, 1972). As previously discussed, carbohydrates are well known factors in the regulation of pectolytic enzyme production, both as inducers and repressors of synthesis and activity. During the first week of colonization of the cereal ovary, C.purpurea would encounter the normal levels of carbohydrates in the walls and middle lamella of the cells. After about a week, when colonization had reached the base of the ovary, honeydew exudation begins. The honeydew contains a high concentration (at least 20% w/v) of sugars, i.e. glucose, fructose, sucrose and a variety of oligosaccharides (Nisbet, 1975), which have been implicated in the

inhibition of certain enzyme activities i.e. β -fructofuranosidase (Nisbet, 1975; Dickerson, 1972) and β -glucosidase (Nisbet, 1975). Since the activity of some pectolytic enzymes is known to be affected by sugar concentrations above a critical level, (i.e. catabolite repression or inhibition), the high concentrations in honeydew might be expected to affect the activity of these enzymes in C.purpurea. This effect has been shown in onion tissue infected with Pyrenochaeta terrestris where treatments which enhanced sugar levels in the host were associated with decreased PG synthesis and less tissue breakdown (Horton & Keen, 1966; Keen & Horton, 1966). Any inhibition or suppression of enzyme activity in C.purpurea might play a role in limiting the extent of its penetration beyond the base of the ovary. (Another factor possibly affecting pectolytic activity and fungal penetration at the base is the presence of phenolic compounds. A brown area of discoloured tissue at the host-parasite interface at the ovary base has often been observed, and it may be fair to speculate that this is due to phenolics and/or their oxidation products. Since phenolic compounds have been shown to inactivate some pectolytic enzymes (Cole & Wood, 1961), it is feasible that their presence might ultimately restrict fungal penetration into the rachilla.)

However, in the present study it was shown that dialyzed parasitic tissue and honeydew had higher PG activity than the healthy control, and that PMG activity increased with age of infection (Table 6). The activity of the other pectolytic enzymes was not measured. Honeydew always showed greater activity than the parasitic tissue. A possible explanation for this is that since these enzymes are produced extracellularly, the leaching effect of honeydew may cause enrichment of the honeydew in terms of enzyme activity, at the expense of the parasitic tissue (especially the

basal portion). It appears therefore, that the synthesis of PG and PMG is not repressed 'in vivo', since if it was, very low levels of activity would be expected in the dialyzed honeydew and mycelial extracts. However, since the extracts were dialyzed, any inhibition by the sugars of enzyme activity rather than of synthesis would not be apparent. Undialyzed extracts were not tested, therefore it remains a possibility that the sugars may repress PG and PMG activity 'in vivo', as they appear to do with β -glucosidase activity (Nisbet, 1975). Interpretation of the 'in vitro' studies allow for these enzymes to be inducible, but could equally indicate that they are constitutive but subject to catabolite repression at glucose levels of 5% or less. Since the concentration of sugars in the honeydew at the host-parasite interface is likely to exceed 20% w/v, very little or no activity might be expected if the enzymes were subject to catabolite repression, whether they be inducible or constitutive. However, again caution should be exercised in extending results from 'in vitro' experiments to the 'in vivo' situation.

By about 10-12 days after infection, the ovary is usually completely shattered and replaced by the invading fungal mycelium, therefore very little pectic material remains 'in situ'. The results show that PG and PMG continue to be secreted as the age of infection increases (oldest sample was 26 days old). This may suggest that these enzymes do not require continuous induction by pectic substrates, and that once induced during the early stages of intercellular penetration (when there is abundant pectic material) production continues. Alternatively, the fungal hyphae at the ovary base, in contact with the host cells, may be continually induced by the pectic components of their walls.

It is apparent that further studies of the 'in vitro' and 'in vivo' activities of these enzymes is necessary before their function and regulation is understood. In addition, it is clear that several lines of investigation should be pursued in order to establish basic facts about the pectolytic enzymes of C.purpurea. For instance, further purification of these enzymes is essential in order to discover their optimum pH value and temperature, and through this, more efficient extraction and assay procedures could be devised. Viscometry and chromatography of reaction products would show whether these enzymes were endo- or exo- in nature. It is important that the effects of these enzymes from infected tissue and culture filtrates be studied on slices of cereal ovary tissue before their activity can be proven. Such studies would reveal any cell separating and killing ability of the enzymes, and further work on isolated cell walls from cereal ovaries may clarify the process of wall degradation. Even so, the ability of these enzymes to degrade cell walls would not prove their presence in the relevant location and in sufficient concentration to cause damage. One way this could be elegantly demonstrated is the use of specific fluorescent-labelled pectolytic enzyme antisera on sections of infected tissue. This method, given the appropriate instruments, should be able to locate each enzyme and estimate its concentration.

The present study therefore, has shown that pectolytic enzymes are produced by C.purpurea both 'in vitro' and 'in vivo'. However, the results are too preliminary to adequately explain the role of these enzymes in parasitism, and how their activity is controlled. Fuller investigation of these enzymes is necessary to substantiate the speculations and conclusions reached in this discussion.

4.3. β -GLUCANASE AND β -GLUCOSIDASE ACTIVITY OF CLAVICEPS Spp.

β -glucan hydrolysing activity was found in C.purpurea during both parasitism and growth in liquid culture. Comparison of such activity with that of Basidiomycete QM806 shows that the enzyme in C.purpurea is relatively less active, although the ergot extract was a less pure enzyme preparation. This does not necessarily reflect upon its effectiveness in pathogenesis. The enzyme produced by B.QM806 is known to be highly active, and in the present work, fulfilled a useful comparative function. The results showed that C.purpurea produced β -glucanase throughout its parasitic development (and in honeydew), its activity increasing with age of infection. Since most of the host tissue is destroyed by 10-12 days after inoculation, this activity can only be attributed to the parasite. By contrast, healthy tissue showed very low activities. The pattern of β -glucosidase paralleled that of β -glucanase. Armed with these 2 enzymes, the pathogen has the capability of degrading at least some of the β -polysaccharides it encounters during parasitism. Activity is not confined to the sphacelial mycelium but was also present in sclerotial tissue, both in parasitic and saprophytic conditions.

During growth in liquid culture, the hyphal morphology at 6 days was typically sphacelial, and 2 days later it started to differentiate into a sclerotial form. This corresponded with a slight decrease in the specific activity of β -glucanase which, however, did not fall below 4.2 μ g glucose/ml/mg protein/min. This activity is higher than that in dialysed honeydew and about 1/15th the activity of B.QM806 glucanase. This relatively high activity in C.purpurea filtrates is presumably sufficient to maintain the fluidity of the culture by hydrolysing any extracellular glucan which may have been produced. Dickerson et al (1970) showed that the sphacelial form of C.fusiformis str. 139/2/1G in culture was associated with the production of an extracellular glucan, which resulted in a viscous cul-

ture. The subsequent glucan hydrolysis was associated with the appearance of sclerotial hyphae in the culture which produced a β -glucanase and restored culture fluidity. Since, in the present study, C.purpurea cultures were fluid throughout growth, glucan accumulation was presumably prevented by the activity of the β -glucanase.

The strain of C.fusiformis, 139/2/1G, had been found as a variant of a parent strain, 139/2/1 (Dickerson et al, 1970). The latter produced highly viscous cultures when first isolated, due to the accumulation of β -glucan and lack of β -glucanase activity. However, when this isolate was used in the present study, culture viscosity was not excessive and β -glucanase activity was detected in the filtrate. This activity was not as high as in the confirmed glucanase-producer, 139/2/1G. The explanation for this may lie in the mutation of strain 139/2/1 during successive vegetative transfers or prolonged storage. This has apparently produced a strain capable of partially degrading the glucan, and forming cultures with a viscosity intermediate between the original strain 139/2/1 and strain 139/2/1G. (The β -glucanase activity of this 'new' strain is 4-5% of that of B.QM806; the activity of strain 139/2/1G was calculated by Dickerson et al (1970) as 5-10% of that of B.QM806.)

Another factor in culture viscosity is the degree of branching in the β -glucan chain. This might partly account for the differences in culture viscosity between C.fusiformis str. 139/2/1, the 'new' strain and C.purpurea. A high degree of branching is associated with high solubility and low viscosity in solution (Buck et al, 1968). Perlin & Taber (1963) found that the glucan of a strain of C.purpurea has β -1-6 branches at every 4th glucose residue. The glucan of a strain of C.fusiformis, F2047, has a similar branching frequency but which increases with age (Buck et al, 1968). The glucan structure of the strains used in the present study has not been determined but any differences which might exist would influence culture viscosity.

In any event, the original intention of using a non-glucanase producing strain for subsequent testing in parasitic conditions was frustrated by the strain alteration, and it was therefore necessary to find a new non-glucanase producing strain. Thus an Indian isolate of C.fusiformis from naturally infected millet (ex Bajra) was selected, since in liquid culture it produced a viscous immobile culture, resembling the original African strain, 139/2/1. Its differentiation into the sclerotial form during saprophytic growth was not associated with effective β -glucanase production. However, in parasitic conditions it did possess β -glucanase activity albeit considerably lower than C.purpurea. Also, polysaccharide (? glucan) was detected in the dialysed extracts of parasitic tissue and honeydew, which did not occur in C.purpurea extracts. This implies that glucan is produced by C.fusiformis during parasitism simultaneous with β -glucanase production. The extent of hydrolysis of this glucan by the detected enzyme is uncertain, since nothing is known about the glucan structure of that particular isolate of C.fusiformis.

