

THE COLONISATION OF CEREAL OVARIES BY
CLAVICEPS PURPUREA, *CLAVICEPS FUSIFORMIS*, *TILLETIA CARIES*
AND *TOLYPOSPORIUM PENICILLARIAE*.

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ABSTRACT

The colonisation of ovaries of millet by *Claviceps fusiformis* and *Tolyposporium penicillariae* and of wheat by *C. purpurea* and *Tilletia caries* was investigated using light and electron microscopical techniques. The tissues with which the pathogens became associated within the ovary were examined by light microscopy and details of the host cells associated with, or invaded by, the pathogens were investigated using specific ^{electron microscopical} staining and enzyme cytochemical techniques. In each instance the development of the tissues after pollination and infection were compared in order to elucidate the nutritional relationship between the host and the parasite. Spore formation was also investigated.

Tolyposporium penicillariae and *Tilletia caries* replace the region within each host where, in pollinated tissue, the embryo and endosperm (seed) would have developed. Both form intercellular and a few intracellular hyphae. Host tissues, similar to the placenta-chalaza in pollinated tissue, develop in *T. caries* infected wheat ovaries suggesting that the parasite utilises the nutrient transport system which is normally established in association with a developing ovule. The placenta-chalazal tissue does not develop in millet infected with *T. penicillariae*. However the majority of the parasite occupies a similar region in the ovary to the seed in pollinated millet and the slight modification of the infected ovary, and the presence of hyphae within the undifferentiated placenta-chalazal tissue, similarly suggests the utilisation of the host's nutrient transport system for uptake of assimilates into the developing parasite.

Claviceps fusiformis and *C. purpurea* hyphae penetrate and colonise the whole of the ovary. In so doing they displace all the special host tissues concerned in the nutrient transport to the seed and associate with living host cells, particularly those of the vascular tissues, in the pedicel region. Some of the host cells within the pedicel were colonised. Osmiophilic bodies in intact host cells of the pedicel of infected ovaries were similar to those in the pedicel of pollinated tissues where they probably cause the host cells to disrupt during the later stages of growth. The intactness of these cells and the absence of callose within the pedicel of the infected tissue probably assists with the flow of nutrients to the fungus.

The development of both *T. caries* and *C. purpurea* within the same ovary is consistent with the results above. *C. purpurea* hyphae establish in the pedicel displacing upward the *T. caries* colony and the associated differentiated maternal transport tissues.

In all infections a few host cells were penetrated by fungal hyphae but there was never any evidence that the host plasma membranes were breached or modified. Thus it seems likely that the efflux of host solutes in infected tissues was controlled by the processes normally occurring in the development of pollinated flowers.

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ABBREVIATION LIST

A	Anthers
Al	Aleurone layer
An	Antipodals
b	Osmiophilic bodies
C	Cavity
Cc	Central cell
Ca	Callose
Co	Collapse region
Cp	Chalazal pad
Cr	Coleorhiza
Ct	Coleoptile
CW	Cell Wall
c	chloroplast
cc	clamp connections
co	conidial pocket
ch	chromosomes
cw	cross wall
cp	cell plate
cu	cuticle
Ec	Egg cell
Ed	Electron dense material
Em	Embryo
EmS	Embryo-Sac
En	Endosperm
ER	Endoplasmic Reticulum
ET	Endosperm Transfer cell
Et	Electron transparent material
e	epicotyl
F	Filiform apparatus
Fn	Fungal nucleus
FW	Fungal Wall
g	Golgi apparatus
Hc	Host cell
Hr	Hilum region (Hilar)
HW	Host wall
h	Intercellular hyphae
I	Interface
IE	Inner Epidermis
II	Inner Integument
Ih	Intracellular hyphae
Ipm	Invaginated plasma-membrane
Lb	Lipid bodies
Lt	Lenticular thickenings
M	Mound region
Mc	Mesocarp
Mp	Micropyle
m	mitochondria
ml	middle lamella
N	Nucellus
Nie	Nucellar inner epidermis
Np	Nucellar projection
n	host nucleus
O	Ovary
OE	Outer Epidermis
OI	Outer Integument

OL	Ovarian Locule
Ov	Ovule
OW	Ovary Wall
Pc	Pericarp
Pd	Pedicel
Ph	Phloem
Pn	Polar nucleus
Ps	Pigment strand
Pt	Protective tissues
pc	parenchyma cell
pd	plasmodesmata
pm	plasma-membrane
R	Rachilla
Rd	Radicle
r	rachis
S	Seed Coat
SB	Sporeball
Sc	Sclerotial tissue
Sp	Sphacelial tissue
St	Stroma
St	Starch grains
Su	Scutellum
Sy	Synergid
s	spore
si	spore initial
sh	sporogenous hyphae
sl	stylodia
sp	septa
sp	sporidia
st	stigma
T	Teliospore
Tc	Transfer cell
tWc	thick Walled cell
V	Vascular tissue
Vh	Vascular associated hyphae
VIh	Intracellular Vascular associated hyphae
Vs	Ventral side
v	vacuole
ve	vesicle
W1	Primary Wall
W2	Second Wall
W3	Third Wall
W4	Fourth Wall
WI	Wall Ingrowth
wb	woronin bodies
wm	wall-like material
Xy	Xylem tracheids

SECTION ONE

INTRODUCTION.

Plant pathogens obtain nutrients from their hosts in a variety of ways. Some (e.g. *Penicillium expansum*, *Botrytis cinerea* and *Erwinia carotova*) are pathogens which destroy the host tissue so that nutrients are released from the host for utilization by the pathogen. This is a destructive process so once the released nutrients have been exhausted the pathogen must destroy further host tissue to release more nutrients. Other pathogens obtain their nutrients directly from the living host; that is they can absorb nutrients from the host tissues without first destroying the host cells.

Nutrients are available in the living tissue either within the symplast, the living part of the host bound by plasma membranes and interconnected by plasmadesmata, or within the apoplast, that region outside of the plasma membranes and encompassing cell walls and intercellular spaces. There are a few pathogens which contact the host symplast e.g. *Mycoplasmas* (Whitcomb, 1979) and *Olpidium brassicae* (Temminck and Campbell, 1968), where the major barrier to nutrient transfer from host to pathogen is the pathogen's membrane. The majority of pathogens are, however, in the host's apoplast. Even when the host wall is penetrated and the pathogen grows within the lumen of the cell it is surrounded by an invaginated host plasma membrane which represents the division between the apoplast and the symplast.

It has been argued that the concentration of nutrients within the general apoplast is sufficient to support pathogen growth for several days without destroying the host cells (Hancock and Huisman, 1981). Ectomycorrhizal fungi have an extensive intercellular

mycelium and mantle which develop without the benefit of haustoria. Thus nutrients almost certainly are available in the apoplast (Smith, Muscatine and Lewis, 1969). Furthermore, *Taphrina deformans* and some smut fungi are entirely or largely intercellular in growth (Cooke, 1977) depending on the apoplast for their nutrients (Bracker and Littlefield, 1973).

Some pathogens develop so that they colonise a nutritive rich region of the host apoplast. Wilt fungi (*Verticillium* sp: *Fusarium* sp.) colonise the conducting vessels of the host thus obtaining access to a potentially continuous supply of solutes. Though beneficially positioned for nutrient uptake these pathogens eventually occlude the conducting pathway causing the plant to wilt and eventually die. Other nutrient rich regions within the apoplast of the plant host are discussed below.

Direct contact with the host plasma membrane enables the plasmodial stage of *Plasmodiophora brassicae* to have immediate access to nutrients secreted by or diffusing from the host cell (Williams and Yukawa (1967); Keen and Williams (1969)). Efficient transfer of nutrients from host to pathogen is evident in pathogens which form haustoria, in particular most *Erysiphales* where only the haustorium contacts the host cells, the mycelium and sporulating structures remaining on the surface of the host's leaf. These and other haustorial pathogens probably obtain their nutrients from an isolated region of the apoplast. It has been shown that haustoria of rusts, powdery mildews and probably downy mildews (Heath (1976); Gil and Gay (1977); Coffey (1983); Manners and Gay (1983); Woods and Gay (1983)) also create an isolated apoplast around the haustorium within the infected cell. Nutrients crossing the extrahaustorial membrane

cannot freely move out of that apoplast because of an impermeable seal created around the haustorial neck. Nutrients can only pass either back into the symplast of the cell, which the haustorium has colonised, or they can pass into the haustorium itself. Therefore a pathogen which colonises or creates an isolated apoplast is at an advantage over one in an unrestricted apoplast.

There are other regions within the host where nutrients are available in large quantities within the apoplast. These occur between two groups of cells which are in close proximity but not connected by plasmodesmata. Solutes travelling within one symplast must therefore be transferred to the apoplast before uptake by the second group of cells. It is probable, and has been shown to be so in many cases, that the presence of transfer cells at such sites aids the transfer of nutrients from the one symplast to the other (Gunning and Pate, 1969). Transfer cells are not one particular sort of plant cell but the term describes an assortment of types which perform the transfer function (Gunning and Pate, 1974). Gland cells, epidermal cells, phloem and xylem parenchyma cells can all be classified as transfer cells, if their walls develop invaginations or infoldings which consequently increase the surface area of the plasma membrane lining the inner surface of the host wall (Gunning and Pate, 1974). Transfer cells have been found to develop at sites where there is a physical restriction to solute exchange imposed by an impervious layer e.g. cuticle, casparian strip, an air gap or discontinuity in the symplast of the host. They are often found as part of the vascular system of many plants, in nodes and at the junction between different generations of the plant (Pate and Gunning, 1972; Gunning and Pate, 1974). Between the generations cytoplasmic continuity is

lacking and consequently all materials exchanged from parent to offspring must pass via the apoplast (Pate and Gunning, 1972). Transfer cell development has been shown to coincide with the onset of intensive solute transport (Pate and Gunning, 1972). An example occurs in the development of giant cells with wall ingrowths in roots of *Helianthemum* induced by invading parasitic nematodes (Jones and Gunning, 1976). Cytological studies have shown transfer cells to be rich in ATPase activity (Maier and Maier, 1972; Bentwood and Cronshaw, 1978) indicating an active transport of nutrients across the extensive membrane. It seems likely that these cells may control the transport of ions and assimilates between the cytoplasm of the two unconnected symplasts.

Many regions in the plant have a plentiful supply of nutrients (e.g. meristematic tissue, vascular tissues and storage tissues (such as tubers, fruits, grains and some stems)). Some of these are colonised by biotrophic fungi which selectively destroy and replace specific regions of host tissue with somatic or reproductive structures of the pathogen. Luttrell (1981) has termed these pathogens replacement diseases and they include ergot (*Claviceps* sp.), Tar spot (e.g. *Phyllachora lespedezae*), Smuts (*Ustilago* sp.), bunt (*Tilletia caries*), false smuts (e.g. *Bipolaris ravenelii* on *Sporobolus*) and rusts. However, the validity of the hypothesis that these fungi occupy a nutritive rich region depends on their exact location with respect to particular cells of their hosts.

The ovaries of cereal crops were chosen as subject for research because not only are they storage organs, which during development act as very powerful sinks, but also there is a break in the symplast

between the maternal tissues and the developing seed (embryo and endosperm). Nutrients must therefore pass into an apoplast surrounding the seed before they can enter it. Both these features make the ovary a nutritive rich region and a potential site for biotrophic development.

Pearl millet and wheat were chosen for study. Species of *Claviceps* which infect the flowers of each and *Tolyposporium penicillariae* and *Tilletia caries* which respectively infect millet and wheat were selected as pathogens. It was first necessary to establish the development of the host tissues after pollination before examining the colonisation of the tissues by the respective pathogens. A detailed survey of the host cell types associated with the parasitic hyphae was carried out so that possible pathways for nutrient transfer into the developing fungal mycelium could be proposed and compared with the pathway of nutrient flow into the developing ovule.

SECTION TWO

GENERAL MATERIALS AND METHODS.

2.1. Cultures and Culture Media.

2.1.1. *Claviceps fusiformis* Loveless.

Cultures were maintained by aseptically transferring mycelial mats onto Medium-T slopes and incubating at 27°C. Honey-dew containing fungal conidia collected from the previous year's infection, was also stored in small ampoules under liquid nitrogen until required for inoculation.

2.1.2. *Claviceps purpurea* (Fr)Tul.

Cultures were maintained as *C. fusiformis* but incubated at 15°C.

2.1.3. *Tilletia caries* (DC.)Tul.

Bunt balls collected from wheat infected with *T. caries* in the previous year, were stored at room temperature in a dry place and used as a teliospore inoculum when planting the wheat seed.

The original inoculum and cultures were kindly supplied by Dr P.G. Mantle.

2.1.4. *Tolyposporium penicillariae* Bref.

(syn. *Moesziomyces penicillariae* (Bref) Vanky (1977))

Cultures were maintained by inoculating malt agar plates with sporeballs, collected from infected millet ovaries, and subsequently aseptically sub-culturing the developing sporidia and mycelium onto

further malt agar plates. All cultures were incubated at 18°C and stock cultures were kept at 4°C.

Mature infected millet ovaries, containing sporeballs, collected from infected greenhouse plants, were stored at room temperature in a dry place.

This culture (IMI 292500) was initially supplied by Dr E. Mordue of the Commonwealth Mycological Institute, Kew.

2.1.5. Medium T.

A medium used by B.Shaw (1979) on which *Claviceps fusiformis* and *C. purpurea* were grown.

Reagents

The medium, per litre of distilled water, consisted of;

Sucrose,	100.000g
L-asparagine,	10.000g
Ca(NO ₃) ₂ .4H ₂ O,	1.000g
MgSO ₄ .7H ₂ O,	0.250g
KH ₂ PO ₄ ,	0.250g
KCl,	0.125g
FeSO ₄ .7H ₂ O,	0.033g
ZnSO ₄ .7H ₂ O,	0.027g
L-cysteine hydrochloride,	0.010g
Yeast extract (Oxoid),	0.100g
Oxoid Agar No.3,	15.000g

Adjusted with NaOH (10% w/v) to pH 5.2 and sterilized at 121°C for 20min. The cooling medium was aseptically poured into sterile test-tubes to make slopes and when cold stored at 4°C.

2.1.6. Malt Agar.

Reagents

Malt Extract	5% w/v	Oxoid
Agar	2% w/v	Oxoid
Distilled water	1l	

The ingredients were mixed together and dispensed into 200ml medical bottles. After being autoclaved at 121°C for 20 min the cooled agar was aseptically poured into sterile plastic petri-dishes or stored for later use.

2.2. Plants and Inoculations

2.2.1. Millet (*Pennisetum americanum* (L.) Leake)

Seeds of the commercial variety BJ104 and male sterile 5141A were kindly supplied by Dr J. Willingale in conjunction with the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

Seeds were germinated in moist, covered petri-dishes at 30°C for 3 days, transplanted into a mixture of peat : John Innes No.2 (2:1 v/v) and grown for 60 days in a Fison growth cabinet (model no.600G3/TTL) set at 12h light (23°C) and 12h dark (18°C). After this daylength sequence, which was required to initiate flowering, the plants were repotted into 20cm diameter pots using a peat : John Innes No.2 (1:2 v/v) mixture and transferred to a glasshouse where plants flowered approximately 90 days after-germination. The conditions within the cold glasshouse, maintained at a minimum temperature of 18°C, varied with the seasons, though most work was completed during the summer months when the temperature reached 30-

35°C/day : 18-22°C/night, conditions similar to those during the monsoon growing season in Hyderabad, India. (See Appendix A)

The millet inflorescence matures basipetally, see PLATE 2:1, the stigmas protruding from the floral cavity for approximately 6 days before constricting and withering due to aging (Willingale and Mantle, 1985). Cultivar BJ104 is highly protogynous, the stigma being extended 7 days before self-anthesis. To prevent cross-pollination the emerging inflorescences were covered with small paper bags. *C. fusiformis* infections were obtained by spraying a suspension of conidia, prepared by agitating tap water on the surface of a 10 day old slope culture, decanting through two layers of muslin and spraying, using a Shandon spray gun, the millet inflorescence when most of the stigmas were fully extended, approximately 4 days after the first stigma emergence. After inoculation the small paper bag was replaced.

As very little information was available on the development and infection of *T. penicillariae* it was first necessary to evaluate the conditions required to consistently establish infection. Variations of the inoculum source (sporidia, sporeballs or mycelial fragments); times of inoculation with respect to the flowering sequence; cultivars, (cultivar 5141A (male sterile) was used in the initial infection studies so that competition from pollen grains was eliminated) and variations in temperature proved unsuccessful until it was recognised that high humidity ($\geq 90\%$ R.H.) was required for infection to occur. *T. penicillariae* infections were obtained by decanting, through 2 layers of muslin, a sporidial suspension prepared from a 5 day old sporidial culture. The suspension was then sprayed, using a Shandon gun, onto the fully extended stigma

approximately 4 days after the first stigma emerged. The inflorescence was then covered with a clear polythene (PVC) bag in order to maintain a high relative humidity. Further variations with the inoculum source and temperature are discussed in section 4.2.