The importance of the structure of a particular glucan on enzyme activity has been clearly demonstrated in this study. All the extracts tested for β -glucanase activity showed a higher activity on laminarin than on S.sorghi glucan (Table 7). The structure of the laminarin used in this study has been shown, by chromatography, to be composed mainly of β -1-3 linked glucose with very few β -1-6 branches. S.sorghi glucan however has a β -1-3 linked main chain with β -1-6 branches at about every 5th point (i.e. glucose:gentiobiose = 4:1) (Buck et al, 1968). These differences probably account for the observed differences in enzyme activity. Thus, the low activity in the present parasitic extracts of C.fusiformis may reflect the relative 'unsuitability' of the 2 β -glucans used as test substrates. Possibly higher activities may have been obtained had the glucan been of a more appropriate origin e.g. the pathogen's natural host, millet; similarly for C.purpurea extracts with β -glucans from

wheat and rye.

Electrophoresis of the crude extracts from B.QM806, honeydew of C.purpurea and C.fusiformis (ex Bajra), and parasitic tissue of the latter showed that the position of at least one protein band corresponded in all four extracts. Although these extracts were impure, the evidence suggests that this band may represent the β -glucanase enzyme, since such activity is common to all four extracts.

To summarise therefore, this study has shown that C.purpurea produces β -glucanase and β -glucosidase when growing saprophytically and parasitically. The fluorimetric study of callose degradation by parasitic extracts suggests that β -glucanase may play a role in pathogenesis (as discussed later). If such a role is to be implicated in ergot pathogenesis generally (i.e. other ergot fungi) then such fungi should possess a comparable β -glucanase activity. C.fusiformis usually does not produce such an enzyme during saprophytic growth in sufficient quantity to prevent submerged liquid cultures from becoming extremely viscous. In parasitic conditions, a typical C.fusiformis strain did however produce β -glucanase, although with lower activity than in C.purpurea. However, since the millet floret is smaller, and may possess β -glucans with different structures from those in wheat, the fungal β -glucanase activity may be sufficiently high to fulfil its suggested role in pathogenesis.

4.4. FLUORESCENCE STUDIES OF CALLOSE (β -1-3-GLUCAN) HYDROLYSIS BY
C.purpurea.

The present fluorescence studies established that healthy ovaries, when mechanically wounded, produced a wound reaction involving callose deposition which was visualised by yellow fluorescence after staining with aniline blue. Both wounded and control ovaries showed a similar type of fluorescence in the phloem, due to the natural occurrence of callose in that tissue. In infected ovaries, before wounding, there was no evidence of wound callose, although fluorescence of the hyphae was observed. This is probably due to the β -glucans in the walls, which is also supported by the observed diminution of hyphal fluorescence after incubation of infected sections in the β -glucanase of B.QM806. The difference in the pattern of fluorescence between saprophytically grown and parasitic hyphae could be due to several factors. In the parasitic situation, the composition of hyphal walls or distribution of certain wall components may vary from that in saprophytic hyphae. This might be possible since their nutrient supplies and immediate physical and chemical environments may differ. In addition, enzyme activity may be affected, and the observed variability of hyphal fluorescence may result from non-uniform activity of fungal β -1-3-glucanase. Alternatively, the possibility exists that the host cells may be attempting to immobilise the hyphae by direct secretion of callose, which would further contribute to hyphal fluorescence.

When ovaries at an early stage of infection were mechanically wounded, some wound callose was evident. However, as the infection progressed, the cells appeared to lose their ability to produce wound callose when mechanically wounded. Therefore, when the ovary becomes infected, the cells do not appear to produce callose (or it is rapidly hydrolysed), although for a few days they retain the ability to do so when mechanically wounded.

Callose deposition occurs as a response, not only to mechanical injury, but also to infection by fungi and other agents. Its main function

is thought to be the sealing and plugging of injured cells, and in some cases it may be deposited as a continuous layer. Since pressure applied to tissues (Ziegler, 1964; Umrath, 1932), and intercellular hyphal penetration (Skipp et al, 1974; Heath & Heath, 1971) cause the wound response, the extensive intercellular colonization and cell wall invagination in C.purpurea infections might initiate such a response. The trauma which the pistil undergoes during parasitism by ergot is considerable since the whole ovary is destroyed. Since callose is produced in response even to minor stress, such major injury may be expected to produce a correspondingly larger response. A considerable impedance to fungal penetration could be envisaged if the cells reacted in this way. In addition, further development of the fungus might be prevented at the ovary base if the tissue in the 'plate' was sealed off by deposition of callose. Since Heslop-Harrison (1966) showed that callose may be more impermeable than normal cellulose cell walls, the nutrient flow into the infected floret might be effectively cut off. As a consequence of such restricted nutrition, the establishment of the 'compatible relationship' (Luttrell, 1976) necessary for ergot development would be in serious jeopardy.

The present study showed, however, that although the cells in infected ovaries are, at least initially, able to produce callose, they do not do so. Such an absence of reaction has been observed by other workers in other host-parasite combinations, but has remained a puzzle (Bushnell, 1972). It is proposed in this thesis that the explanation for the apparent lack of callose deposition in ergot infections may lie in the β -glucanase activity of the parasite. C.purpurea produces β -glucanase during parasitic growth and its activity increases just before or coincident with honeydew exudation. Thus this enzyme also occurs in the honeydew. Both honeydew and parasitic extracts were found to be capable of degrading mechanically-induced wound callose. In addition, visual assessment of fluorescence suggests that both phloem and pollen tube callose were also degraded.

The implications of these observations in the pathogenesis of this parasite are fourfold:

- 1) During stigma infection and fungal growth in tissues which are normally routes for pollen tubes, secretion of fungal β -glucanase may cause digestion of pollen tube callose or callose deposited in the stigma cells. This may result in a disturbance of tube growth, during which time fungal hyphae would continue to grow. In addition, any callose deposited in the stigma cells as a result of trauma, or a type of 'incompatibility reaction' similar to that seen between incompatible pollen and stigma cells (Knox, 1973) may also be degraded. β -glucanase activity therefore, might be a considerable advantage at such a critical period of fungal penetration. This is discussed in detail later.
- 2) The parasite may secrete β -glucanase during the initial colonization of the ovary, which enzyme may digest any callose formed by the host as a reaction to the physical or chemical stimulus of the growing intercellular hyphae. (Such enzyme activity need only be localised and thus difficult to detect in whole tissue extracts.)
- 3) Once infection reaches the ovary base, the further secretion of β -glucanase may prevent these basal tissues from sealing and cutting off the nutrient flow from the rachilla, by the deposition of wound callose. The nutrients probably flood from the sieve tubes and transfer cells around the core of thick-walled cells, into the 'plate' of cells. (The remnants of the ovarian vascular traces may be ineffectual at this stage.)
- 4) Any callose in the sieve tubes surrounding the core of thick-walled cells (or around plasmodesmata conducting nutrient between transfer cells and the sieve tubes (see 1.1.)) may be degraded by the fungal β -glucanase. Since callose in phloem has been shown to affect the regulation of the nutrient flow (Webster & Currier, 1968), its degradation would, in effect, remove any obstacles in the conducting tissue, resulting in a continuous unimpeded flow. In addition, any wound callose in the sieve tubes, which

is produced in reaction to the trauma, would also be degraded. The prevention of tissue sealing and 'unblocking' of sieve tubes would account for the large volumes of honeydew which began to exude about a week after inoculation, the stage when fungal colonization has reached the ovary base. Such large volumes are clearly in excess of the pathogen's initial nutritional requirements. Since β -glucanase activity does not decrease, the gradual decline in honeydew exudation as the infection ages is presumably due to increased uptake by the developing ergot.

During parasitism, the fungus receives sucrose from host translocate and converts it to monosaccharides and a variety of oligosaccharides by enzymatic activity i.e. β -D-fructofuranosidase (Dickerson, 1972; Mower & Hancock, 1975a). Mower & Hancock (1975b) suggested that this conversion creates a sucrose deficit, which causes a further movement of sucrose towards the parasite. They also showed that there is a significant difference in water potential between fungus and host which also encourages flow towards the parasite. They suggest that the 'sucrose sink' thus produced is the major driving force for the observed increase in translocation rate to the fungus. However, sucrose accounts for over 90% of the total soluble carbohydrate in freshly exuded honeydew. Oligosaccharides are found only when the honeydew has been in contact with the parasitic tissue for several hours (Dickerson, 1972). This observation, together with the sudden manner of onset of honeydew exudation, suggests that a sucrose sink does not operate the initial release of honeydew. Thus some other mechanism(s) is required, and it may also be involved in maintenance of nutrient flow. The present work suggests that fungal β -glucanase may constitute such a mechanism. This enzyme therefore, may be intimately involved in the establishment of the parasitic mycelium and in the initiation and maintenance of the nutrient supply to the developing sclerotium. Since callose deposition has been interpreted as a possible host resistance mechanism (Aist, 1976), fungal β -glucanase appears to be a factor in

overcoming this potential resistance to infection.

The significance of the degradation of pollen-tube callose by enzymes of *C.purpurea*

Most of the work on pollen tube growth and fertilization has been done on plants whose pollen is easily grown 'in vitro', (Linskens & Kroh, 1970) e.g. *Petunia* (Cresti & van Went, 1976), *Lilium* spp. (Rosen, 1961; Kroh & Helsper, 1974; Reynolds & Dashek, 1976) and cotton (*Gossypium* spp.) (Jensen & Fisher, 1970). Pollen of the *Graminae* is more difficult to grow 'in vitro', hence 'in vivo' studies have been more common. Among the cereals wheat, barley and rye, although investigations on wheat and barley have appeared predominantly in the literature, it appears (Batygina, 1974) that the fertilization process is similar in all these cereals. The flowering pattern in cereals is also broadly similar, but there may be differences in the timing of events (see 1.6a).

Infection by *C.purpurea* normally occurs during flowering, and therefore it is likely that fungal spores are present on the stigma at the same time as pollen grains. This may be even more significant in ergot infection of millet (caused by *C.fusiformis*) in which stigma infection is even more common than in wheat or rye, due to the pattern of events at anthesis (Thakur & Williams, 1978). It is known that once fertilisation is accomplished, the incidence of infection decreases within a few days (Shaw, 1975). The period during which infection can occur, therefore, is relatively short, and even then the processes of pollination and fertilization may interfere with fungal penetration. Generally, the incidence of natural ergot infection is greater in rye than in wheat, largely as a result of the difference in flowering (and therefore pollination) habits (see 1.6a). However, male-sterile varieties of wheat are particularly threatened by infection since the flowers can remain open for several

days, waiting to be pollinated. Male-sterile varieties are used for the production of hybrid seed, and therefore ergot infection may cause serious losses of the valuable seed (Shaw, 1975). In rye and male-fertile varieties of wheat however, it seems that, on balance, the chances of pollination are considerably higher than those of infection. Pollen tube growth rate may be 30-40 times greater than that of the fungal hyphae (see later), yet the incidence of infection is often surprisingly high. These two facts are not easy to reconcile. To be completely successful (i.e. colonize the ovary to such an extent as to ensure an adequate nutrient supply for further development to yield a sclerotium), the pathogen may either:

- a) need to become established within the ovary before fertilization occurs, or
- b) be capable of a sufficiently high colonization rate in relation to pollen tube growth to prevent fertilization, or
- c) be able to colonize the ovary despite the rapid tissue changes initiated in the ovule by fertilization.

In such circumstances as above, there must be a critical period when the fate of the ovary is in the balance, and the chances of successful fertilization occurring are equal with those of successful fungal infection.

A hypothesis is therefore tendered proposing that fungal hyphae can actively compete with pollen tubes for access to and growth in the ovary tissue. This hypothesis is based on the present microscopic (3.1.) and enzymatic (3.2.,3.3.) studies, together with previously published data on hyphal and pollen tube growth, anatomy of the cereal ovary and stages in the fertilization process. The following comparison between pollen tubes and hyphae illustrates some striking resemblances between the two, and shows that competition between them may be possible for several reasons.

Both fungal hyphae and pollen tubes exhibit apical growth. This is thought to involve, in both cases, secretory vesicles, which contain the new wall material and/or lytic and synthetic enzymes necessary for inser-

tion of new material into the wall (Van Der Woude et al, 1971; Roggen & Stanley, 1969; Cresti & van Went, 1976; Helsper et al, 1977; Bartnicki-Garcia, 1973; Fevre, 1977a,b). The different types of vesicles originate from the rough endoplasmic reticulum cisternae (Cresti & van Went, 1976) in pollen tubes, or Golgi apparatus (Van Der Woude et al, 1971; Grove et al, 1970) in both pollen tubes and hyphae. The vesicles migrate towards the apex and coalesce with the plasma membrane thereby accomplishing two functions: a) extending the plasma membrane surface and b) discharging enzymes and/or material contributing to wall growth (Bartnicki-Garcia, 1973).

β -1-3-glucans are a feature of the walls of Ascomycete hyphae, and callose (a β -1-3-glucan) occurs in pollen tube walls. In hyphae, the glucan is found in the outermost amorphous layer, together with other wall materials, while in pollen tubes it is found in the innermost wall layer and as plugs within the tube (1.8.).

Several enzymatic activities appear to be common to both pollen tubes and C.purpurea hyphae. β -1-3-glucanases present in the pollen grains are thought to degrade the callose in the intine below the germ pore thus stimulating germination, while cellulases and pectinases increase the rate of elongation (Roggen & Stanley, 1969; Linskens & Kroh, 1970). C.purpurea has been shown, in the present study, to possess β -1-3-glucanase and pectolytic activity. β -fructofuranosidase activity, found in pollen tubes (Paton, 1921) has also been shown in C.purpurea (Dickerson, 1972). During pollen tube growth in stylar tissue, enzymatic degradation by the pollen tube and utilization of the intercellular material, or outer wall layers of the transmitting tissue has been shown to occur in some plants (Kroh & Munting, 1967; Linskens & Kroh, 1970; Kroh et al, 1970; Kroh & Helsper, 1974). A similar system may be operating during the intercellular penetration of C.purpurea through ovarian tissue, but although degradation of middle lamella material and pectolytic activity have been demonstrated in

the present study, there is no other evidence that this material is utilised by the fungus for growth.

Another important factor which may have a bearing on competition between pollen tubes and hyphae of C.purpurea is that initially they grow intercellularly in the same tissues. In cereals, the pollen tube grows between the cells of the stigmatic hairs to the transmitting tissue (Arber, 1937; Esau, 1965) within the style, and continues to the cone-shaped apex of the outer integument at the top of the ovule (Fig. 3). From there it usually leaves the transmitting tissue and grows between the inner epidermis of the ovary wall and the outer integument of the ovule (Pope, 1946), although one account suggests growth within the outer integument (Lermer & Holzner, 1888). Since the ovule is anatropous, the micropyle is located towards the rachillar end of the ovary, so that the pollen tube must grow around the ovule in order to reach the micropyle and fertilise the ovum (Fig. 3). In the present work, hyphae of C.purpurea have been located in the stigma and style (Plate 7) growing in the transmitting tissue (Plate 8,9). Hyphal growth proceeded to the cone-shaped apex of the outer integument, and hyphae surrounded the upper part of the ovule. Penetration into the ovule did not usually take place via the micropyle: instead hyphae often penetrated the ovule at the stigma (distal) end. Growth occurred between the inner epidermis and outer integument cells, (the latter often collapsing and allowing fairly extensive colonization) before invasion of the inner integument layers and the ovule. The degeneration of the outer integument is a normal occurrence in ovaries soon after fertilization (2-3 days), and these cells may therefore be primed to disintegrate after a certain stimulus, which in the infected ovary may be supplied by the hyphae. Therefore, one advantage that the hyphae have over the pollen tubes is that they need to grow a shorter distance before penetrating the ovule. Since the ovule is the prime target for pollen tubes, and a target of hyphae, the route taken to reach it is

important. The proliferation of hyphae, therefore, in the spatially limited pathway usually taken by the pollen tubes could physically obstruct pollen tubes and retard their growth.

Finally, the size of the pollen tubes in relation to the hyphae is significant if the possibility of competition is to be inferred. From work done on pollen tubes growing within stylar tissue, the diameter of the wheat pollen tube was shown to be about 6.6μ (Batygina, 1974). When measured while growing 'in vitro', it was found to be only half as wide (Loo & Hwang, 1944). Barley pollen tubes growing 'in vivo' are $5-6\mu$ in diameter, (Pope, 1946). Pollen tubes of other plants are of a similar size ($5-8\mu$) e.g. Petunia (Linskens & Kroh, 1970; Cresti & van Went, 1976), Nicotiana (Tupy, 1959) and Lilium (Reynolds & Dashek, 1976). However, during growth in the ovary tissue, the diameter of the pollen tubes may vary considerably and this is probably due to resistance of the surrounding tissue (Pope, 1946). In the present study, pollen tubes growing in the stigma had a diameter of about 5μ (Plate 38b). The average diameter of hyphae growing in ovary tissue was about 3μ . Therefore dimensional similarities between pollen tubes and hyphae make it likely that hyphae could easily grow in tissues which are proscribed routes for pollen tubes.

It appears, therefore, that there are several similarities between cereal pollen tubes and hyphae of C.purpurea. In order for the preceding comparison of hyphae and pollen tubes to be a valid contribution to the hypothesis that competition occurs between them, the timing of events in the pollination/fertilization sequence must be related to those of the infection process. First, growth-rate of pollen tubes and hyphae must be an important consideration. From the present work, the hyphal growth rate during initial infection of the ovary tissue can be calculated at roughly $1\mu/\text{minute}$. This is based on the extent of colonization after a certain number of days, and assuming that infection began about half-way

along the stigma. It is therefore only a rough estimate.