Pollinated millet samples were obtained by tapping pollen from an anthesising inflorescence into the surrounding paper bag and transferring this bag to an inflorescence where the stigma were fully extended, again tapping the bag to disperse the pollen grains. Samples were subsequently taken at the required intervals.

2.2.2. Wheat (*Triticum aestivum* L. (*T. vulgare* Host.) cv. Avalon and Rapier.

Undressed wheat seeds grown for infection by *Claviceps purpurea* and *Tilletia caries* were generously supplied by Mr W.B.Gow of West Farm, Eaton, Oxfordshire.

Wheat seeds, either inoculated or uninoculated with *T. caries*, were sown in rows approximately 30cm apart and 10cm deep at Chelsea Physic Garden, Chelsea, London during November of 1983 and 1984. The wheat seeds were inoculated with *T. caries* by immersing them in a concentrated suspension of teliospores prior to sowing and subsequently covering the planted seeds with more teliospores before burying. Infected wheat plants were not distinguishable until late July when control plants were flowering.

During early July of the following year the wheat inflorescences emerged from boot. Uninfected plants were still unpollinated as anthesis and self-pollination occurred about one week later. However, the ovaries of infected plants were already filled with developing or fully mature teliospores. It was therefore necessary to sample much earlier to obtain the stages of development within the

ovary. Samples were taken throughout the period of rachis extension, between the appearance of the 5th node and the time the inflorescence was in boot (stages 35-45 of the decimal code [Zadok's (1978)], see Figure 2:1). Cutting the rachis promoted the development of tillers and if any of the new tiller heads showed bunt symptoms the corresponding sample, stored in glutaraldehyde (2.5% v/v) in 0.1M sodium cacodylate buffer, was processed further. Samples obtained after emergence from boot (stages 45-80, see Figure 2:1) were processed immediately as infection symptoms were visible with the naked eye. A large number of florets (30-40) were sampled at each stage because of the uncertainty of whether the sampled host tissue, particularly at an early stage, was infected. Also in some inflorescences only some of the florets were infected, a phenomenon termed partial bunting.

Some maturing bunted and control wheat inflorescences were inoculated, prior to anthesis, with a suspension of *C. purpurea* conidia prepared by either agitating tap water on the surface of a 10-day old culture and filtering this through two layers of muslin or from an aqueous suspension of defrosted honey-dew. The suspension was injected, through the glumes, into the floral cavity using a hypodermic needle (Shaw, 1979).

Pollinated wheat samples were obtained by labelling the self-pollinating florets at anthesis and subsequently taking samples at the required intervals.

2.3. Transmission Electron Microscopy.

2.3.1. General Fixation Procedure.

A standard fixation and embedding procedure was adopted for all specimens for transmission microscopy (T.E.M.).

Reagents

Glutaraldehyde	5%(v/v)	Agar Aids Practical Grade.
Sodium cacodylate buffer	0.4M	Taab
Osmium tetroxide	4%(w/v)	Taab
Ethanol	100%	James Burrough Ltd
Epoxypropane	100%	British Drug Houses(BDH)
Epon resin(Luft,1961)	1A:7B	Agar Aids

Double distilled water.

Ovaries dissected from the surrounding protective tissues so that the ovary remained attached to the pedicel, were left whole, or cut longitudinally or transversely into two halves, under a fixative of glutaraldehyde (2.5% v/v) in 0.1M sodium cacodylate buffer. The samples were left in the fixative, in stoppered bottles or covered embryo cups for a minimum of 2h before washing in three subsequent changes of fresh buffer (20min each). Transferred to a fume cupboard the samples were post-fixed in osmium tetroxide (1% w/v) in 0.1M sodium cacodylate buffer for 1-2h, depending on the size of the tissue. They were then dehydrated through a 10%-step series of ethanol (10min each) to 100% ethanol. After being taken through the following sequence:

- 2 changes in 100% ethanol for 15min each
- 30min in a mixture of ethanol:epoxypropane (2:1 v/v)
- 30min in a mixture of ethanol:epoxypropane (1:2 v/v)
- 30min in 100% epoxypropane

the samples were left in epoxypropane : Epon 1A:7B (1:1 v/v) for at least 2h at room temperature. The lids were then removed allowing the epoxypropane to evaporate prior to storing overnight at 4°C. The ovaries were subsequently transferred through 4 changes of fresh Epon resin during the following 2 days and then polymerized at 60°C for 60h in flat PVC embedding dishes.

C.purpurea and *C.fusiformis* samples taken 7 d.p.i. or later were washed over a period of 2 hours in 6 successive changes of the initial fixative before processing further so that the sugary honey-dew, which causes poor fixation of the tissue, was removed. ←

Samples containing *T.caries* teliospores were embedded in Epon all B resin, a harder consistency enabling the teliospores to be sectioned.

2.3.2. Sectioning and Staining.

Embedded material, selected by light microscopy, was sawn out of the resin disc and mounted on a 10mm wooden dowell using Durofix glue (Rawplug Co.). Block-faces were trimmed into flat-topped pyramids (3x2mm) using single-edged razor blades and thick sections (0.5-1.0 µm), obtained with glass knives and a Reichert Ultracut microtome, were mounted onto glass slides and stained with toluidine blue (see section 2.4) before observation with a light microscope. This enabled both a detailed survey of the tissue and a precise location of cells for ultrathin-sectioning.

Block-faces trimmed to the selected areas (approx. 1 x 0.5mm) were cut using an IVIC diamond knife on either Reichert or LKB 2 microtomes. Sections with silver or gold interference colours were picked up on 75 mesh, 100 mesh or slotted copper grids which had been previously filmed with formvar (a 0.6%(w/v) solution in

chloroform). Most sections were subsequently stained (see sections 2.3.2.i and 2.3.2.ii) before observation using a Philips 301G TEM operated at 80KeV. Images were recorded on Agfa-Gevaert RA7 10P or F071P line-lith film and printed on Agfa-Gevaert rapidoprint paper using a Rapidoprint processing machine.

2.3.2.1. Uranyl Acetate and Lead Citrate Staining.

Reagents.

Aqueous uranyl acetate	2% (w/v)	Sigma
Reynolds lead citrate	Reynolds(1963)	Agar Aids
Double distilled water		
Sodium hydroxide	(Pellets)	BDH

Thin sections picked up on copper grids were immersed for 25min in drops of uranyl acetate (2% w/v) at 60°C, washed in a stream of double-distilled water (d.d.H₂O), by running the water down the forceps holding the grid, and further stained in drops of Reynolds lead citrate solution (Reynolds 1963) for 10-15min at room temperature. In the last treatment the grids were placed in a covered plastic petri-dish which also contained a concentrated sodium hydroxide solution to absorb any CO₂, in order to prevent precipitation of lead hydroxide. The grids were washed in a sodium hydroxide solution (0.1M) before finally being rinsed in a stream of d.d.H₂O, as described above. The sections were dried under the heat of a bench lamp and stored in capsule cards.

2.3.2.ii. Cytochemical Staining.

Periodic Acid-Thiocarbohydrazide-Silver Proteinate (PATCH-SP)

a stain for carbohydrates adapted from Thiéry(1967).

Reagents

Periodic acid	1%	(v/v)	BDH
Thiocarbohydrazide	0.2%	(w/v)	Sigma
Acetic acid	20%	(w/v)	Analar-BDH
Silver proteinate	1%	(w/v)	Gurr
Double distilled water			

Ultrathin sections, showing gold interference colours and mounted on unfilmed 200 mesh gold grids were immersed in periodic acid (1% v/v) for 30min, washed in d.d.H₂O and dried before being placed in a solution containing thiocarbohydrazide (0.2% w/v) in acetic acid (20% v/v) for 72h. The grids were then washed in a descending 5%-step series of acetic acid solutions from 20% to water and then dried under a bench lamp before being placed in an aqueous solution of silver proteinate (1% w/v), in the dark, for 30min. Finally the grids were washed in d.d.H₂O and air-dried before observing in the transmission electron microscope.

Controls omitted the periodic acid, thiocarbohydrazide or silver proteinate steps.

Periodic Acid Chromic Phosphotungstic Acid (PACP)

a stain for glycoproteins in plasma-membranes adapted from Roland, Lembi and Morré (1972).

Reagents

Periodic acid	1% (v/v)	BDH
Chromium trioxide	10% (w/v)	BDH

Phosphotungstic acid 1% (w/v) Agar aids

Double distilled water

Ultrathin gold sections mounted on unfilmed 200 mesh gold grids were immersed in periodic acid (1% v/v) for 30min, washed in d.d.H₂O and air dried before placing in an aqueous solution containing phosphotungstic acid (1% w/v) and chromic acid (10% w/v) for 20min. The grids were washed in d.d.H₂O and dried before observation in the transmission electron microscope.

Controls omitted either the periodic acid or the phosphotungstic/chromic acid steps.

2.3.3. Electron Microscopical Localization of
Adenosine Tri-phosphatase (ATPase).

Reagents

Formaldehyde*	16%(w/v)	BDH
Glutaraldehyde	8%(v/v)	Polaron Equip. Ltd.
Sodium cacodylate buffer(pH 7.2)	0.4M	Taab
Adenosine 5-triphosphate(ATP)	10mM	Sigma
Sodium-β-glycerophosphate	10mM	Sigma
Magnesium nitrate Mg(NO ₃) ₂	10mM	BDH
Lead nitrate Pb(NO ₃) ₂	10mM	BDH
Sodium orthovanadate	2mM	BDH
Tris maleate buffer(pH 7.2)	0.5M	BDH
Osmium tetroxide	4%(w/v)	Taab

Double distilled water

*The formaldehyde was prepared by heating a solution of paraformaldehyde neutralised with drops of 1.0M sodium hydroxide.

All stock solutions were prepared the previous day and stored at 4°C, except for ATP and sodium-β-glycerophosphate which were prepared immediately before use.

Dissected ovaries were cut under fixative, either in half or in thin slices, using a Wilkinson Sword razor blade. The samples were left in the fixative, comprising a mixture of formaldehyde (4% w/v) and glutaraldehyde (0.5% v/v) in 0.1M sodium cacodylate buffer, for 2.5h. The samples were washed in 6 successive changes of 0.1M tris maleate buffer for 2h at 4°C before being transferred to one of the following incubation media, for 2.5h at room temperature:

ATP substrate. ATP (2mM); Mg(NO₃)₂ (2mM) and Pb(NO₃)₂ (2mM) in 0.1M tris maleate buffer, pH 7.2 .

Alternative substrate. Sodium-β-glycerophosphate (2mM); Mg(NO₃)₂ (2mM) and Pb(NO₃)₂ (2mM) in 0.1M tris maleate buffer, pH 7.2.

ATPase inhibitor. ATP (2mM); Sodium orthovanadate (2mM); Mg(NO₃)₂ (2mM) and Pb(NO₃)₂ (2mM) in 0.1M tris maleate buffer, pH 7.2 .

No substrate control. Mg(NO₃)₂ (2mM) and Pb(NO₃)₂ (2mM) in 0.1M tris maleate buffer, pH 7.2 .

The samples were then washed in 4 subsequent changes of 0.1M sodium cacodylate buffer for 1h before being post-fixed in osmium tetroxide (1% w/v) in 0.1M sodium cacodylate buffer for 1h, dehydrated and embedded in resin, as set out in the procedure described in section 2.3.1.

Ultrathin gold sections (approximately 90nm thick) were mounted onto formvar coated grids and observed unstained by transmission electron microscopy.

2.4. Light Microscopy

Toluidine blue staining.

Thick resin sections, mounted by air drying on glass slides, were covered with a drop of toluidine blue (1% w/v) in borate buffer, pH 12.0, and heated gently until steam was evident and the edges of the drop had started to dry. The stain was rinsed off using d.d.H₂O and air dried before permanently mounting in Canada balsam.

Light micrographs were obtained using a Reichert Zetapan microscope with bright field illumination. Images were recorded on 35mm film (Ilford Pan F (ASA 50)), which was developed for 9min in promicrol (standard strength).

2.5. Fluorescence Microscopy.

Thick sections (0.5-1.0µm) cut from Epon embedded material were mounted and air dried onto glass slides. A drop of aniline blue (0.1% w/v) in 0.07M tri-potassium phosphate buffer, pH 12.0, was placed on the section and left for 1-3hr (Allen and Friend, 1983). Observations were made with a Reichert Zetapan Binolux II with illumination provided by a mercury-vapour lamp (HBO200W/2) with an exciter filter E2 (transmitting with a peak at 365nm) and a barrier filter Sp2 (reflecting ultraviolet up to 425nm). Fluorescence was enhanced by prestaining the sections with toluidine blue (section 2.4) which quenched the surrounding autofluorescent regions (Smith and McCully, 1978a). Images were recorded on 35mm film (Ilford FP4 (ASA 125)) which was developed for 11 minutes in promicrol (standard strength).

Aniline blue: a stain specific for callose material.

2.6. Scanning Electron Microscopy.

To examine the growth of the fungi within the host material it was necessary to either cut or fracture open the ovaries. A number of methods were tested.

2.6.1. Following the general procedure for tissue preparation specimens were cut using a sharp razor blade or frozen and fractured at one of several stages (Table:2.1).

The cutting action, at any stage of the procedure, caused the soft tissue to compress so that when observed in the scanning electron microscope (S.E.M.) the tissue was distorted and cell contents were smeared across the cut surface. If the cut tissue was treated by chemical hydrolysis (Kinden and Brown,1975) or by enzymatic digestion (Honneger,1985) the cell contents were washed away during the subsequent treatments, leaving only the cell walls of the host and parasite intact. Unfortunately these techniques produced distorted images when used on the delicate ovary tissues and charging, under the electron beam in the S.E.M. resulted in unsatisfactory images.

The electrical conductivity was improved by treating the hydrolysed or digested specimen alternately with solutions of osmium tetroxide (1% w/v) in buffer and saturated thiocarbonylhydrazide solution over a number of days (Murphy,1978). Tissue preparation now took two weeks and the image remained distorted.

Specimens were also frozen and fractured at various stages of the preparative procedure. If fractured after fixation (Plotnikova et al,1979) the tissue at the centre of the specimen shrivelled during critical point drying as the fixatives had been unable to penetrate them. It was therefore necessary to fracture prior to

fixation. Following the procedure devised by Barnes and Blackmore (1984), modified from Tanaka and Mitsushima (1984), the tissue was frozen in a cryoprotectant under liquid nitrogen, fractured and left in buffered osmium tetroxide (2% w/v) for at least two weeks, before continuing the general preparative procedure. This gave good electrical conductivity. If observed with a high resolution S.E.M. details such as golgi apparatus and chloroplasts could be seen as the osmium etches away at the fractured surface leaving the membrane-bound organelles intact. High resolution was not attainable with the Philips 500 S.E.M. so although successful, this method was unnecessarily long. It was also difficult to hold the ovaries under the liquid nitrogen so that they could be fractured along a particular plane. An alternative method was therefore developed (section 2.6.2) incorporating parts of the above methods that proved to be successful with the delicate ovary tissue.

2.6.2. Routine Preparation of Specimens for Scanning Electron Microscopy.

Reagents

Glutaraldehyde	5%(v/v)	Agar Aids(Practical grade)
Sodium cacodylate buffer	0.4M	Taab
Dimethylsulphoxide	50% (v/v)	BDH
Tissue-Tek		O.C.T. 4583 R.Lamb
Osmium tetroxide	4% (w/v)	Taab
Acetone	100%	May and Baker
Durafix		The Rawplug Co. London
Silver dag		Agar Aids

Specimens were fixed in glutaraldehyde (2.5% v/v) in 0.1M sodium cacodylate buffer at pH 7.2, for 1h and taken through a 10%-step series of dimethyl sulphoxide (DMSO), a cryoprotectant, to 50% before mounting and accurately orientating in Tissue-Tek (a gel-like substance) on the head of an aluminium rivet. After rapidly freezing, by plunging into slushed Arcton 22 (ICI), the rivet was inserted into a hole in a liquid nitrogen cooled brass block and the tissue fractured with a Wilkinson Sword razor blade, previously cooled with liquid nitrogen. After thawing, by placing in d.d.H₂O for 30min, and washing in six successive changes of 0.1M sodium cacodylate buffer over a period of 2h, to remove residual cryoprotectant, the material was post-fixed in osmium tetroxide (2% w/v) in 0.1M sodium cacodylate buffer for at least 2h, or overnight at 4°C. It was then dehydrated in a 10%-step series of acetone, for 10min each, to 100% acetone and the specimens processed in a Polaron E3000 critical point drier. The specimens were mounted onto stubs using Durafix glue or liquid silver dag, coated with gold, using a Polaron E5000 sputter coater, and stored in a dessicator. They were observed in a Philips 500 S.E.M. operated at 12 KeV, with a spot size of 320nm. Images were recorded on 35mm film (Ilford Pan F (ASA 50)) and printed on Agfa-Gevaert paper using a Rapidoprint processing machine.

PLATE 2:1

A SERIES OF PHOTOGRAPHS SHOWING THE FLOWERING SEQUENCE

IN PEARL MILLET (*Pennisetum americanum*) var. BJ104

- a Inflorescence emerging from the boot sheath.
- b White feathery stigma emerge at the top of the inflorescence.
- c Stigma extending at the top and emerging towards the base of the inflorescence.
- d Stigma fully extended at the top of the inflorescence continuing to emerge and extend basipetally.
- e All stigma are fully extended - Full protogynae

Artificial pollination or pathogen inoculation was done at this flowering stage.

NOTE: The stigma are beginning to wither at the top of the inflorescence due to their age.

- f Yellow-brown anthers emerge at the top of the inflorescence.
- g Anthers emerge basipetally shedding pollen onto the lower stigma.
- h A self-pollinated inflorescence. Maturing grains are white.
- i A self-pollinated inflorescence. Mature grains are silvery in colour.

Flowering Sequence in Pearl Millet



Pollination
Infection
Stage

FIGURE 2:1

THE DECIMAL CODE FOR GROWTH STAGES OF ALL SMALL GRAIN CEREALS.

(Zadok's and others)

In the code, ten principal growth stages are distinguished and each is sub-divided into secondary stages.

CODE	0	Germination
CODE	1	SEEDLING GROWTH
CODE	10	1st leaf through coleoptile
CODE	11	1st leaf unfolded
CODE	12	2nd leaf unfolded
CODE	13	3rd etc....
CODE	2	TILLERING
CODE	20	Main shoot only
CODE	21	Main shoot and 1 tiller
CODE	22	Main shoot and 2 tillers
CODE	23	Main shoot and 3 etc.....
CODE	3	STEM ELONGATION
CODE	30	Leaf sheath erect and exceeds 5cm in length
CODE	31	1st node detectable
CODE	32	2nd node detectable
CODE	33	3rd node etc.....
CODE	37	Flag leaf just visible
CODE	40-49	BOOTING stages in development of ear
CODE	50-59	Stages in development of the inflorescence.
CODE	60-69	Stages in development of anthesis (pollination)
CODE	70-79	Milk development stages in grain
CODE	80-89	Dough development stages in grain
CODE	90-99	Ripening stages in grain (including dormancy)

A Decimal Code For Growth Stages Of All Small Grain Cereals

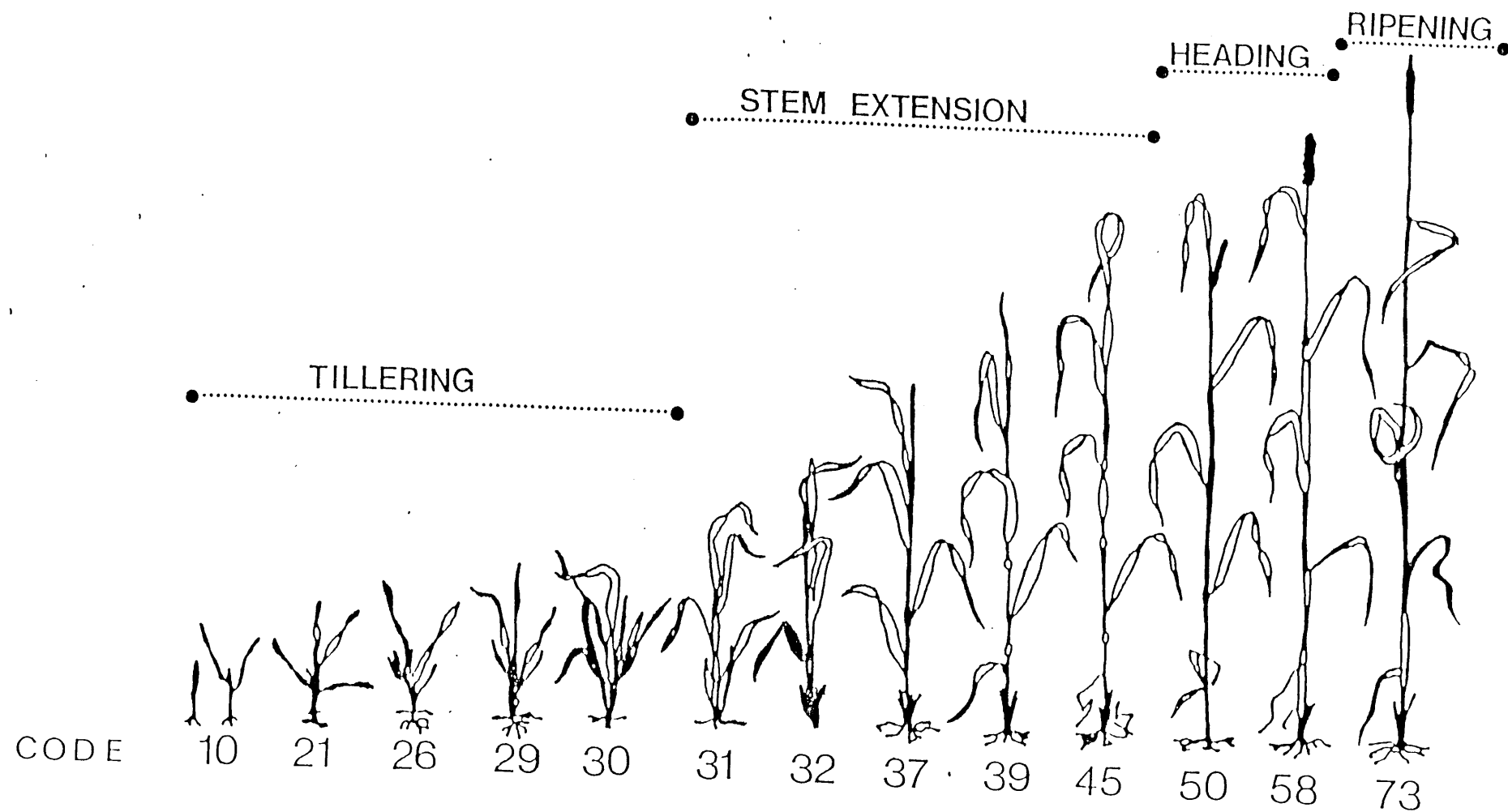


TABLE 2:1

PREPARATIVE PROCEDURES PREVIOUSLY EMPLOYED FOR
SCANNING ELECTRON MICROSCOPY

<u>Procedure</u>	<u>Specimens Cut</u>	<u>Specimens Fractured</u>
Fix tissue in glutaraldehyde (2.5% v/v) in 0.1M sodium cacodylate buffer, pH7.2, for 2 hours.	Honneger (1985)	Barnes and Blackmore (1984)
↓		
Wash, 3 times, in 0.1M sodium cacodylate buffer for 20min each.	Murphy (1978)	
↓		
Post-fix in osmium tetroxide (1% w/v) in 0.1M sodium cacodylate buffer for 1 hour.	Kinden and Brown (1975)	
↓		
Dehydrate in 10% step-series of acetone (10min each) to 100% acetone.		Plotnikova et al (1979)
↓		
Critical point dry.		
↓		
Mount, sputter coat with gold and observe in the microscope.		

SECTION THREE

THE STRUCTURE AND DEVELOPMENT OF THE MILLET OVARY BEFORE AND AFTER POLLINATION.

3.1. The unpollinated millet ovary and the initial development of the tissues associated with the developing embryo after pollination have not been investigated previously.

Samples of millet, unpollinated, 3, 7 and 15 days post pollination (d.p.p.) were processed and examined by light and electron microscopy (sections 2.4.1 and 2.6.1.).

3.2. RESULTS.

Within the unpollinated ovary the embryo-sac lay at an angle of 30-45° to the axis of the ovary. Therefore serial longitudinal sectioning gave rise to sections each containing part of the embryo-sac. Figure 3:1 shows a diagram of the unpollinated ovary derived from a number of these serial thick sections (Plates 3:1, 3:3 and 3:5-8).

The unpollinated ovary contained an embryo-sac which was composed of two synergids, with the filiform apparatus (Plate 3:9), an egg cell (Plate 3:7), a central cell with two polar nuclei (Plate 3:5) and many antipodal cells, at the upper end of the embryo-sac (Plates 3:3, 3:6 and 3:8). The wall of the embryo-sac was thin with small protuberances of cell wall and membranous material (Plates 3:10 and 3:11). The embryo-sac was in turn surrounded by the nucellus, the inner and outer integuments and then the ovary wall which on one side was developed as the hilum (Plates 3:1 and 3:3). The embryo-sac was surrounded except in the region termed the hilum, by three cuticles which lay on the outer surfaces

of the nucellar inner epidermis, the inner integument and the outer integument (Plate 3:2). Between the outer integument and the inner epidermis of the ovary wall lay the ovarian locule a space containing either air or fluid (Plate 3:4).

Xylem tracheids and phloem sieve tubes were visible in the pedicel, an extension of the pericarp tissue which attaches the ovary to the rachilla of the floret (Plates 3:1 and 3:3). Two strands of xylem diverging at an angle of 120° were visible in whole ovaries, cleared overnight in sodium hypochlorite (10% v/v), and each extended from the pedicel through the ovary wall and upwards through the length of the stigma.

Pollination has been described by Willingale and Mantle (1985). Pollen grains landing on the exerted stigmas germinate and penetrate into the intercellular spaces between stigmatic cells forming tubes which travel down the stylodium in specialised intercellular tracts (Willingale and Mantle, 1985). After 4-5 hours a constriction zone becomes visible near the base of the style causing the fused stylodia to wither and eventually become detached from the ovary (Willingale and Mantle, 1985). The pollen tube continues to grow tangentially around the ovarian locule heading for the micropylar tissue (Plate 3:4) (Willingale and Mantle, 1985). Fertilization of the ovary has not been investigated.

Structural changes were evident within the tissues at 3 d.p.p.. The embryo-sac had enlarged as the endosperm multiplied. The surrounding nucellus had disrupted as had the antipodal cells. Transverse sections of material 3 d.p.p. showed that on one side of the ovary nucellar cells (or cells differentiated from tissue adjacent to the hilum in the unpollinated ovary), had remained,

forming a crescent-shaped pad, the chalazal pad (Plate 3:13). The endosperm cells were large and highly vacuolated, except where they abutted the chalazal pad. These cells had smaller vacuoles, more cytoplasm and their cell walls, adjacent to the chalazal pad, had invaginated to form small ingrowths (Plate 3:13). These cells are the endosperm transfer cells (Plate 3:14). Between the chalazal pad and the compacted cells of the hilum a dark band of cells had been formed, the pigment strand (Plate 3:15). Phloem sieve tubes were visible within the cells of the hilum region. Endosperm cells in the upper portion of the endosperm had nuclei, plasma-membranes and they were producing starch grains (Plates 3:16 and 3:18), whilst the endosperm cells in the lower portion were still forming after the rapid coenocytic division of the primary endosperm nucleus (Plate 3:17).

By 7 d.p.p. very few nucellar cells were visible, most having disrupted as the seed (embryo and endosperm) enlarged (Plate 3:19). The endosperm cells continued to divide and enlarge filling the space within the ovary provided by the expanding ovary wall. Starch grains were numerous in the upper portion, basipetally filling the endosperm (Plate 3:19). Endosperm transfer cells remained distinct the wall thickenings extending further into their cells and also developing on neighbouring endosperm cells so that a layer, approximately 3 cells deep of cells with wall ingrowths, was formed (Plate 3:20). The wall thickenings of the endosperm transfer cells were fluoresescent when thick sections were stained with aniline blue (Section 2.5) (Plate 3:21). This stain indicates the presence of callose (Smith and McCully, 1978b). The chalazal pad now extended along the whole

length of the endosperm transfer cells and its cells contained few or no contents (Plate 3:22).

Of the original three cuticles two became distinct, thickening on the outer surfaces of the nucellar epidermis and inner integument (Plate 3:23). The outer integument collapsed after pollination and any remaining cells were disrupted. As previously, these two cuticles isolated the tissues of the ovary wall from those of the developing seed except in the hilum region (Plates 3:13 and 3:22). The cuticles remained very thin at the edges of the hilum and were absent within it. Phloem sieve tubes were visible in the hilum region amongst the compacted tissue of the vascular pericarp and the pigment strand adjacent to the chalazal pad. Plasmodesmata connected all the cells of the pericarp but none were visible between the pericarp tissue and the developing seed (ie. between the chalazal pad cells and the endosperm transfer cells), the remainder of the seed was surrounded and isolated from the ovary wall by both cuticles and a gap (Plate 3:19). Cells within the embryo were small with large nuclei. The embryo continued to enlarge though no differentiation into axial organs was observed (Plate 3:22). The cells immediately surrounding the embryo were disrupted and the cells of the embryo were not symplastically connected to any of the endosperm cells (Plate 3:22).

Within the pedicel phloem sieve tubes and xylem tracheids were separate from each other, the xylem terminating in short lengths of un lignified tracheary elements at the base of the ovary. Xylem transfer or phloem transfer cells were not seen in the glume, lemma or palea traces of the pedicel and phloem transfer cells were absent from the pedicel. Xylem transfer cells were visible in the pedicel

tissue. Figure 3:2 is a diagram showing the various tissues in a developing millet ovary approximately 7 days post pollination.

By 15 d.p.p. the ovary had enlarged further to a diameter of approximately 1.4mm. The embryo was much larger, had differentiated to form axial organs and had extended towards the chalazal pad, though it remained symplastically isolated from the surrounding endosperm tissue (Plate 3:24). The placenta-chalazal tissue (chalazal pad, pigment strand and hilum tissues) was still visible, as were phloem sieve tubes within the hilum region (Plate 3:24).

Cells within the pericarp adjacent to the chalazal pad and those surrounding the vascular tissue in the pedicel stained heavily due to the presence of variously sized spherical bodies within their vacuoles (Plates 3:25 and 3:26). Electron microscopy showed these bodies to be osmiophilic and contained within a membrane. In cells with the largest bodies there were few or no others suggesting that enlargement was by a process of fusion. At the upper end of the pedicel this darkly staining material was not contained by a membrane or a wall and therefore bathed the region directly adjacent to the aleurone layer and endosperm transfer cells (Plate 3:26).

The aleurone layer remained distinct, staining darker than the neighbouring endosperm or ovary wall cells (Plate 3:27). The placenta-chalazal region was now quite small in comparison to the enlarged ovule (Plate 3:28). Cells of the chalazal pad abutting the endosperm transfer cells were devoid of contents and their walls were thicker and stained heavily (Plate 3:28).

As the pollinated ovary reaches maturity the embryo enlarges so that it approaches the endosperm transfer cells (Russell and Dwarthe, 1980) and by 36 d.p.p. these cells, the chalazal pad and the

adjacent pericarp tissues are crushed by the mature embryo (Fussell and Dwarthe, 1980). The diagram of the mature caryopsis (Figure 3:3) was compiled from observations of dry millet seeds using the scanning electron microscope (Plate 3:33) and from other studies of mature caryopses within the literature (Irving, 1983; Zeleznak and Varriano-Marston, 1982).

Localization of ATPase activity within the tissues of the developing ovary was unsuccessful. The deposition of lead within the tissue was more dependent upon the penetration of the substrate media into the tissues than could be attributed to enzyme activity (Plates 3:29 and 3:30). Precipitates were heavy where cells had disrupted or near cut surfaces (hand sections were cut so that during the primary fixation penetration of the substrates was improved) (Plates 3:29 and 3:30). When no damage was apparent to the cell being observed precipitates were found both on the plasma-membrane and in the cell lumen between the plasma-membrane and the cell wall (Plate 3:31). In all the samples observed the chalazal pad tissue did not stain intensely, even when the tissue either side of it had abundant ← precipitates. ATPase activity was observed in the endosperm transfer cells and in the pericarp cells adjacent to the chalazal pad (PLATE 3:32). However the intensity of the staining reaction at these sites was dependent upon how close the tissues were to a cut or disrupted area. No activity was observed in the pedicel vascular tissues and any staining reaction within the pedicel was confined to the outermost layer of cells.