Data on the 'in vivo' growth rates of cereal pollen tubes is fairly limited, but Percival (1921) states that fertilization occurs 30-40 hours after pollination. From the dimensions of the stigma (3-4mm long) and ovary (1mm long), the growth rate of the wheat pollen tube may be calculated as about $1.5\mu/\text{minute}$. Loo and Hwang (1944) obtained a maximum growth rate for wheat pollen tubes 'in vitro' of $0.167\mu/\text{minute}$. Barley pollen tubes have been known to grow at $100\mu/\text{minute}$ at 30°C 'in vivo' (Pope, 1943) but in normal field conditions growth would probably be slower. Growth rates vary between species and are dependant on temperature and other conditions (Pope, 1943; Hoshikawa, 1960), and 'in vivo' growth rates may not be comparable to those 'in vitro' (Stanley, 1971). However, more recent studies on wheat (Batygina, 1974), have shown that the time taken from germination of the pollen grain to entry of the tube through the micropyle may be as short as 45 minutes. This would give a growth rate of about $55\mu/\text{minute}$. Batygina also showed that once fusion of the male gamete has occurred with the nucleus of the egg cell (about 4 hours after pollination), the zygote remains dormant for about 16-18 hours, and only about 24 hours after pollination does it start to divide. By this time the endosperm has divided several times already. Percival (1921) therefore, may have regarded division of the zygote as the first indication of fertilization and hence his figure of 30-40 hours may be a sizable overestimate.

From these figures it is clear that if a pollen grain and a fungal spore land on a stigma together, the likelihood of successful fertilization is much greater than of successful fungal infection, since the growth rate of pollen tubes could be as much as 35 times that of fungal hyphae. The hyphae therefore, apparently cannot compete successfully with pollen tubes solely on a growth-rate basis: infection would need to be established in the ovary wall before pollen tube growth begins,

for the fungus to have any chance of successful parasitism. However, if the fungus could secrete enzymes capable of retarding or stopping the progress of the pollen tubes, the requirement for prior colonization might not be obligatory, and therefore timing of infection in relation to pollination would not be so critical.

It has been shown in the present work that an extract from parasitic ergot tissue is capable of hydrolysing pollen tube callose, both from the walls and plugs, and it may be that this activity is a significant weapon in terms of establishing infection. Callose is thought to act as a plugging and sealing agent whether it is found in phloem, wound regions or pollen tubes. The callose plugs in pollen tubes separate the older parts from the younger region towards the tip, and Brink (1924) was one of the first to suggest that they maintain the integrity of the 'vegetative pollen cell' (i.e. the region between the pollen tube tip and the first callose plug behind it and in which activity is centred). Brink also showed that this 'active vegetative cell' could be separated from the pollen grain and still be capable of independent existence. The callose in the wall of the pollen tube, however, may be involved in the imbibition of water (Cresti & van Went, 1976), thereby maintaining the osmotic conditions of the pollen tube. Stanley (1971) suggests that callose in pollen grains may act as a storage carbohydrate.

It seems very likely therefore, that if callose was removed from the pollen tube, the processes dependent on callose function would be considerably disrupted. Callose plug dissolution, especially of the one nearest the tip, would cause an increase in the size of the 'active vegetative cell', which may alter its wall-synthesising and growth activity. It may fail to confine the cytoplasmic organelles and sperm cells to the tip region, and it is possible that contact between the older disintegrating parts of the pollen tubes, and the younger active tip could no longer be prevented. It is likely that the disruption of the normal organiza-

tion of the pollen tube cytoplasm would adversely affect its growth. Degradation of wall callose causes a structural weakening of the wall, as was shown by Roggen & Stanley (1971), who treated pollen tubes 'in vitro' with a β -1-3-glucanase. In addition, if wall callose plays a part in the osmotic relations of the pollen tube, its removal would have serious consequences on pollen tube turgidity and growth.

Therefore, if hyphal and pollen tube growth occurred together within the stigma and ovary, the degradation of pollen tube callose may temporarily or permanently suspend pollen tube growth and hence fertilization. The valuable extra time gained by the fungus at this critical period could enable it to proliferate further within the ovule, whose structure it rapidly shatters, thereby making fertilization highly improbable. This ability of hyphae to hydrolyse callose, therefore, may provide it with an important method of competing successfully with pollen tubes, especially since the odds appear to weigh heavily against the parasite under field conditions when inoculum only reaches the ovary at the time of flowering.

5. SUMMARY

The rate of infection was subject to environmental conditions (particularly temperature) but once it was established, colonization of the ovary proceeded very rapidly. Initial infection commonly occurred via the stigma. However, in the cases where this was not evident, infection presumably took place directly through the ovary wall. Penetration was intercellular and the main initial targets appeared to be the ovule and the vascular traces. The ovary was usually completely destroyed by the pathogen about a week after inoculation, and release of honeydew coincided with this stage. In the early stages, the fungus proliferated between the top and base of the ovary, pushing it apart and destroying its structure. It grew mainly along the vertical axis of the floral cavity, since the glumes exert a constraint on lateral expansion. Fungal growth never extended into the rachilla, but was restricted to the area immediately distal to a 'plate' of cells and core of thick-walled cells at the ovary base. Sclerotial hyphae were first detected by light microscopy 12-13 days after inoculation, but electron microscopy revealed that they were already developing at 10 days. Proliferation of the sclerotial cells resulted in the formation of a hard ergot sclerotium. Honeydew exudation gradually declined as the ergot matured.

During growth in liquid culture, the fungus produced a range of pectolytic enzymes whose activity was affected by the level of glucose in the medium. Cellulolytic enzyme activity was not detected. At least three of the pectolytic enzymes were also produced throughout the parasitic growth of the fungus. Their activity is thought to help in intercellular penetration and killing of the host cells during colonization.

Parasitic growth of C.purpurea was also accompanied by extracellular β -1-3 glucanase and β -glucosidase activity. The activity of both enzymes increased about a week after inoculation, coincident with fungal coloni-

zation reaching the ovary base and with the onset of honeydew exudation, which secretion also possessed this enzyme activity. The activity in the parasitic tissue did not decline during subsequent sclerotial development. β -glucanase was also produced during saprophytic growth. Its activity probably accounted for the low culture viscosity, due to the prevention of glucan accumulation. C.fusiformis however, normally produces viscous cultures containing relatively large amounts of glucan. The extracellular β -glucanase activity is usually low. An isolate of C.fusiformis, producing typically viscous saprophytic cultures, did however produce β -glucanase during parasitic growth.

Parasitic tissue and honeydew extracts of C.purpurea had the ability to significantly reduce the fluorescence of wound callose, demonstrating the degradation of that β -1-3 glucan. This effect was comparable to that produced by a highly active β -1-3 glucanase from Basidiomycete QM806. Visual assessment of fluorescence intensity indicated that phloem and pollen tube callose was similarly degraded by the parasitic extracts.

The action of parasitic extracts on all 3 forms of callose has been interpreted as constituting an effective mechanism in initiating and maintaining a nutrient supply to the developing parasite. In addition, it may serve to overcome host resistance reactions, in the form of callose deposition, at various stages of colonization. This proposed function for β -glucanase of C.purpurea is not necessarily restricted to that species, since C.fusiformis, which normally produces little β -glucanase in culture, does produce the enzyme during parasitism.

APPENDIX I

Development of a method for visualising wound callose in wheat and rye ovaries

Several stains are available for light microscopy which various authors claim will stain callose. These stains include lacmoid, first used by Tsvett (1911) (Cooper, 1928; Bradbury, 1929; Reynolds & Dashek, 1976) used alone or in combination with other dyes (Nebel, 1939; Vernon & Gifford, 1953). Resorcinol blue (Currier, 1964), Ponceau S (Ulrychova et al, 1976) and aniline blue (Currier & Strugger, 1956; Martin, 1959; Currier, 1957, 1964; Jensen, 1962) have also been used. Other less commonly used stains are coralline, azurine, and benzazurine (Sprau, 1955).

Callose stains blue with aniline blue (Johansen, 1940), and can be observed by light microscopy, but more recently UV fluorescence has been used as a very sensitive technique to detect callose. This method was first developed by Arens (1949) and the characteristic yellow fluorescence has been widely used in work on pollen tube, phloem and wound callose (see 1.8.).

In past work various procedures have been used which differed from each other over the whole range of histological investigation from the preparation of tissues through to visualising the stained material. Therefore, in the present work, different methods of tissue preparation were tested along with various combinations of fixing, staining and ultimate viewing of the material. This led to the selection of the method used in the actual experimental work which has been described in 2.5.

1. Whole Ovary and Lacmoid Tests

Wounds were made by stabbing ovaries vertically and horizontally with a hypodermic syringe needle, a glass needle and a thin wire. The latter produced small wounds and did not break, and was used thereafter for wounding the ovaries. After wounding the ovaries (both healthy and infected) were either left on the plant, or were detached and placed in a covered dish. They remained so for varying periods in order that wound callose production should occur.

The effects of two fixatives and no fixing were compared: (a) Carnoy's solution (abs. EtOH: conc. acetic acid = 3:1 (Johansen, 1940) and (b) Formalin - acetic acid alcohol (FAA) - 40% formaldehyde: glacial acetic acid: 50% EtOH - 5:9:90 (Jensen, 1962).

After fixing and washing, ovaries were stained with 1.5% lacmoid in 50% EtOH for 30 mins. (Reynolds & Dashek, 1976).

The effect of incubating the ovaries in β -glucanase from B.QM806 on staining was also investigated.

The results showed that healthy wounded ovaries stained blue around wounds and the remaining tissue was unstained. Infected ovaries stained blue only in the infected regions - the uninvaded areas were unstained.

Varying the period of callosing after wounding did not appear to affect staining intensity around the wound. The stain was picked up very rapidly i.e. within one minute of wounding, which appears suspect for although the wound callose reaction is known to occur rapidly, it is unlikely that it could be visualised within a matter of seconds. Further tests were carried out therefore, on ovaries which were fixed before wounding and then stained. Ovaries were fixed either by boiling for 10 minutes or placing in ice-cold Carnoy's solution for 1 hour. They were then wounded as previously and stained with lacmoid immediately or after 30 minutes. It was found that in all cases the wounds stained blue, and undamaged tissue remained unstained. Clearly, dead tissue cannot produce wound callose, so that lacmoid must have been staining some other substance, or staining was due to a characteristic of wounded tissue present in death as well as in life, e.g. physical topography.

There was no apparent difference between the two fixatives on subsequent stainability of the tissue. However, a difference was detected between fixed and unfixed tissues, in that in unfixed ovaries the stained areas were more clearly visible. This was especially so in infected ovaries where there was a more sharply defined correlation between the tissues invaded by fungal hyphae and the retention of the stain. Infected areas, therefore, could be more clearly identified by omitting fixation.

Detachment of ovaries from the plant also appears to have minimal effects on subsequent staining of wounds by lacmoid. If however after detachment the ovaries were kept in a warm, dry atmosphere, they became dehydrated and died. Also, the process of detachment often led to inadvertent wounding, and totally undamaged ovaries were more infrequent with this procedure. It was found that undamaged, turgid ovaries were best obtained by leaving the ovaries on the plant and standing the ear in a humid chamber during the period of callosing.

Incubation of wounded ovaries in β -glucanase of B.QM806 did not remove the stain from around the wounds. It is possible that callose was degraded to some extent but that the remnants were still attached to the walls, possibly by hydrogen bonds, revealed the persistence of the stain. This evidence together with that of stain retention by dead tissue suggests that lacmoid is picked up and retained by substances or struc-

tures other than callose. Reynolds & Dashek (1976) however, found that B.QM806 glucanase eliminated lacmoid staining from Lily pollen-tube walls. In their study single cells were observed, not whole tissues or organs as in the present study, and of course, the tissues had not been wounded. Differences in conditions and tissues play an important part in staining, and binding of the stain could be influenced by factors other than its affinity for callose.

In conclusion, it seems likely that lacmoid might not be staining solely callose, and that some other factor is at least partly responsible for the retention of stain around the wounds. Staining with lacmoid therefore, is not advisable for gross staining of whole wounds in tissues.

2. Whole Ovary and Aniline Blue Tests

Healthy ovaries of wheat and rye were wounded and left to produce callose either on or detached from the plant for varying periods as described for lacmoid tests. They were either fixed in Carnoy's solution or just washed, and then stained or treated with β -glucanase from B.QM806, followed by staining. Ovaries were incubated for 18 hrs at 40°C in 1 mg β -glucanase/ml 0.01M acetate buffer pH 5.2. Control ovaries were incubated in buffer. They were stained with 0.5% aniline blue in 50% EtOH for 30 mins.

The results showed that there was no apparent difference in staining between detached ovaries and those retained on the plant, unless detaching caused dehydration. To minimise this risk, all ovaries hereafter were retained on the plant.

In ovaries which were fixed immediately after wounding, the wounds of both glucanase-treated and control ovaries retained some of the stain. In all ovaries which had been stained before incubation in glucanase and where the period of callosing ranged from 30 minutes to 3 hours, the treated ovaries lost some of their stain to the surrounding solution but some stain remained around the wounds, the cut surface at the ovary base and the stigmas. Control ovaries retained their stain around wounds, cut surfaces and stigmas, and did not lose any to the buffer solution which remained colourless. Ovaries which had been incubated in glucanase prior to staining retained less stain than the controls. As in lacmoid-stained ovaries, the wounded areas picked up aniline blue very rapidly, even when the period of callosing was extremely short. Glucanase treatment did not remove the stain completely.

When the period of callosing was extended up to 3 hours and glucanase treatment was subsequently carried out, most of the stain was removed

from the wound area and the incubation solution became blue. Since control ovaries retained the stain, this implies that glucanase treatment caused some removal of the stain presumably by callose breakdown. The stain which persisted after treatment could be due to several factors including wound callose being partly inaccessible to the enzyme or requiring longer or different incubation conditions, adsorption onto cell debris or leached materials, or one or more physical characteristic of the wound area as suggested for lacmoid-stained ovaries.

On the basis of these tests, it was concluded that gross staining of whole wounded organs was an unsatisfactory method for studying wound callose deposition and its subsequent degradation by various glucanase samples. Had it been successful, it would have been a rapid and simple method to use, requiring little manipulative technique. Evaluation of the degree of callose breakdown would have been a major problem since one method of doing this would have been arbitrary and subjective, thus leading to inaccuracy. A spectrophotometric technique could have been developed but its results would have depended on variable factors giving very wide confidence limits, too great to be of use.

It was decided that a far more accurate method of studying this was one based on microscopic examination of the wounded region. Sections of wounded ovaries, stained and treated with glucanase would yield information about the reactions of individual cells around the wounds. Light microscopy of sections stained with lacmoid or aniline blue was one possibility and these together with another stain, Ponceau S, were tested.

3. Tissue sections + lacmoid, aniline blue or Ponceau S

Initially, sections were cut by hand with a razor blade but these were found either to be too thick or the lesioned area tore easily. Wax embedding also gave rise to problems in tissue coherence, especially in infected tissue where there is no continuous network of cells to hold the tissue together. Therefore, the ovaries were frozen, and frozen sections were cut. Ovaries were placed in a drop of 2% gum acacia onto a freezing stage where they froze solid. Sections were then cut, 60-100 μ thick, using a Reichert sledge microtome as described in 2.5, and stained in one of the three stains. This method of obtaining sections was found to have several advantages, not least of which was its speed. The reduction in manipulation and chemical treatment of the ovaries caused less damage to the tissue and staining was facilitated. All future sections were to be cut in this way.

All sections were observed using a Leitz Ortholux microscope. Those

stained in lacmoid (1.5% in 50% EtOH for 30 min, destained in 4 x 50% EtOH and mounted in glycerol) gave variable results. Cells surrounding the wounds stained blue but often cells of undamaged sections also stained blue. Sections stained with aniline blue (0.5% solution in 50% EtOH for 30 min.) were stained pale blue around the wounds. Ponceau S - stained sections gave variable results. Sections were stained in the following solution for 4 min: 1g Ponceau S; 100ml 50% EtOH; 5ml glacial acetic acid; 0.3g phosphomolybdic acid. They were washed in 50% EtOH and counter-stained with 1% aqu. light green solution for 10 min. (Ulrychova et al, 1976). According to Ulrychova et al, callose stains an intense red. In the present study however, red staining was not confined to wounded areas and tended to be diffuse throughout the section. Ponceau S is normally used as a protein stain and was found to be unsatisfactory as a callose stain in this study.

Another method, reported (Currier, 1957) to be far more sensitive and selective, entailed combining aniline blue staining with fluorescence microscopy, and was then tried. Advantages of this method include a short staining time and tissue can be living or fixed. The stain is not fluorescent and so tissues can be mounted in it directly. Aniline blue can be used in low concentrations and has low toxicity. This method was tested and, being found to lend itself more to quantitative (fluorimetric) estimation of the target material, was adopted for subsequent use (see 2.5.).

APPENDIX II

versus

Epi-illumination vs dia-illumination (i.e. transmitted light)

There are several advantages of using epi-illumination instead of dia-illumination. In epi-illumination, the exciting light passes through the objective onto the specimen and fluorescent light passes back, whereas in dia-illumination, the exciting light passes through the condenser to the specimen and is emitted, together with the fluorescent light, from the opposite surface of the specimen. If dark-field fluorescence is used, the darkfield condenser prevents any exciting light from reaching the objective, and only the fluorescent light enters the objective. Thus the intensity of fluorescence is dependent on the thickness of the specimen when dia-illumination is used, but in epi-illumination, fluorescence intensity is independent of section thickness. Therefore fluorescence intensity is usually greater in epi- than in dia-illumination since there is no light loss due to scatter or absorption by the specimen. In epi-illumination, since the light passes through the objective onto the specimen, the objective simultaneously acts as a condenser. Focusing and centring presents no problem, as it can do in dia-illumination where even illumination may be difficult to achieve. The dichromatic beam splitter selects wavelength by reflecting light below a certain wavelength (420 nm here) and transmitting longer wavelengths. It thus acts as a filter reflecting short-wave exciting light onto the specimen, restricting any reflected short-wave light to the incident light path and transmitting the long-wave fluorescence emitted from the specimen. The observer, therefore, sees only the fluorescence image.