3.3 NUTRIENT TRANSPORT WITHIN THE MILLET CARYOPSIS.

The structural development of the pollinated millet ovary is similar to that of maize (Kiesselbach and Walker, 1952; Felker and Shannon, 1980) and in sorghum (Sanders, 1955; Giles et al, 1975). Physiological data obtained from investigations of these similar grains is presented to propose a possible mechanism for nutrient transport into the developing millet caryopsis.

Radiolabelling studies have shown that assimilates accumulate in the cytoplasm and vacuoles within the pedicel tissues of maize. They move symplastically through the pedicel tissues and enter the apoplast of the placento-chalazal region where it is thought that the main constituent, sucrose, is hydrolysed to hexose sugars prior to or during uptake by the basal endosperm transfer cells. The hexose sugars now freely available in the apoplast can be taken into the symplast of the seed via the endosperm transfer cells where they will be utilized for growth and storage.

The mechanisms proposed to be involved with the movement of the assimilates into the developing caryopsis will be discussed, in the subsequent chapters, in relation to the transport of nutrients into the colonising pathogen.

PLATE 3:1

A light micrograph of a longitudinal section in the dorsal-ventral plane through an unpollinated millet ovary. The embryo-sac is not visible in this section. The ovary wall (OW) encloses the outer integument (OI), the inner integument (II) and the nucellus (N). The hilum region (Hr) is composed of small cells with dense cytoplasm. Phloem sieve tubes (Ph) lie between the hilum and the ovary wall and vascular tissue (V) is visible in the pedicel (Pd).

Magnification X 250

Staining: Toluidine Blue

PLATE 3:2

An electron micrograph of an unpollinated millet ovary showing a longitudinal section through the ovary wall.

Cuticles (cu) are visible on the surface of the nucellus (N) and the inner integument (II) and between the latter and the ovary wall (OW). The outer integument is not visible in this section.

Magnification X 3200

Staining: Uranyl acetate and lead citrate.

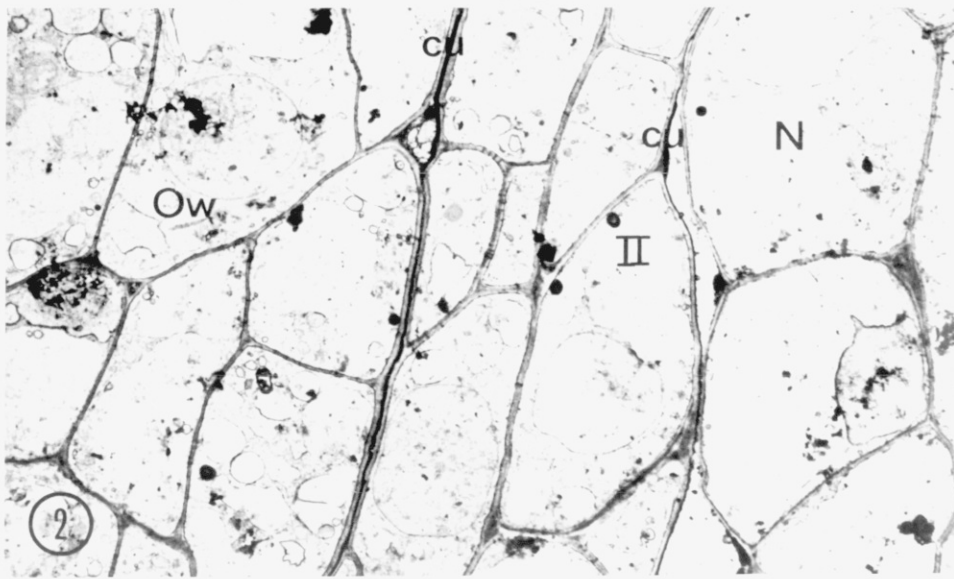
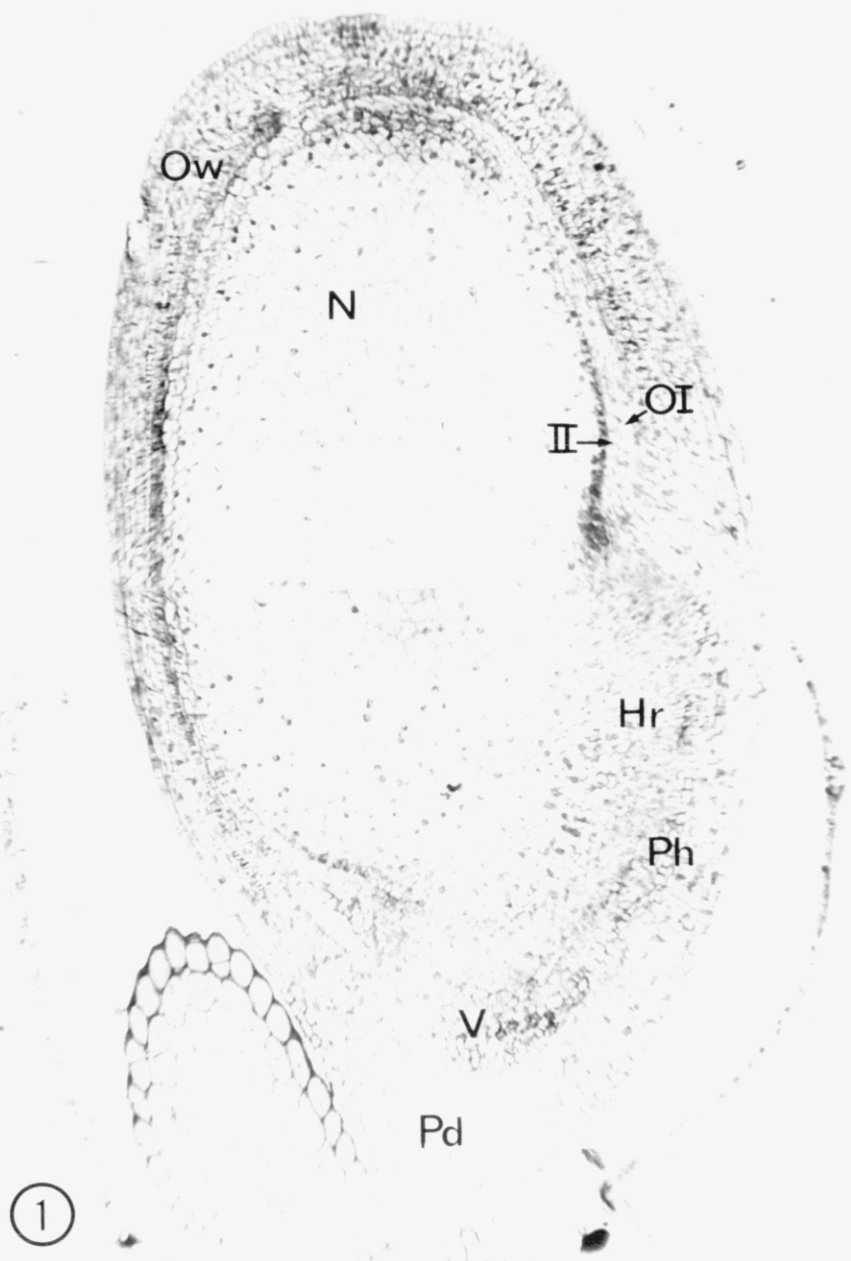


PLATE 3:3

A light micrograph showing a longitudinal section through the basal region of an unpollinated millet ovary in which part of the embryo-sac (EmS) is visible. The nucellar cells are arranged in columns in the region of the micropyle (Mp) at the base of the embryo-sac [Plate 3:4].

No plasmodesmata are seen connecting the embryo-sac to the nucellus (N) and the nucellus and integuments are isolated from the ovary wall by the cuticles which line them except in the hilum (Hr). tissues inside it.

Magnification X 230

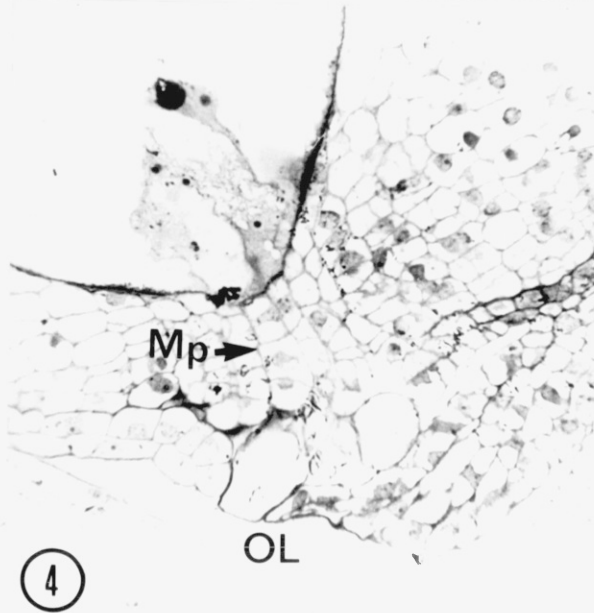
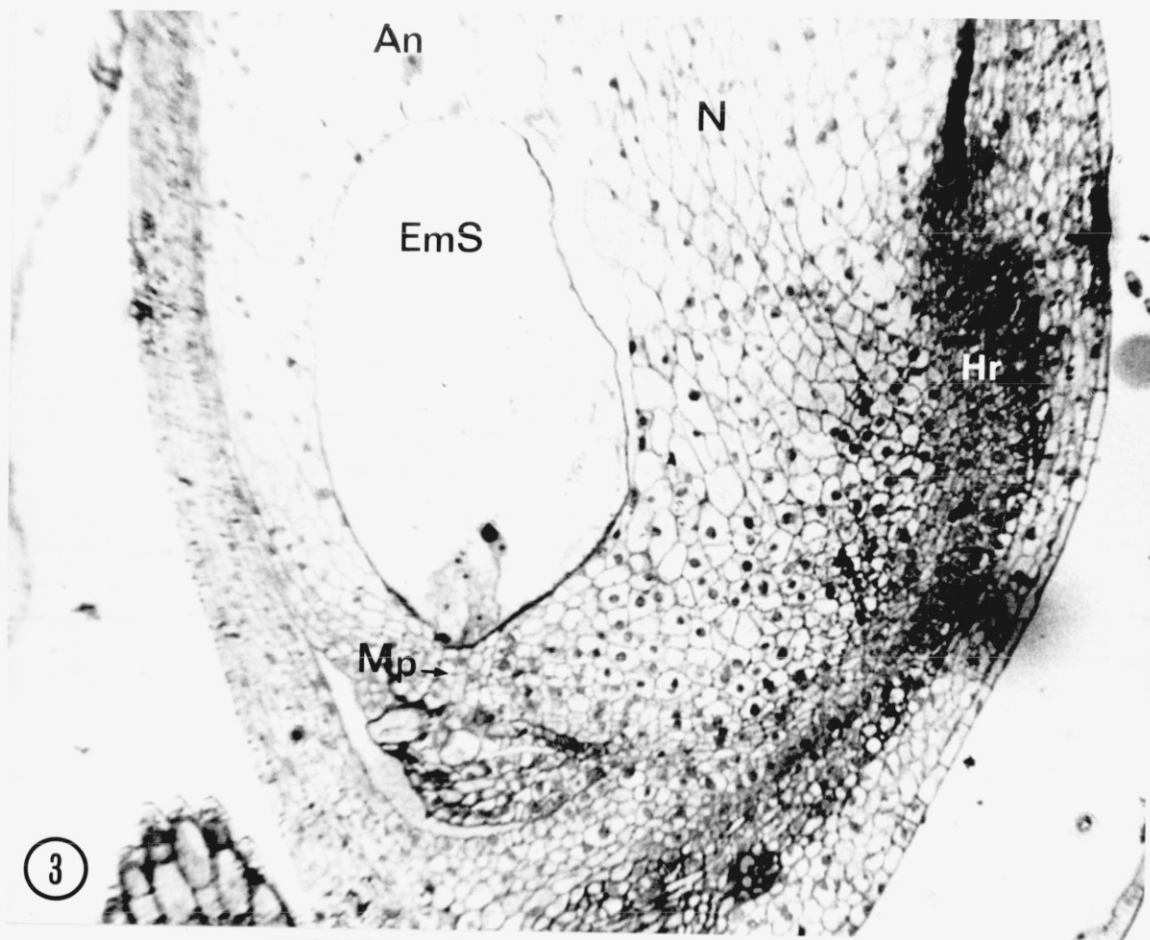
Staining: Toluidine blue

PLATE 3:4

A higher magnification of the micropyle region shown in Plate 3:3. The gap, observed here at the base of the micropyle is the ovarian locule (OL) [Willingale et al 1986]. It is a small air filled space which lies between the ovary wall and the outer integument which unless punctured in some way prevents fixation of the tissues inside it.

Magnification X 780

Staining: Toluidine blue



PLATES 3:5-3:8

Light micrographs of sections taken from millet ovaries prior to pollination. They were all stained with toluidine blue.

PLATE 3:5

This section shows two polar nuclei (Pn) within the embryo-sac (EmS) which is surrounded by the nucellus (N).

Magnification X 340

PLATE 3:6

Several antipodal cells (An) are visible at the top of the embryo-sac. The embryo-sac wall (W) is distinct from the surrounding nucellus (N) and the membrane (m) of the central cell. One of the polar nuclei (Pn) is visible within the central cell.