In transmitted light darkfield microscopy, there must be a layer of oil or water between the slide and condenser, a procedure obviated in epi-illumination. If it is necessary to scan large areas of tissue for a long time, the transmitted light method is the most useful. However, since epi-illumination is easier and more convenient to use and involves fewer variables, it yields more reproducible results, better photomicrographs and more reliable quantitation of fluorescence.

APPENDIX III

Fluorescence Photomicrography

The problems most frequently encountered in fluorescence photomicrography arise largely from the low level of light intensity emitted by the fluorescing material. This necessitates the use of long exposure times which results in photographic images of poor quality. Long exposure times cause reduced contrast since the silver grains become less sensitive on long exposure. Fading of fluorescence is another consequence of long exposure times but this varies with fluorochrome, specimen and excitation light intensity. Brightly fluorescing objects are known to fade more rapidly than those less bright, and insertion of a grey filter is considered useful when selecting an area of the section to photograph, since this retards fading. Alternatively, if the microscope is equipped with several light sources, transmitted light may be used during searching and also orientating the camera, and epi-illumination for actual photography. The fluorochromes commonly used in immunofluorescence e.g. FITC (fluorescein isothiocyanate), RB 200 (Rhodamine B 200), and DANS (1-dimethylaminonaphthalene-5-sulphonic acid) have rapid fading rates with continued exposure to UV (e.g. fluorescence emission from FITC conjugates is reduced by half in 30 seconds under darkfield conditions (Nairn, 1969)). The fluorescence from the material used in the present study, however, was not subject to such fast fading, and only after 1-2 minutes exposure was it noticeably reduced. Dry unmounted preparations have been observed to fade rapidly by some workers (Nairn, 1969), but this was not found to be a problem in the present work. Fading is sometimes accompanied by colour change e.g. RB 200 changes from orange to golden yellow on exposure to intense illumination, but again this phenomenon was not observed with aniline blue-induced fluorescence of callose. Another disadvantage of long exposure times is that the chances of vibration are increased, causing blurring of the image, and this was frequently encountered in the present study.

It was important therefore to reduce exposure time as much as possible, and this was done in several ways. First, the microscope was set up so that the best image contrast and best yield of fluorescence was obtained. The light source was centred and stable with no flickering, and objectives with a high N.A. and low power eyepieces were used where possible. The specimen was illuminated as near the wavelength of optimum excitation (400 nm) as possible and observed at the peak wavelength of the fluorescence spectrum (540 nm). This was achieved by selection of

appropriate excitation (UG1) and barrier (K530) filters. The detail measurement setting was used on the microscope camera and exposures were timed automatically.

Secondly, the choice of photographic film was important. Fast films, such as Kodak High Speed Ektachrome (ASA 160), have been recommended (Nairn, 1969; Koch, 1972; Schuit, 1970; Holz, for Zeiss), and it is thought advantageous to set the camera speed higher than that of the film e.g. ASA 400 instead of 160. In the present work, three colour and one black and white films were tested. These were: (a) Kodak High Speed Ektachrome daylight film, ASA 160 (set at ASA 200 and 400) for colour transparencies, (b) Kodak Vericolour II Type S Professional film, ASA 100 (set at ASA 200) for colour negatives, (c) Kodacolor II, ASA 100, also for colour negatives, (d) Ilford Pan F, ASA 50 for black and white negatives. The best results were obtained with High Speed Ektachrome film (ASA set at 400 and film developed as if it were ASA 160), where the colours were comparable to those of the specimen, and contrast was good. With the two colour negative films, the correct colours were not always reproduced on the prints and this variability reduced the usefulness of these films. (Colour negative films are not generally recommended because of their low sensitivity and the subjectivity of positive production, (Pl. 38a) (Koch, 1972). Pan F film yielded prints with good contrast and of a similar standard to those reproduced from High Speed Ektachrome transparencies. With all four films, and using transmitted light microscopy, the exposure times were generally between 5 and 10 minutes depending on magnification and amount of fluorescing material in the area photographed. The fluorescence intensity faded after 1-2 minutes quite rapidly and remained at a low level. This fading was irreversible. However, when epi-illumination was used (and High Speed Ektachrome film), the exposure time was reduced to around 2-5 minutes (i.e. by about half) which led to improved contrast and a sharper image.

The advantages of epi-illumination over dia-illumination have already been mentioned and the former's superiority in fluorescence photomicrography is one of its major assets, since without it many low intensity fluorescent objects could not be photographed clearly. Since accurate photographic records of fluorescent preparations are widely required, this type of microscopy coupled with high speed colour transparency film is considered to give best results. Colour reproduction is good, colour copies and black and white prints are easily obtained. Plates 37, 38b were reproduced from colour transparencies taken with High Speed Ektachrome daylight film.

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A rôle for β -glucanases in the parasitism of cereals by *Claviceps purpurea*

A. G. DICKERSON, P. G. MANTLE, L. J. NISBET and BARBARA I. SHAW

Department of Biochemistry, Imperial College, London SW7 2AZ, U.K.

A rôle for β -glucanases in the parasitism of cereals by *Claviceps purpurea*

A. G. DICKERSON, P. G. MANTLE, L. J. NISBET† and BARBARA I. SHAW

Department of Biochemistry, Imperial College, London SW7 2AZ, U.K.

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Histological investigation of parasitism of *Claviceps purpurea* on rye and wheat revealed the close proximity of the fungus to host vascular elements at the host-parasite interface. Extracts of infected tissue and developing ergots possessed glucanase activity, and honeydew, which exudes from infected florets, showed about ten-fold greater activity. Callose, a β -1-3-glucan which occurs normally in sieve tubes and is deposited by wounded cells in response to injury, fluoresces bright yellow when stained with aniline blue and irradiated with u.v. light. Infected tissue extracts, and honeydew, hydrolysed wound and phloem callose, as shown by an almost total elimination of fluorescence due to this material in wounded ovary sections. It is suggested that the glucanases of *Claviceps purpurea* play a rôle in maintaining a nutrient supply to the pathogen by hydrolysing callose and preventing its further deposition since none is found in infected ovaries.

INTRODUCTION

The main carbon source available for parasitic growth of *Claviceps purpurea* is sucrose [6, 15]. This and other sugars are contained in the honeydew which exudes, often in copious amounts, from florets during the early stages of infection of the host grasses and cereals. The parasite modifies and utilizes the sucrose initially present to yield the oligosaccharides characteristic of "stale" honeydew [8]. It is clear that honeydew is intimately connected with ergot parasitism and may be regarded as the source of nutrition for growth and differentiation of the fungus. The phenomenon of honeydew formation has previously received little study. Enzymes in the developing ergot and honeydew have, therefore, been investigated concerning a possible mechanism by which the parasite could initiate and maintain this supply of exudate.

MATERIALS AND METHODS

Organism

The *Claviceps purpurea* strain (29/4D) is the same as that used by Dickerson *et al.* [8].

Growth of host plants

Svalof's Fourex (Tetraploid) rye (Swedish Seed Association, Svalof, Sweden) and Maris Dove wheat (Plant Breeding Institute, Cambridge, England) were grown

† Present address: Roche Products Ltd, Welwyn Garden City, Herts AL7 3AY.

both in pots in a glasshouse and also in open plots at the Chelsea Physic Garden, London.

Inoculation of host plants

Plants were inoculated by procedures similar to those described by Corbett *et al.* [4] and Mantle & Nisbet [14]. These procedures ensured that infection would be followed by copious production of honeydew and eliminated carry-over of any factors from axenic culture.

Sampling

Infected rye ears were sampled at appropriate stages of ergot development and dissected to remove the fungal tissue intact. Approximately 20 developing ergots were combined for analysis at each stage. About 12 days after inoculation, sclerotial mycelium was observed to have displaced acropetally the earlier formed sphacelial tissue. From this time, sampled ergots were normally divided into older and younger segments which were analysed for enzyme activity. Honeydew was collected for analysis from the first day of its appearance under glasshouse conditions.

Preparation of purified glucan

Purified glucan was prepared by the methods described by Buck *et al.* [2] and Dickerson *et al.* [7]. Isotopically labelled glucan was prepared from cultures to which filter-sterilized, [U-¹⁴C]sucrose solution had been added, before inoculation, to give a final concentration of 1 μ Ci/ml. Purified samples of glucan were freeze-dried from the aqueous state to 1% w/w moisture.