Magnification X 340

PLATE 3:7

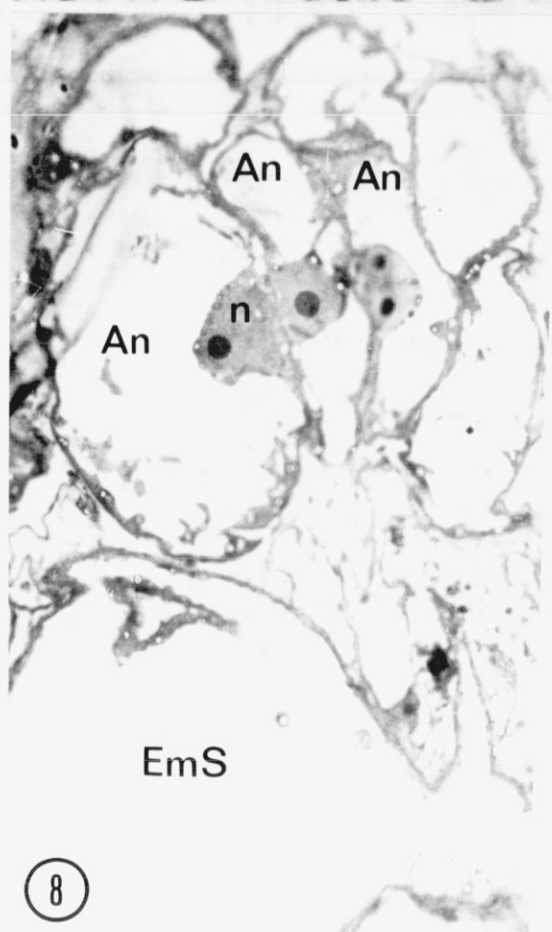
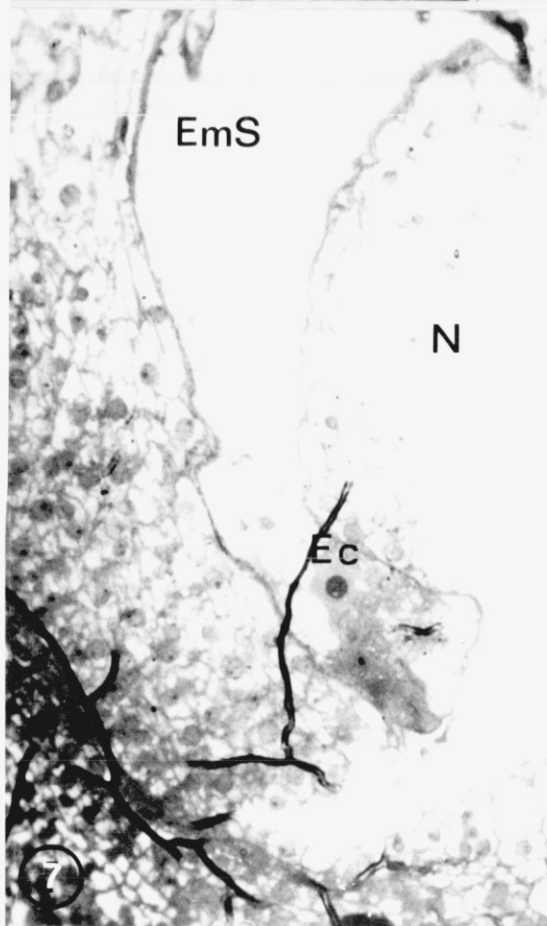
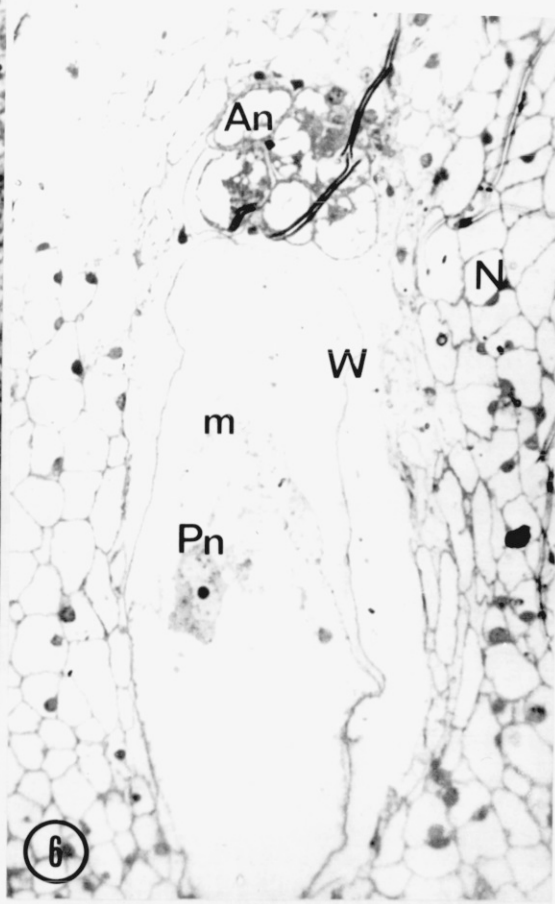
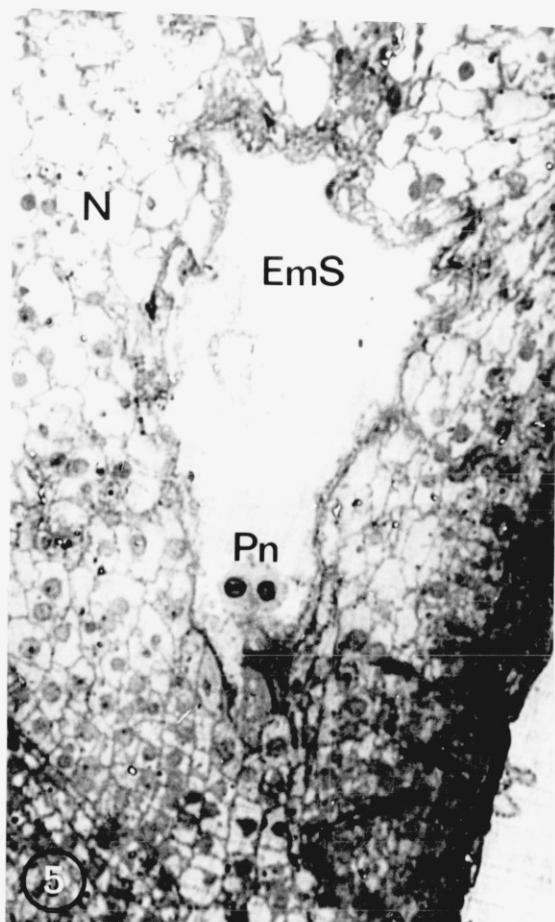
The egg cell (Ec) is visible at the base of the embryo-sac (EmS) in this section.

Magnification X 340

PLATE 3:8

The antipodal cells (An) are large and highly vacuolated situated at the upper end of the embryo-sac (EmS) furthest from the micropyle.

Magnification X 890



PLATES 3:9-3:12

Electron micrographs taken from sections of millet ovaries prior to pollination. They are all stained with uranyl acetate and lead citrate stains.

PLATE 3:9 At the base of the embryo-sac, adjacent to the micropyle, two synergids (Sy) are visible. The filiform apparatus (F) is distinct in one of these cells. Small wall ingrowths (Arrowed) on the embryo-sac wall are also visible on the left of the first synergid. Magnification X 2700

PLATE 3:10 The polar nucleus (Pn) from Plate 3:6. The cytoplasm associated with this nucleus is less dense than that associated with the egg cell. The central cell membrane (m) has bound particles associated with it though many are not easily distinguishable from it. Magnification X 2700

PLATE 3:11 The embryo-sac wall (W) and inner membranes (m) are spacially isolated from the surrounding nucellus (N). Magnification X 2400

PLATE 3:12 The micropyle (Mp) is made up of nucellar cells which form columns directed towards the base of the embryo-sac. The embryo-sac wall at the top of the micropyle (base of the embryo-sac) has thickenings resembling small wall ingrowths (arrows). Magnification X 1400

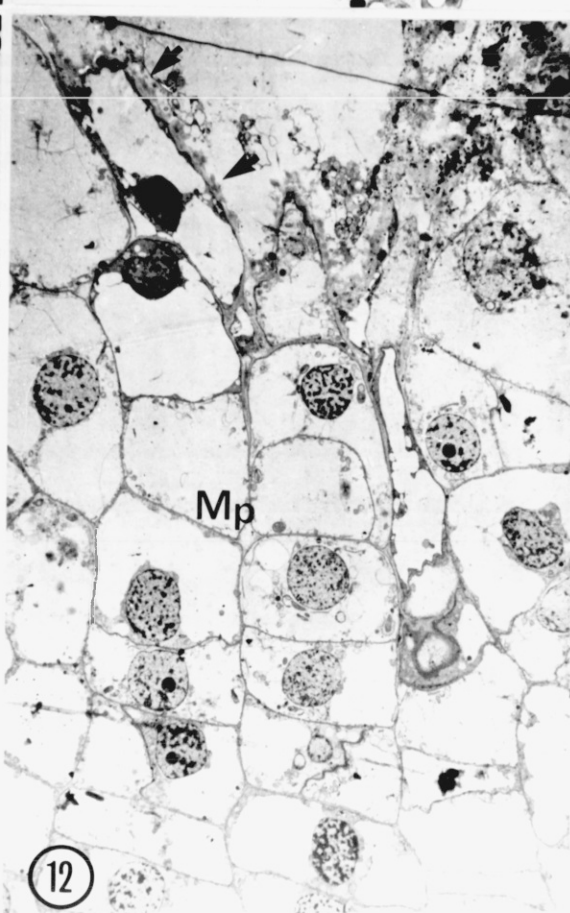
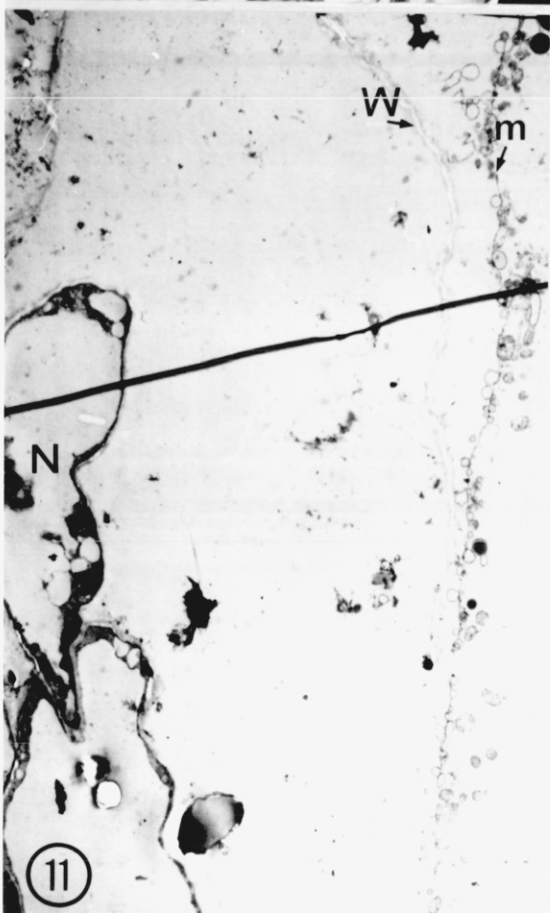
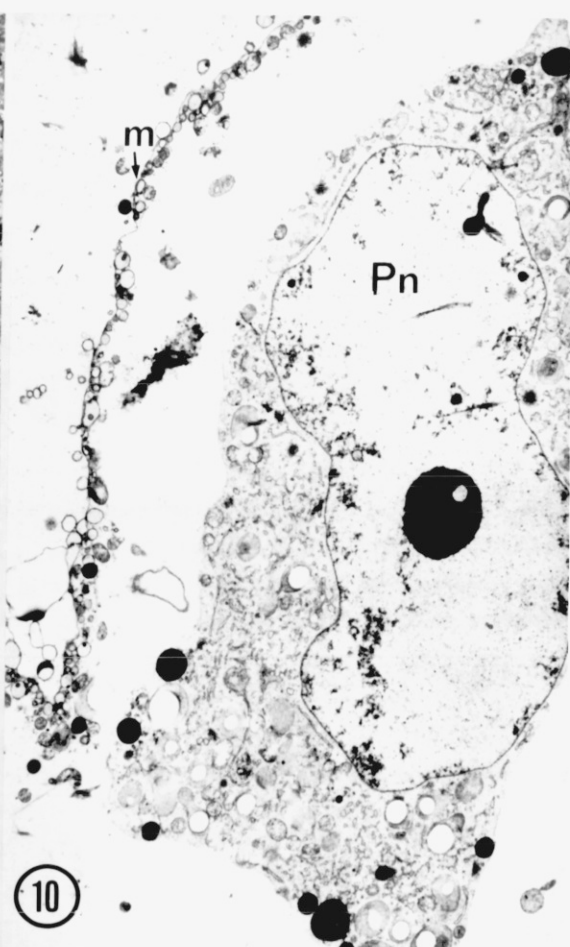
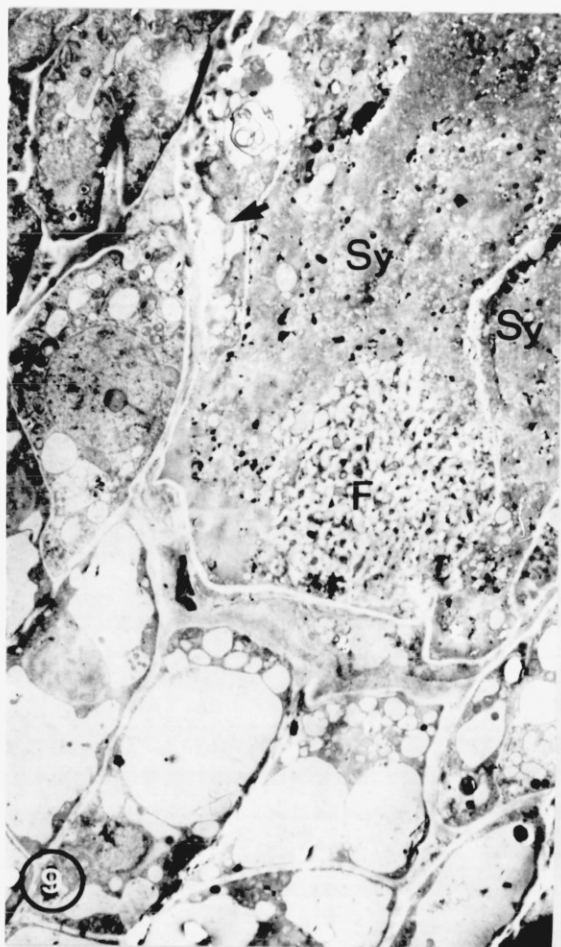


PLATE 3:13

A light micrograph of a transverse section through a millet ovary 3 days post pollination. The embryo-sac has enlarged and the endosperm (En), formed from the primary endosperm nucleus, fills most of the expanding ovule. The ovary wall (OW), inner integument (II) and outer epidermis of the nucellus (N) remain. The outer integument and majority of the nucellar cells have collapsed. The hilum region (Hr) remains as a dense area of cells and a tissue, presumably derived from nucellar cells, forms the chalazal pad (Cp) between the endosperm cells and the hilum.

Magnification X 230

Staining: Toluidine blue

PLATE 3:14

An electron micrograph of part of a millet ovary 3 days post pollination showing that where the endosperm cells abutt the chalazal pad (Cp) they form small wall ingrowths (WI). These endosperm cells are termed the endosperm transfer cells (ET).

Magnification X 9800

Staining: Uranyl acetate and lead citrate

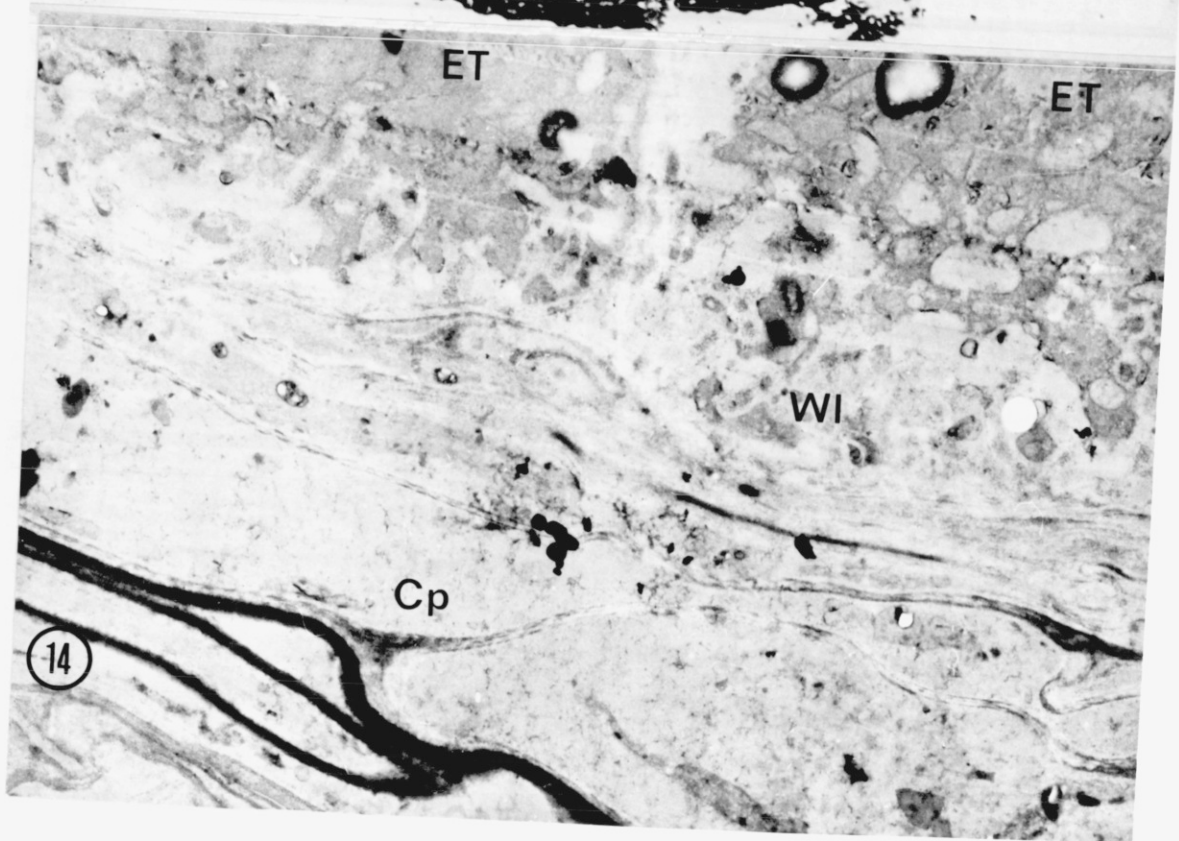
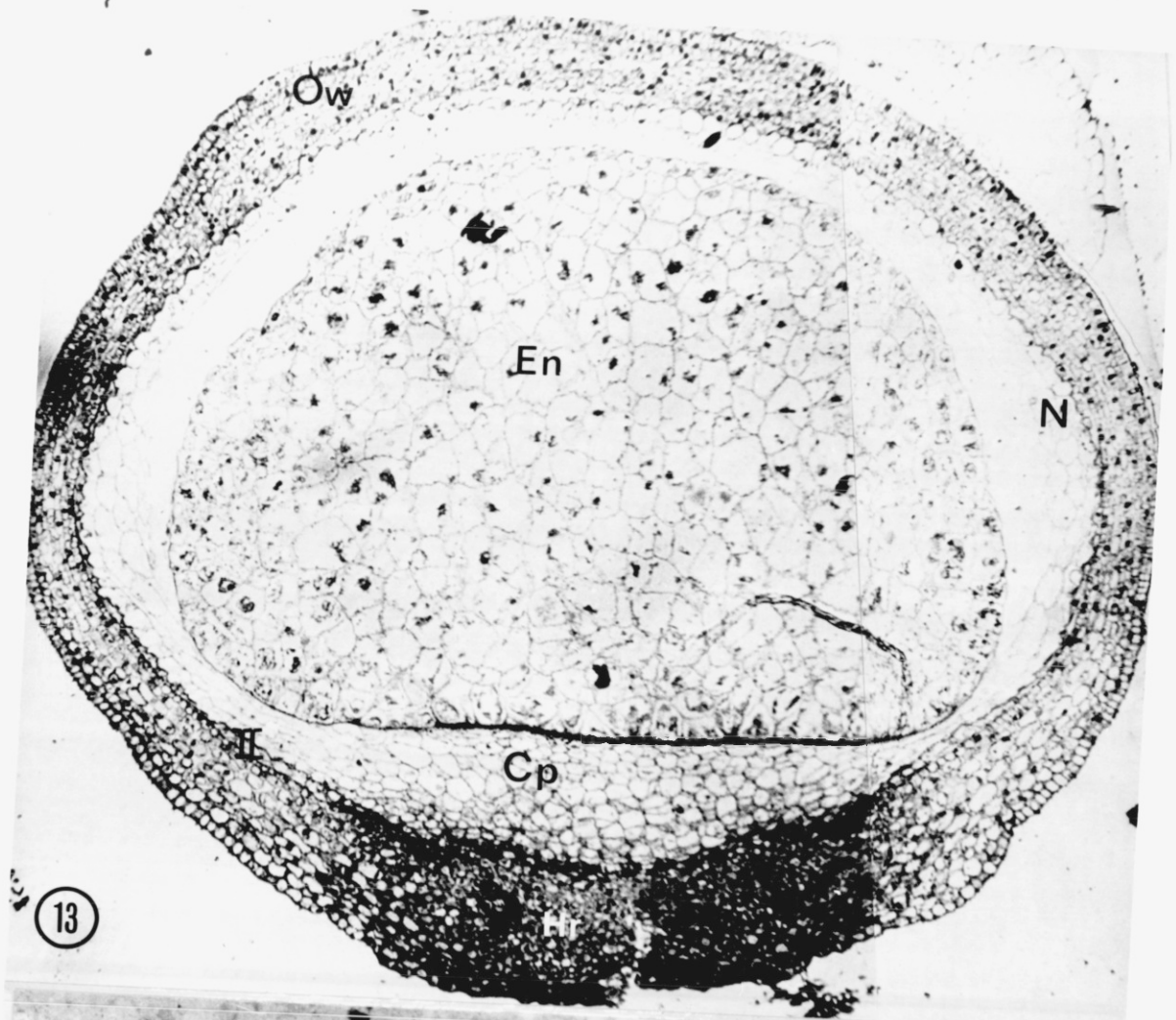