Glucanase determination

Ergot tissue (200 mg) was extracted at room temperature by grinding for 1 h in a pestle and mortar with 1 ml of the acetate buffer; the extract was centrifuged at 15 000 *g* for 20 min and 500 μ l of the supernatant was incubated with 5 mg [U-¹⁴C]glucan. Honeydew incubations contained: 200 μ l of honeydew used directly (spores removed by centrifugation) or after dialysis for 18 h at 4 °C against 0.001 M sodium acetate buffer, pH 5.2, using 10 l/10 ml honeydew; 5 mg [U-¹⁴C] glucan and 200 μ l 0.01 M sodium acetate buffer, pH 5.2. All incubations were performed at 30 °C. Activity of glucanase was determined by incubation of the sample with [U-¹⁴C]glucan and determining the radioactivity of the products or by determining its residual levels of glucan at stages during its hydrolysis [2]. Glucose liberated during the reaction was also estimated by the methods described by Dickerson *et al.* [7].

The glucanase activity of ovaries and young seeds from healthy ears was also analysed.

With honeydew, replicated experiments did not show more than 10% variation. With tissue extracts, difficulties in precisely standardizing experimental conditions with respect, particularly, to the age of both plants and developing ergots resulted in an overall variation of 30%. Results are presented, in all cases, as the mean of at least three replicates.

Glucosidase determination

Gentiobiose (7.5 mg) was dissolved in 0.5 ml of 0.01 M sodium acetate buffer, pH 5.2. Tissue extract (1.0 ml, see above), in the same buffer, was added. The mixture was incubated at 30 °C and 2 × 0.1 ml aliquots were taken at intervals over 5 h and the liberated glucose estimated as above.

Protein estimation

Protein in solution was estimated by determining the extinction of the sample at 260 and 280 nm and reading the concentration from a nomograph compiled by Adams (California Corporation for Biochemical Research) from the data of Warburg & Christian [27].

Microscopy and histochemistry

(a) Infected ovaries and developing ergots were fixed in 2.5% v/v glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and post-fixed in 1% w/v osmium tetroxide in the same buffer. They were embedded in Spurr's resin [26] and sectioned (1 to 2 μ m) using an LKB Mk III ultramicrotome. Sections were stained with 0.1% w/v alkaline toluidine blue.

(b) *Callose induction*. "Wound" callose formation was induced in attached ovaries of rye or wheat by inserting a thin-wire needle (0.125 mm diameter) 3 times longitudinally into the ovary, to a depth of 2 mm. After wounding, ears were placed, with the stem in water, in a humid atmosphere in the light for 4 h to stimulate callose deposition. Ovaries were then carefully removed, frozen on a stage and sectioned (60 to 70 μ m) using a Reichert sledge microtome. Sections (20; approximately three ovaries) were placed in each of the following and incubated at 45 °C for 2 h.

- (i) Distilled water (1 ml);
- (ii) glucanase (5 mg/ml) from Basidiomycete QM806 [20];
- (iii) dialysed honeydew (freeze-dried; 5 mg/ml);
- (iv) freeze-dried extract (see above and Table 1) from the sphacelial caps of 30-day-old ergots (top 3 mm; 5 mg/ml).

After incubation, the sections were stained in excess 0.01% w/v aniline blue in 0.067 M K_2HPO_4 , pH 8, for 10 to 15 min, mounted on microscope slides and heated briefly on a heating block (50 °C) to stretch them and ensure adherence to the slides.

(c) *Fluorescence microscopy and microspectrofluorimetry*. Aniline blue-stained sections were examined with a Leitz Ortholux fluorescence microscope using dark-field illumination. Illumination was provided by an HBO 200 W mercury arc lamp, and exciter filters UG1 (2 mm thick) and BG38 (4 mm thick) were used in conjunction; the barrier filter was a K530.

Quantitative measurement of fluorescence was obtained using a microspectrofluorimeter, based on the Leitz Microspectrograph using monochromatic epi-illumination and photon counting [18]. Sections were illuminated at a wavelength of 400 nm, and the emitted fluorescence was measured at λ_{max} 540 nm. The total fluorescence on each side of the long axis of the wound, which appears oval in the

section, was measured, giving a total standard area in each case of 3920 sq. μm . Each area was counted twice and results, statistically analysed, were obtained from measurements made on between five to ten different sections for each treatment.

Histology

At a stage when the parasitic sphacelial mycelium had grown to occupy about twice the volume of the ovary at inoculation [Plates 1(a), (b)], fungal hyphae did not extend below the base of the ovary, but were in close proximity to the host vascular tissues as indicated by xylem vessels (Plate 2). Sections of later stages in ergot parasitism confirmed that the fungus remained restricted to the tissues distal to the central core of thick-walled cells of the rachilla described by Zee & O'Brien [28, 29]. Although the fungus was producing conidia [Plate 1(a)], a characteristic of sphacelial tissue, honeydew was not yet evident. Thus it was concluded that, up to this stage, the parasite had grown by utilizing cellular components of the ovary supplemented by nutrients supplied through the ovarian vascular traces.

Hydrolysis of fungal and plant polysaccharides

(a) *Hydrolysis of fungal glucan.* In a preliminary experiment parasitic sphacelial tissues were shown to hydrolyse purified glucan indicating glucanase activity.

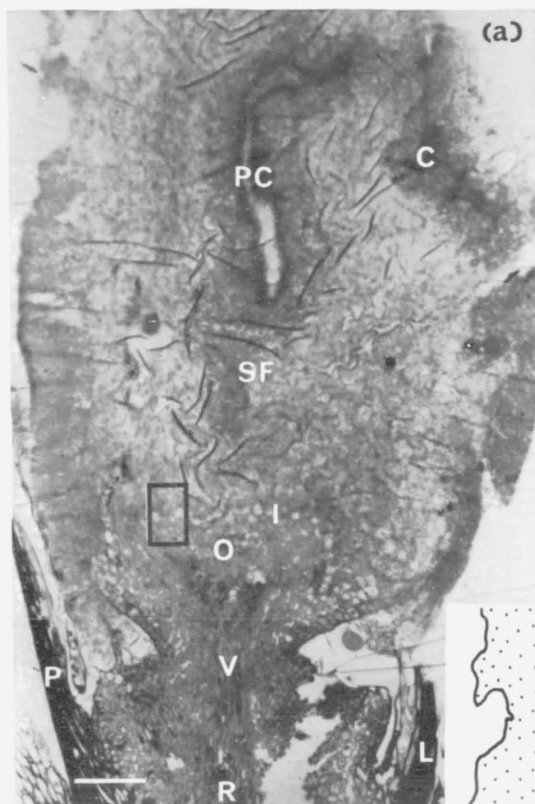
TABLE 1
Action of enzymes in parasitic mycelia of Claviceps purpurea strain 29/4D on glucan—for experimental details see text

Morphology of developing ergot	Time after inoculation (days)	Rate of glucan hydrolysis ^a		Glucosidase ^b activity of extracts
		Mycelial extract	Extract of uninfected ovary/young seed	
Sphacelial	5	26	22.5	1.6
Sphacelial	7	24	22.6	— ^c
Sphacelial	9	65	30.0	9.6
Sphacelial	12	86	33.5	—
Apical half ergot (sphacelial)	14	153	—	—
Basal half ergot (mostly sclerotial)	14	27	—	—
Apical half ergot (33% sphac., 67% scler.)	19	75	18.6	28.1
Basal half ergot (sclerotial)	19	34		6.8
Apical half ergot (20% sphac., 80% scler.)	22	205	—	31.9
Basal half ergot (sclerotial)	22	59	—	9.8
Apical half ergot (15% sphac., 85% scler.)	25	91	—	—
Basal half ergot (sclerotial)	25	86	—	—
Apical third ergot (25% sphac., 75% scler.)	30	314	—	27.9
Middle third ergot (sclerotial)	30	68	—	—
Basal third ergot (sclerotial)	30	119	—	18.5
Mature ergot	60	185	—	—

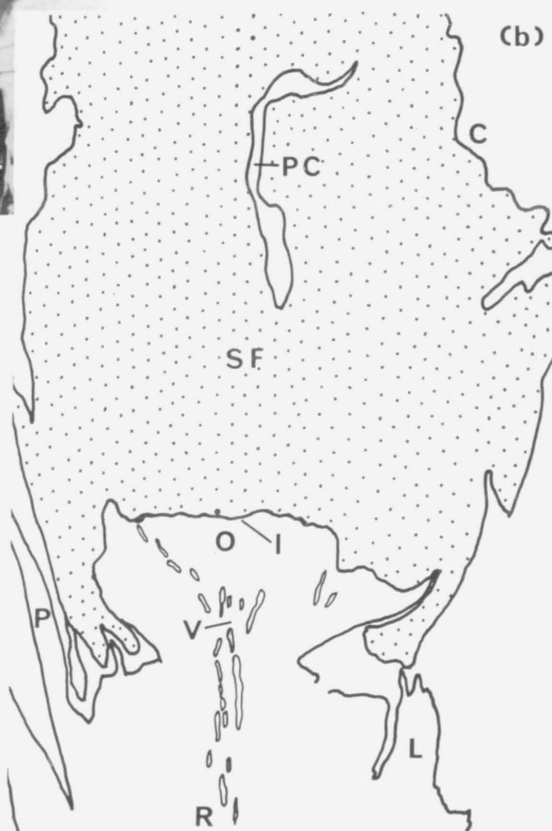
^a μg glucan hydrolysed/mg protein/ml incubate/h; first-order kinetics.