PLATE 3:15

Part of the transverse section shown in Plate 3:13. The maternal tissues associated with the developing seed (embryo and endosperm (En)) include the chalazal pad (Cp), the pigment strand (Ps) and the hilum region (Hr). The endosperm transfer cells (ET) abutt the chalazal tissue, the terminus of the maternal tissue.

Magnification X 630

Staining: Toluidine blue

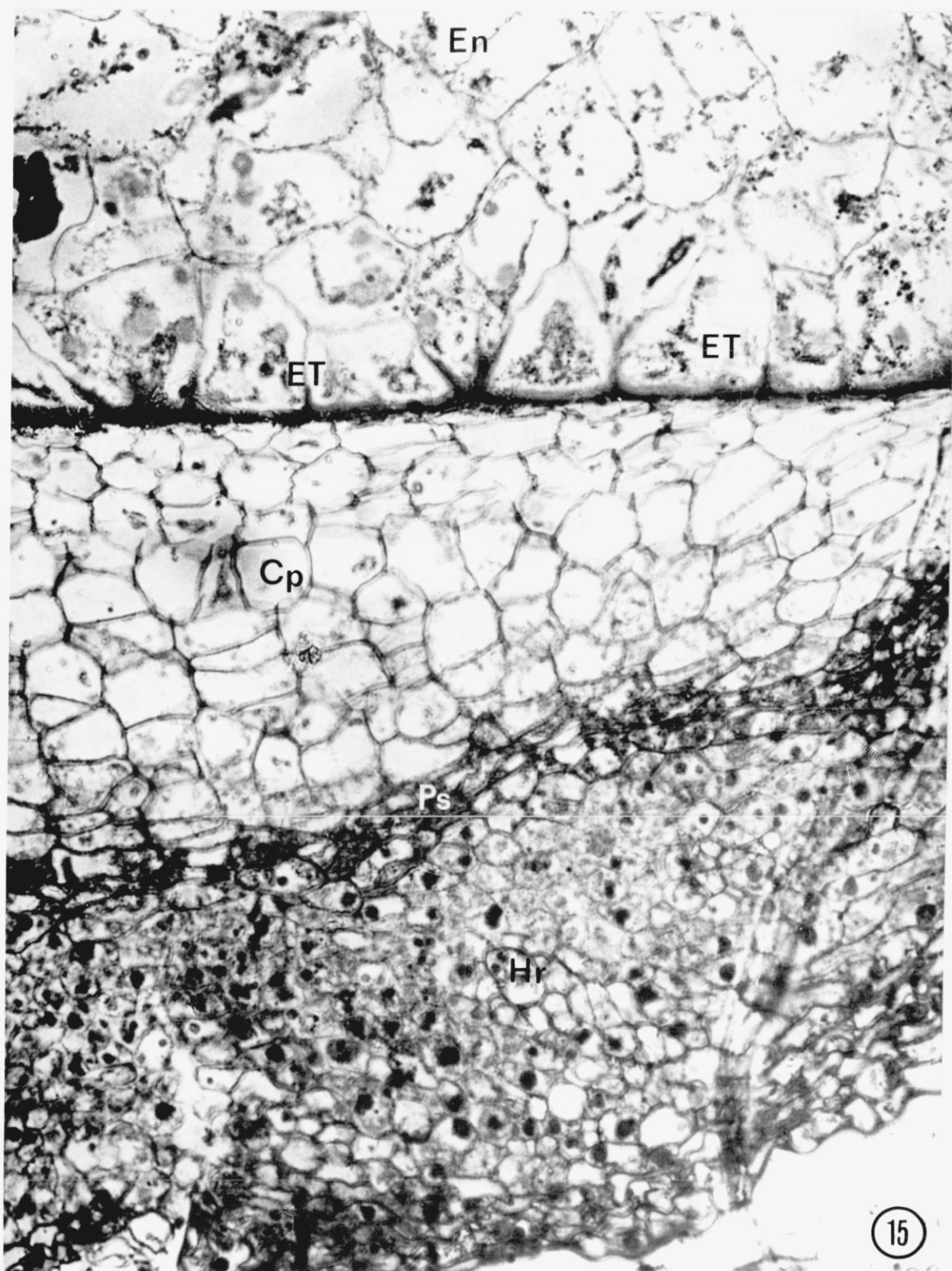


PLATE 3:16

A light micrograph showing endosperm cells (En) distal to the pedicel in millet ovaries 3 days post pollination. Each cell contains a nucleus (n) and many starch grains (St).

Magnification X 1340

Staining: Toluidine blue

PLATES 3:17 ad 3:18

Electron micrographs showing that by 3 days post pollination the cells in the lower portion of the central cell do not contain starch grains (St) (Plate 3:17) whilst those in the upper portion have begun to form them (St) (Plate 3:18).

Plate 3:17

Magnification X 2400

Staining:uranyl acetate and lead citrate

Plate 3:18

Magnification X 1650

Staining:uranyl acetate and lead citrate

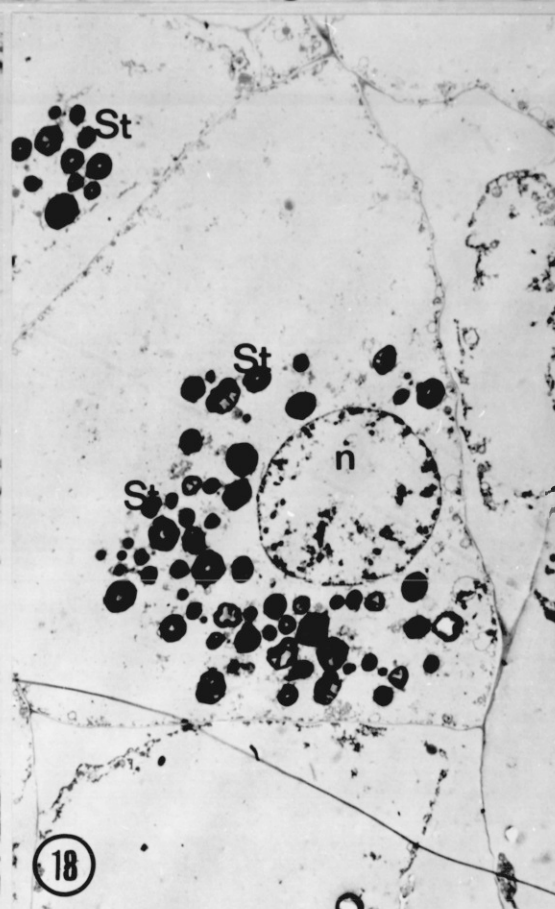
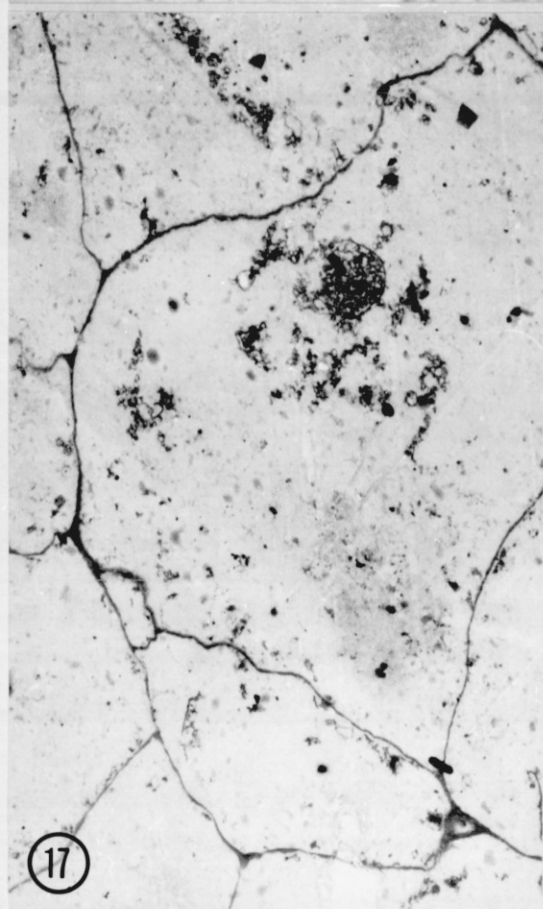
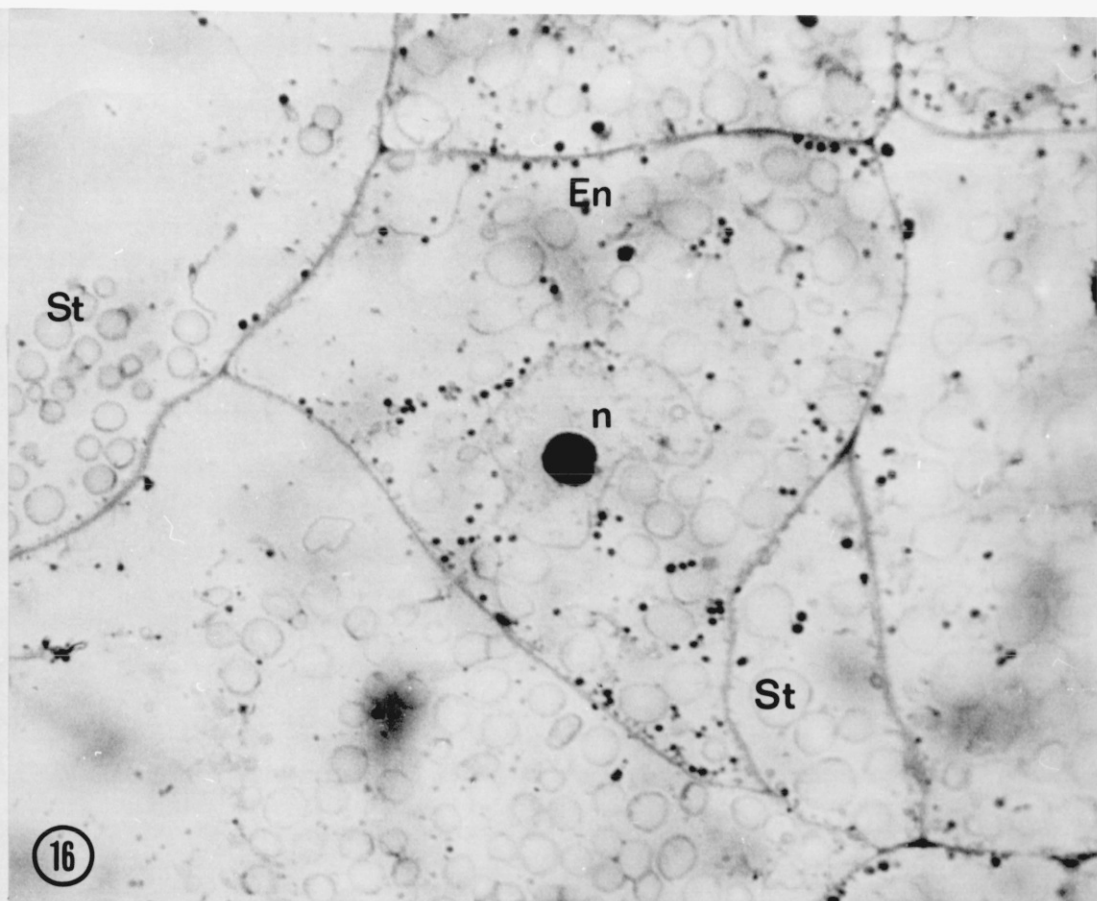


PLATE 3:19

A light micrograph of a longitudinal section through a millet ovary 7 days after pollination, showing the position of the tissues in relation to each other.

Ovary wall (OW), endosperm (En),
endosperm transfer cells (ET), chalazal pad (Cp),
pigment strand (Ps), hilum region (Hr).

The endosperm has increased in size as more cells are produced. Many starch grains are present in the upper portion of the endosperm.

Magnification X 230

Staining : Toluidine blue



PLATE 3:20

An electron micrograph of the wall ingrowths (WI) in the endosperm transfer cells (ET) in a millet ovary 7 days post pollination. The ingrowths have enlarged protruding further into the cell than in Plate 3:14. The chalazal pad (Cp), its cells almost devoid of any contents, extend along the whole interface with the endosperm transfer cells. One of the cuticles (cu) is visible at the edge of the chalazal pad.

Magnification X 1400

Staining: uranyl acetate and lead citrate

PLATE 3:21

A light micrograph of a thick section from a millet ovary 7 days post pollination stained with aniline blue showing bright fluorescence of the wall ingrowths (WI) of the endosperm transfer cells (ET).

Magnification X 1350

Staining: Aniline blue

The fluorescence of the wall ingrowths (WI) was distinguished from autofluorescence (yellow) by its lime green colour.

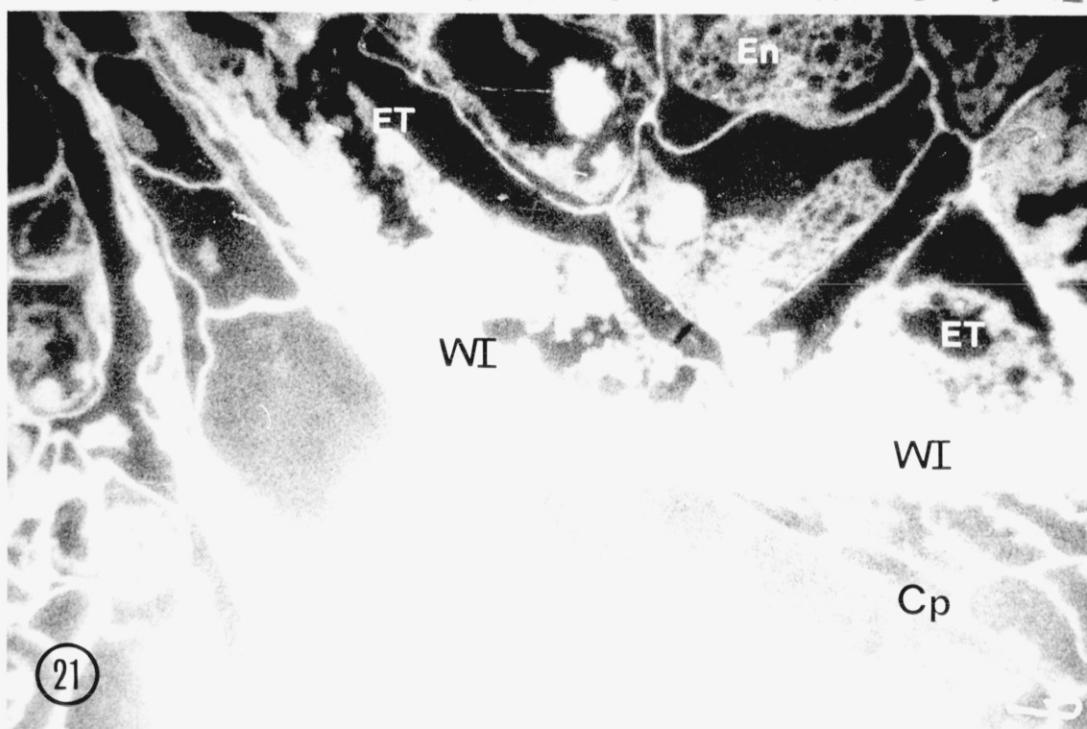
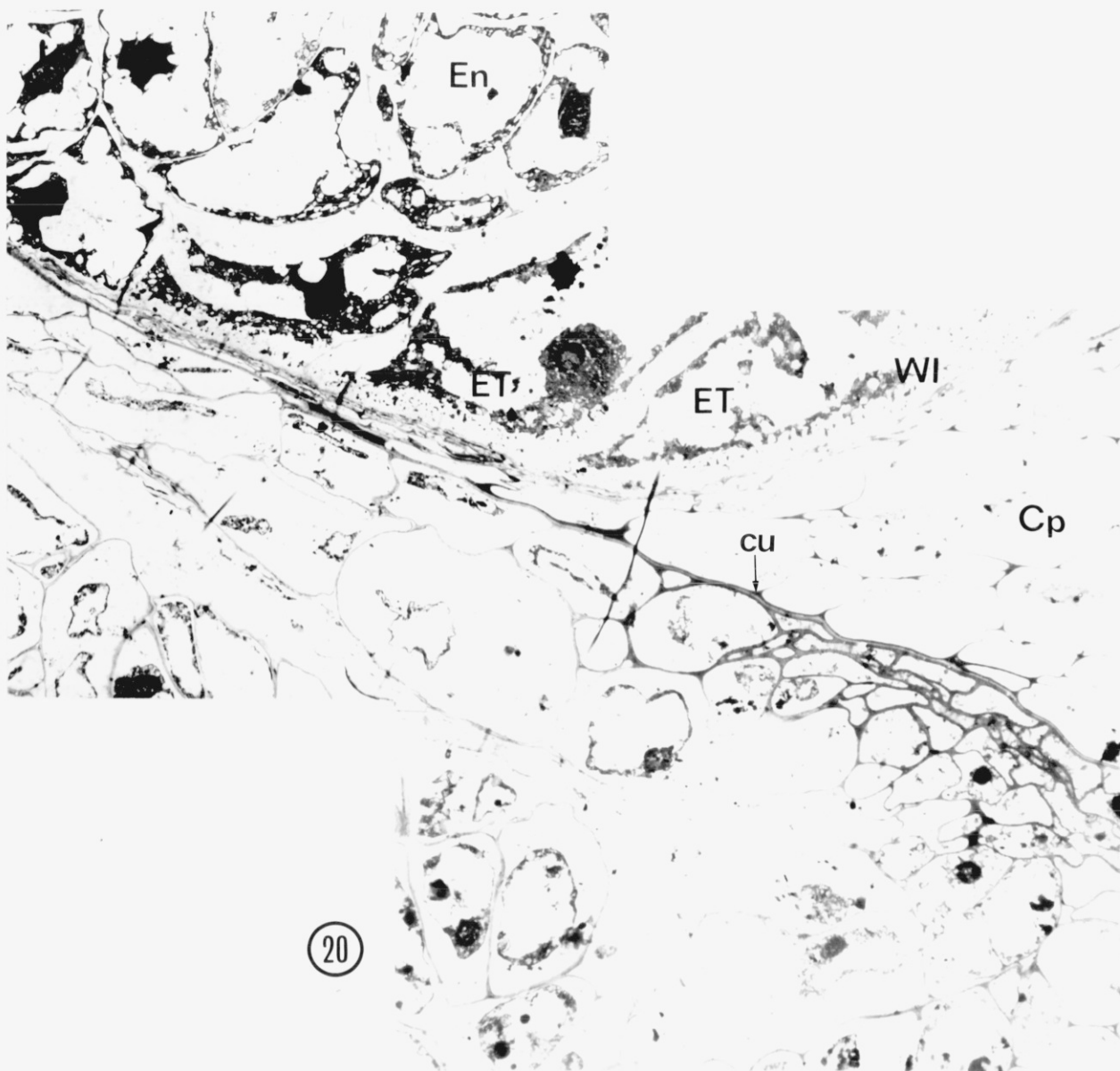


PLATE 3:22

A longitudinal section through the base of a millet ovary 7 days post pollination. Some embryo cells (Em) are visible in the endosperm opposite the chalazal pad (Cp) and hilum region (Hr). The outer layer of endosperm cells (En), except for the endosperm transfer cells (ET) adjacent to the chalazal pad, have become distinct in being darker and smaller than the others. This layer is termed the aleurone layer (Al). Vascular tissues (V) occur in the pedicel (Pd) and include phloem sieve-tubes in the hilum region (Hr).

Magnification X 250

Staining: Toluidine blue

PLATE 3:23

An electron micrograph of a transverse section through a millet ovary 7 days post pollination. Two cuticles (cu) surround the developing ovule, the thicker one covering the outer epidermis of the nucellus (N) and the thinner one lining the inner integument (II). The cells of the ovary wall (OW) have expanded leaving large intercellular spaces within the mesocarp.

Magnification X 2100

Staining: uranyl acetate and lead citrate

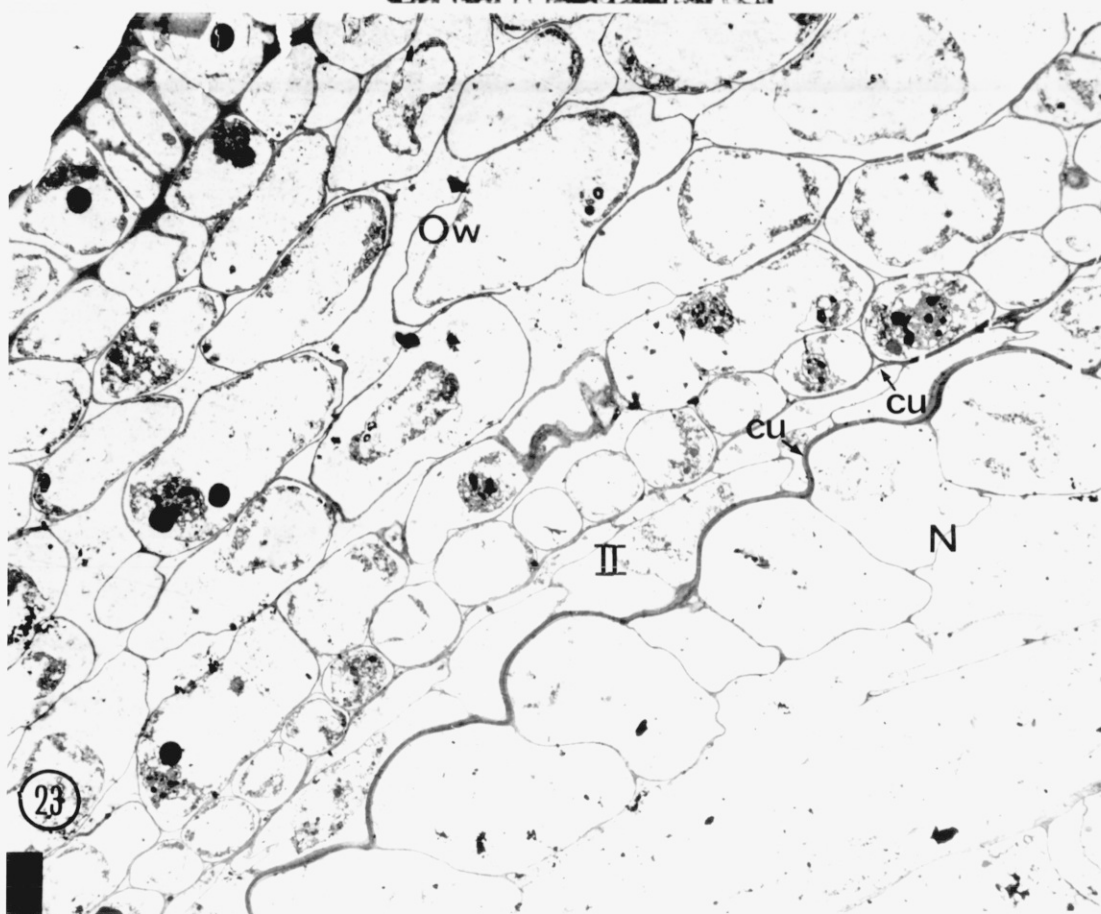
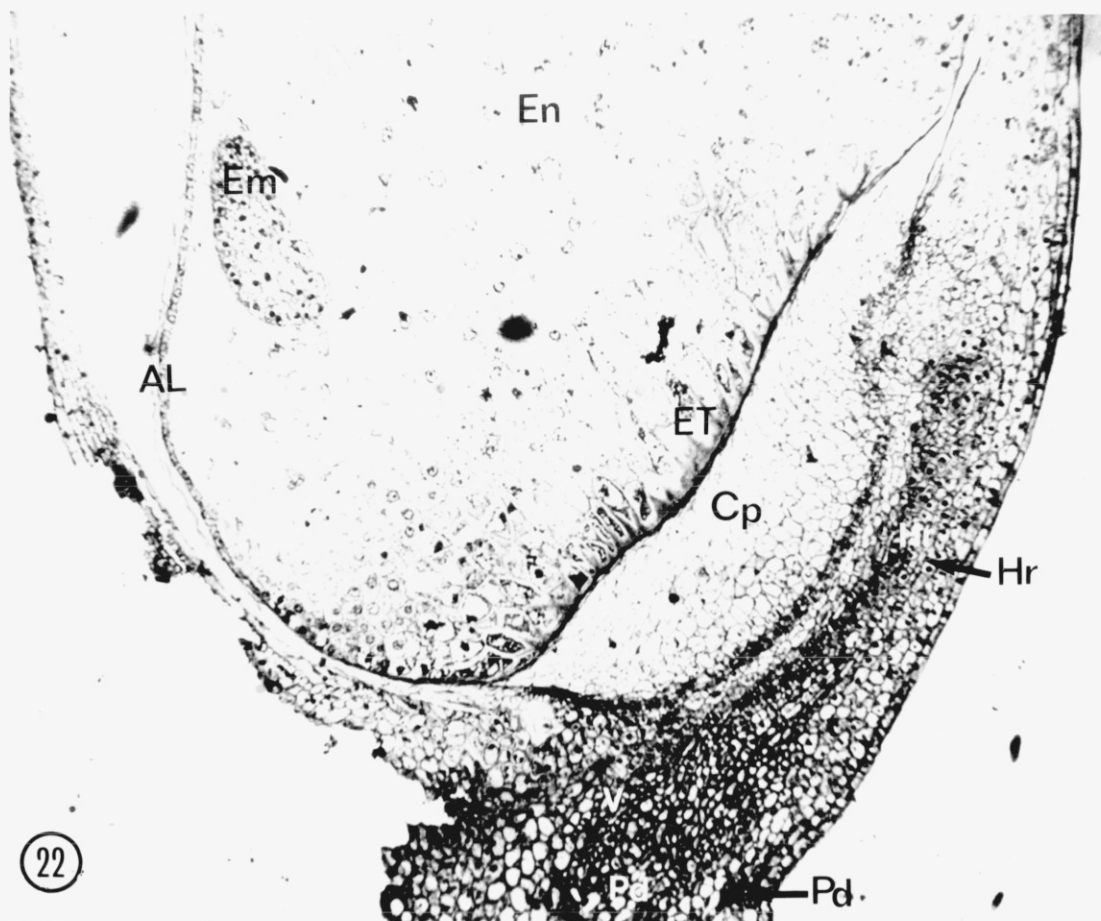


PLATE 3:24

A light micrograph of a longitudinal section through the basal region of a millet ovary 15 days after pollination. The embryo (Em) has differentiated to form the radicle (Rd), the epicotyl (e) and the scutellum (Sc).

Ovary wall (OW), aleurone layer (Al),
endosperm (En), chalazal pad (Cp),
hilum region (Hr), pedicel (Pd),
vascular tissue (V)

Magnification X 230

Staining: Toluidine blue



PLATE 3:25

A light micrograph showing a longitudinal section of the basal region of a millet ovary 15 days after pollination. The embryo (Em) has enlarged and the endosperm cells (En) in the basal portion of the ovule have become filled with starch grains. The aleurone layer (Al) remains distinct as does the vascular tissues (V) in the pedicel (Pd).

Magnification X 175

Staining: Toluidine blue

PLATE 3:26

Part of Plate 3:25 showing the junction at the base of the endosperm (En) and the pedicel (Pd). There has been an increase in the number of dark staining bodies (b) in the vacuoles of cells within the pedicel. Similar staining material also impregnates the cells and the space in the region between the top of the pedicel and the endosperm transfer cells (ET).