^b μg gentiobiose $\times 10^{-2}$ hydrolysed/mg protein/ml incubate/h; first-order kinetics.

^c —, not tested.



(a) Longitudinal section of 7 day-old ergot developing on its host. Only the base of the host ovary (O) remains, the upper part having been displaced by the sphacelial form (SF) of the parasite. The base of the ovary is intact and contains vascular tissue (V). Rectangle indicates area of Plate 2. Scale bar is 100 μ m.



(b) Line drawing of (a) showing extent of ergot development (dotted area) and its proximity to host vascular tissue. P, palea; L, lemma; R, rachilla; C, conidia borne on surface; PC, pocket of conidiation.



PLATE 2. Enlarged detail of Plate 1 showing host-parasite interface (I) in relation to xylem vessel (X). Host cells (H) below the host-parasite interface appear healthy, and the border between host and parasite is quite distinct. N, nucleus; St, starch grains. Scale bar is 5 μ m.

Extracts of this tissue demonstrated that an increase in activity appeared between 7 and 9 days from infection preceding the appearance of honeydew (Table 1). Up to 7 days, infected and uninfected tissues showed the same levels. Activity was well established 12 days after infection and was retained throughout the development of the ergot. Glucosidase activity paralleled that of the glucanase (Table 1) and was confirmed by the absence of gentiobiose which is released from *Claviceps* glucan following hydrolysis by exo-glucanases [2, 7]. As the ergots matured, the levels of glucanase increased (Table 1) and despite the fact that most of the mature tissue was sclerotial, extracts from the apical region of the ergot showed consistently higher activity than those from the basal region. Honeydew extracts showed glucanase activity more than ten-fold greater than that of ergot tissue extracts (Table 2). The difference between the rates for dialysed and undialysed honeydew

TABLE 2
Action of enzymes present in the honeydew exudation from cereals infected with Claviceps purpurea strain 29/4D, on [$U^{14}C$]glucan—for experimental details see text

Host plant	Time after inoculation (day)	Rate of glucan hydrolysis ^a		Protein concentration of honeydew (mg/ml)	
		Exudate not dialysed	Exudate dialysed	Exudate not dialysed	Exudate dialysed
Rye	11	2.3	2.6	1.4	1.2
Rye	17	1.9	2.4	3.4	2.7
Wheat	17	3.0	4.0	3.9	3.0

^a Results expressed as mg glucan hydrolysed/ml incubate/mg protein/h.

might partly be ascribed to the respective protein estimations, since in the undialysed honeydew absorption at 280 nm is due to free tryptophan and tyrosine in addition to protein [17]. The variations in the ratios of glucose to gentiobiose (Table 3) showed that glucose was the major product in dialysed honeydew incuba-

TABLE 3
Ratio of glucose : gentiobiose released from [$U^{14}C$]glucan during incubations with honeydew collected from cereals infected with Claviceps purpurea strain 29/4D—for experimental details see text

Incubation time (h)	Rye: day 11 ^a		Ration of glucose : gentiobiose		Wheat: day 17	
	Undialysed	Dialysed	Undialysed	Dialysed	Undialysed	Dialysed
0	1.2	4.0	2.0	2.7	1.6	2.4
0.5	1.7	8.0	1.8	5.6	2.0	8.7
1	1.8	11.3	2.1	9.0	2.3	11.8
2	1.9	16.4	2.0	12.2	2.8	16.0
3	1.8	13.8	2.2	18.7	3.0	27.3
5	1.8	19.7	2.2	19.7	4.2	32.2
24	3.9	8.3	2.6	3.1	2.3	12.0

^a First day of honeydew appearance.

tions with glucan, as a result of β -glucosidase activity on the gentiobiose released. However, in the non-dialysed honeydew incubations, the ratio of glucose to

gentiobiose was very low, which indicated that β -glucosidase was inhibited by constituents of the honeydew that can be removed by dialysis.

(b) *Hydrolysis of plant callose.* Mechanical wounding of healthy ovaries of rye and wheat resulted in the deposition of callose around the wounded area within 1 h, especially in the cell walls adjacent to ruptured cells. Table 4 shows that the bright

TABLE 4
Effect of exo- β -1 $\rightarrow$ 3 glucanases from various sources on the fluorescence of aniline blue-stained ovarian "wound callose"—for experimental details see text

	Water	Treatment		Exo- β -1 $\rightarrow$ 3-glucanase ^a
		<i>Claviceps</i> extract	Dialysed honeydew	
Fluorescence	19.7 $\pm$ 2.0 ^b	7.8 $\pm$ 2.0 ^b	6.8 $\pm$ 2.8 ^b	5.6 $\pm$ 2.8 ^b
P value ^c	—	0.002	0.01	0.002

^a From basidiomycete QM806.

^b S.E.M. given; results are expressed as photons sq. $\mu\text{m}^{-1} \text{s}^{-1}$.

^c Significance of difference between control and treatment.

yellow fluorescence of callose, deposited 4 h after wounding, with aniline blue was markedly diminished in all three treatments. In the case of the QM806 treatment, there was no residual fluorescence that could be attributed to callose.

The fluorescence observed in phloem of the ovarian vascular tissue was reduced by 80% in these treatments.

In infected ovaries, fluorescence microscopy revealed no evidence for the presence of callose.

DISCUSSION

Callose is a β -D-1 $\rightarrow$ 3 linked glucan whose conformation is considered to be a stable triple helix [19]. It has a wide distribution in plants, occurs naturally in sieve tubes and is deposited rapidly by the plant to plug wounds resulting from non-specific mechanical or chemical injury [5, 10, 11, 16]. It is also formed within structures known as papillae and is deposited between host plasmalemma and cell wall in response to infection by fungi, viruses and nematodes [1, 21, 22]. However, papillae do not always form during penetration of cells by fungi, and the factors controlling the deposition processes are unresolved [1, 3].

Claviceps purpurea parasitizes grasses and cereals successfully without deep invasion of the host plants' tissues and little if any intracellular penetration (Plate 2). Amongst other reactions, wound callose may possibly be deposited by the plant in response to the physical pressure or chemical stimulation of the invading hyphae; moreover, papillae have been shown to occur in response to intercellular hyphae in rusts at the host-parasite interface [12, 23]. Enzymes which hydrolyse callose, e.g. exo- β -1 $\rightarrow$ 3-glucanase [7], have been detected early during infection by *C. purpurea* before the release of honeydew is evident, and glucanase activity is a consistent feature of ergot tissues adjacent to the point of attachment of host and parasite. Activity in this region of the developing ergot appeared to decline coincident

with release of honeydew. However, glucanase activity has been found to be particularly high in dialysed honeydew; this is to be expected since most glucanase activity in *Claviceps* is manifest extracellularly [7]. The activity of some enzymes previously investigated is repressed in non-dialysed honeydew, probably by the high levels of free hexose [8]. The low β -glucosidase activity noted here in honeydew could arise for the same reason, since high levels of glucose in particular have been shown to be an inhibitor of the same enzyme from *Saccharomyces cerevisiae* [9]. Moreover, there may be a low requirement for β -glucosidase activity *in vivo* since callose is unbranched and would yield few β -glucosides following hydrolysis by an α - β -1 $\rightarrow$ 3-glucanase. However, some β -glucosides might arise from the action of the glucanase on other glucan-type polysaccharides known to be present in cereals [25]. These glucans contain mixed linkages as well as β -1 $\rightarrow$ 3, and accumulation of the β -glucosides from this source could be limited by the presence of a β -glucosidase.

In cereals, a core of thick-walled cells interrupts the continuity of xylem between the rachilla and ovary or seed [13, 28, 29]. This central core is ringed by sieve tubes and transfer cells which form the only direct pathway between rachilla and ovary. In infected ovaries, penetration of hyphae does not proceed beyond the central core, and it is at this location that fungal glucanase might have its greatest effect. The present fluorescent studies suggest that the enzyme could not only digest any wound callose that may form in sieve tubes and surrounding cells, but, since no callose was detected in infected ovaries, also degrade normal sieve plate callose. This unblocking of sieve tubes and possible degradation of wound callose could maintain a flow of nutrients to the pathogen.

Smith *et al.* [24] have discussed the concept of "source" and "sink" between heterotroph and autotroph in fungal parasitism of plants, and Mower & Hancock [15] suggest that a "sucrose sink" is the major driving force behind the translocation from host to parasite in *Claviceps* spp. The volume of honeydew released to the pathogen is normally far in excess of its requirements. This suggests that the flow of nutrients is not necessarily regulated solely by means of a "sink", and that other factors are operating to initiate and maintain the exudation. The present studies suggest that glucanases have a rôle in establishing parasitic sphacelial mycelium, and sustaining the development of sclerotia by ensuring an abundant supply of nutrients.

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