Magnification X 1120

Staining: Toluidine blue

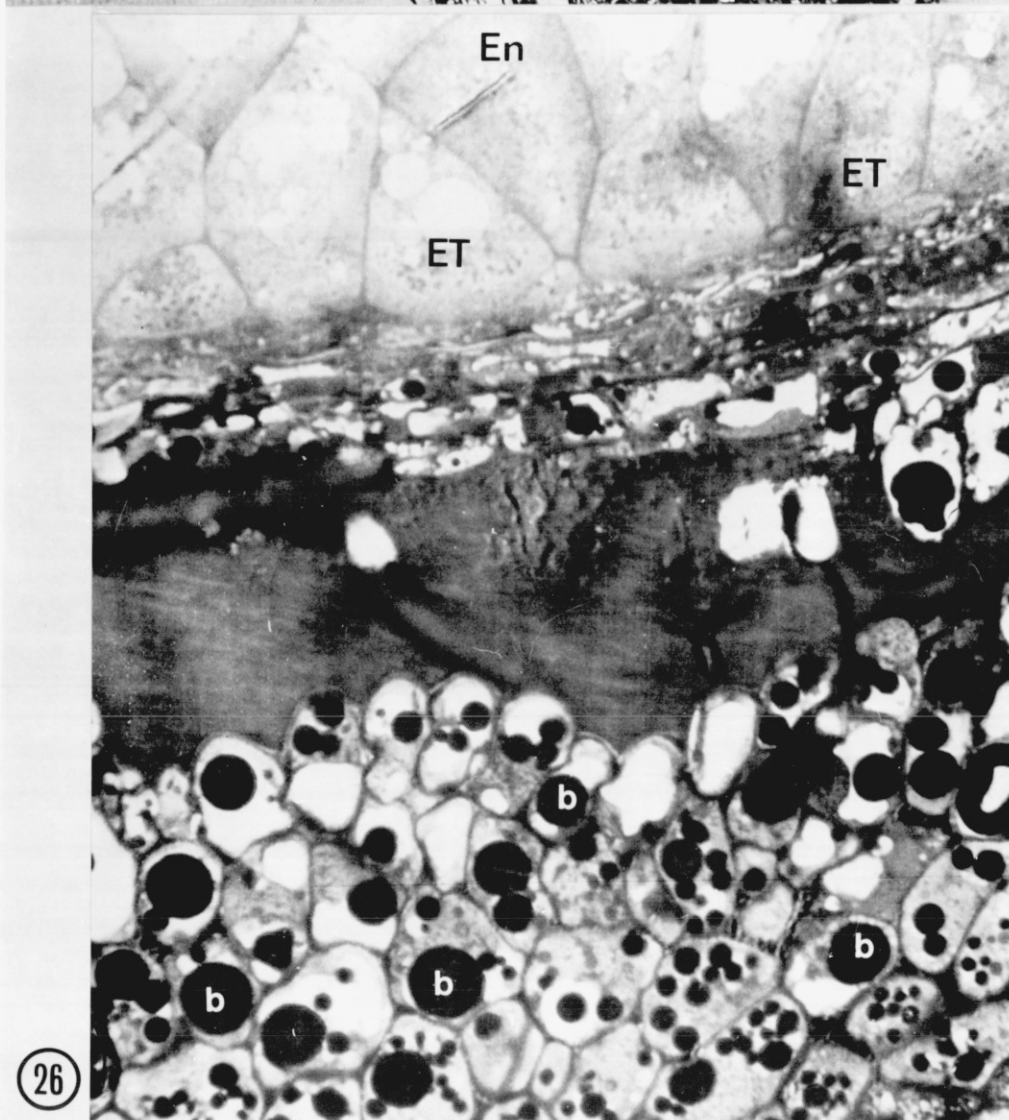
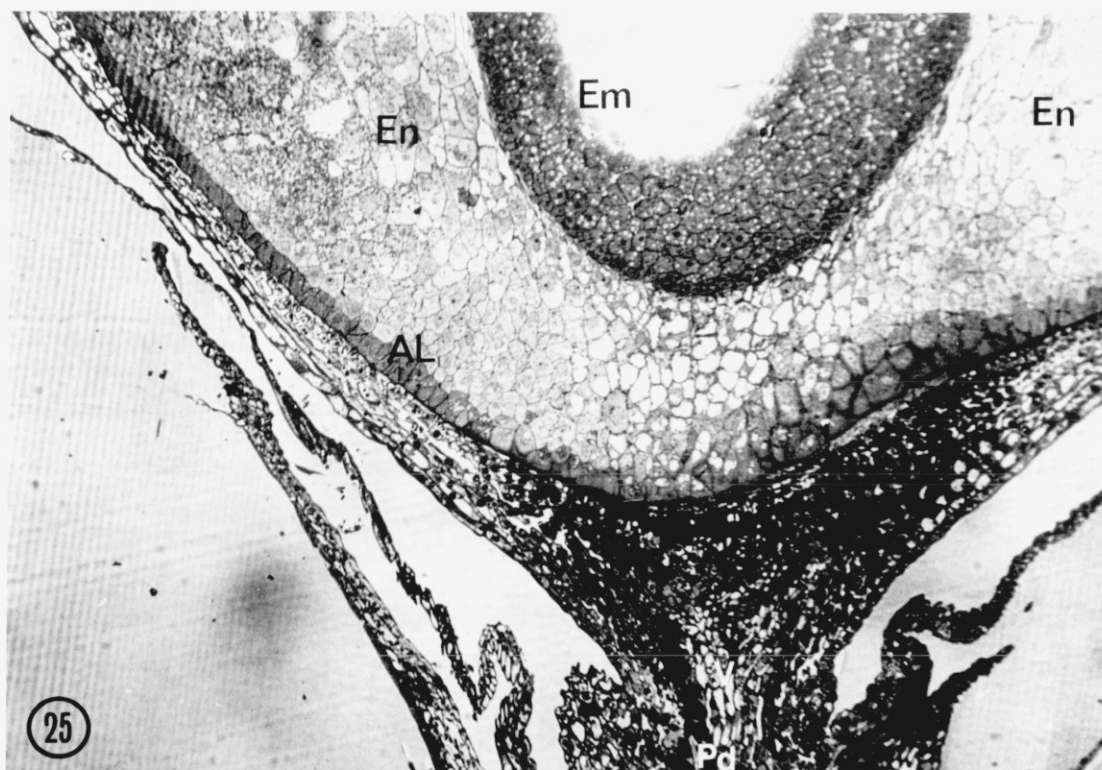


PLATE 3:27

A light micrograph showing the distinctive aleurone layer (Al) lying between the ovary wall (OW) and the endosperm cells (En).

Magnification X 1340

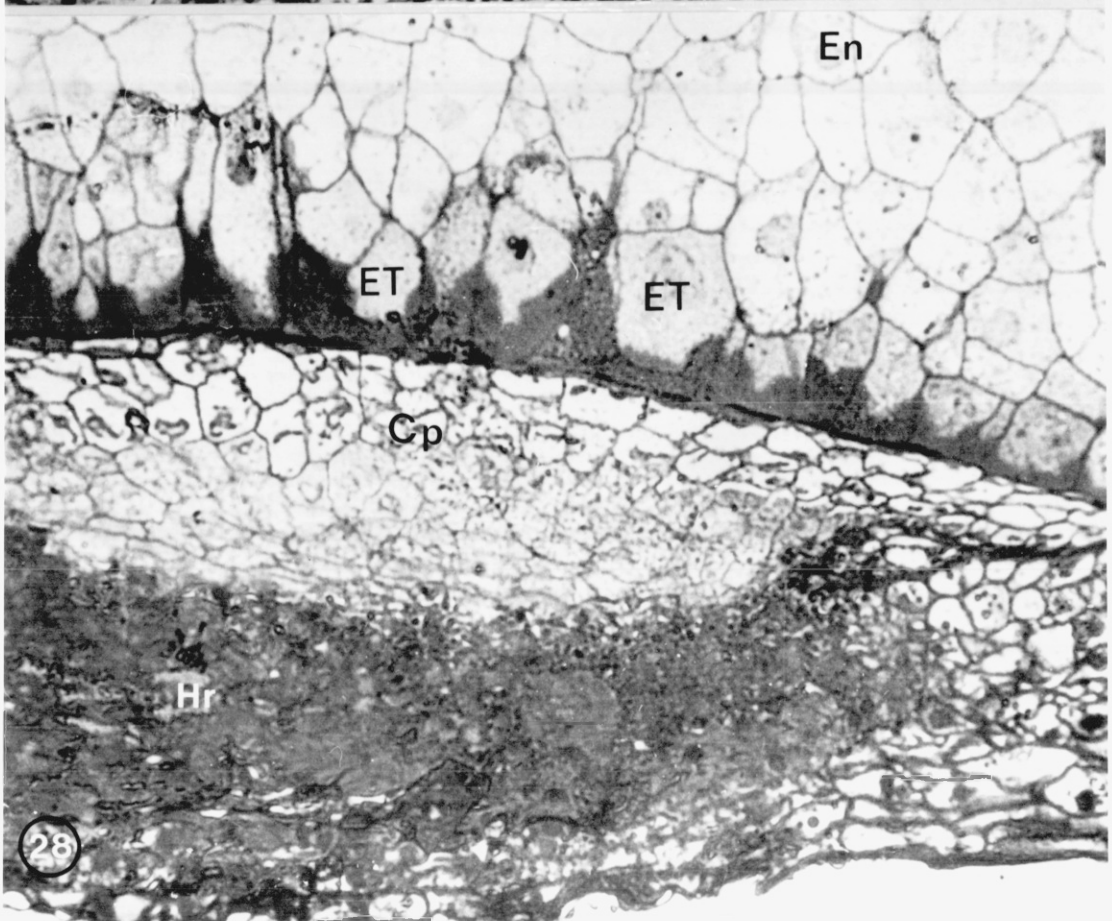
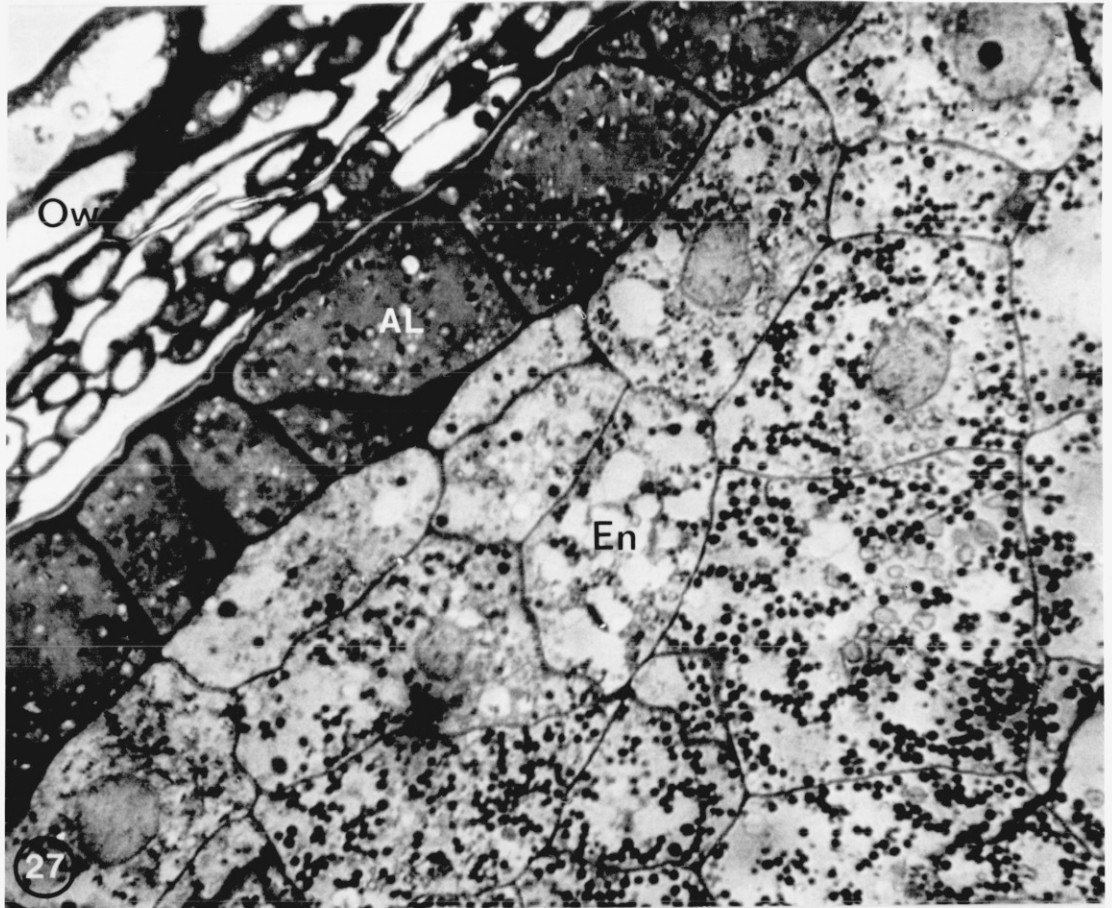
Staining: Toluidine blue

PLATE 3:28

A transverse section through a millet ovary 15 days after pollination showing the seemingly empty cells of the chalazal pad (Cp), the thick wall ingrowths of the endosperm transfer cells (ET) and the compacted densely staining cells of the hilum region (Hr).

Magnification X 510

Staining: Toluidine blue



PLATES 3:29 and 3:30

Electron micrographs of millet ovaries 7 days post pollination treated for the localization of ATPase activity.

PLATE 3:29

This micrograph shows the limited penetration of the substrate (ATP) and other media. Lead precipitation is limited to within a few cells from the cut surface. *(cut surface is the right hand side)*.

Magnification X 1320

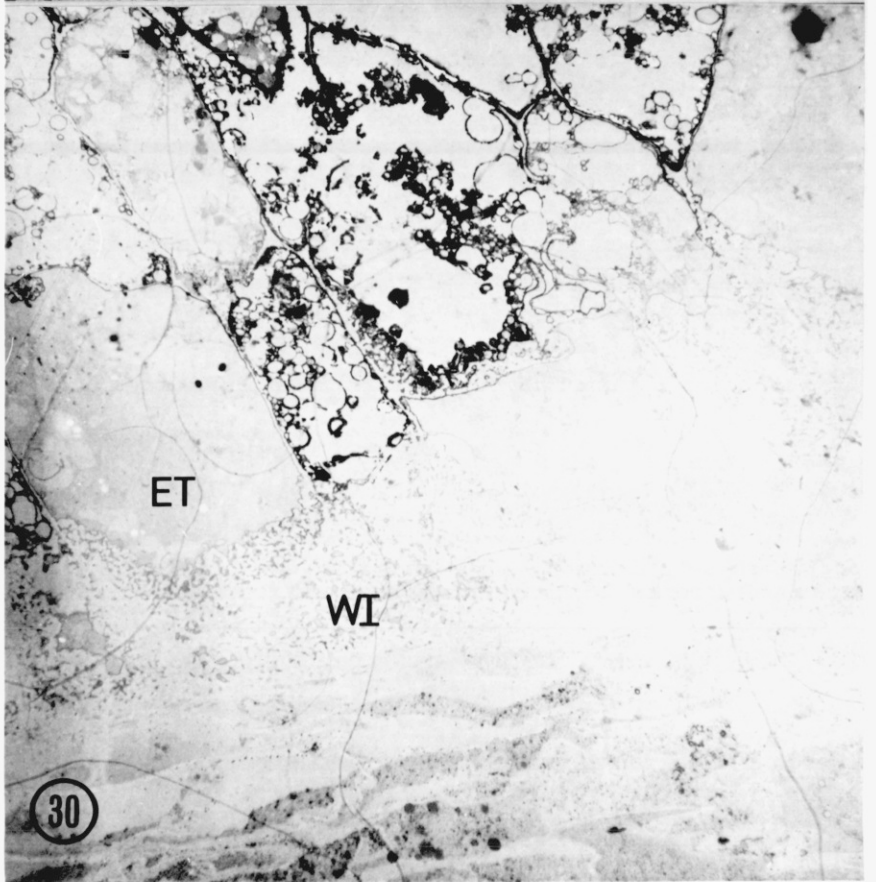
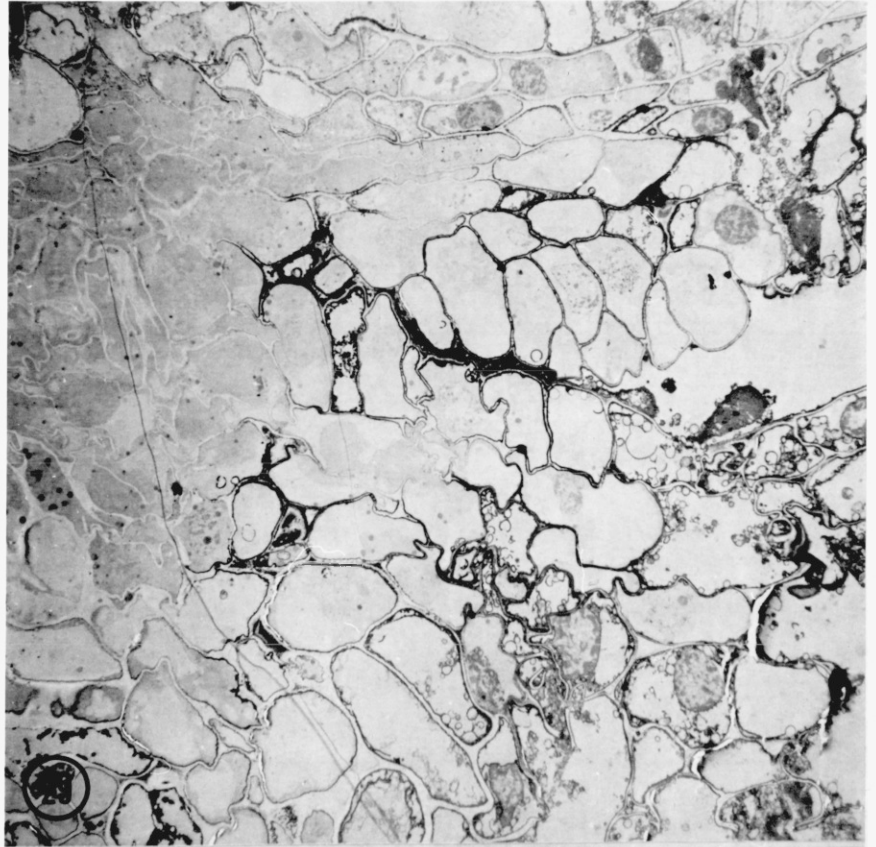
Staining: ATP substrate

PLATE 3:30

Deposits of lead are located on membranes within the endosperm cells that have disrupted. Precipitates occur on the membranes lining the wall ingrowths (WI) of the endosperm transfer cells (ET) but the intensity seemed to depend on how near they were to the cut surface.

Magnification X 1650

Staining: ATP substrate



PLATES 3:31 and 3:32

Electron micrographs of millet ovaries 7 days post pollination treated for the localization of phosphatase (3:31) or ATPase (3:32) activity.

PLATE 3:31

Cells at the top of the pedicel have lead deposits on their membranes. The region between these cells and the developing ovule are often covered in non-specific lead deposits.

Magnification X 4250

Staining: β -glycerophosphate substrate

PLATE 3:32

Lead deposits are most abundant near the cut surface (lower left). Generally cells below the chalazal pad (Cp) have heavy deposits within, and sometimes between, the cells though the chalazal pad has none or only light deposits. The endosperm transfer cells (ET) have lead deposits both within the cytoplasm and on the membrane associated with the wall ingrowths (WI)

Magnification X 2150

Staining: ATP substrate

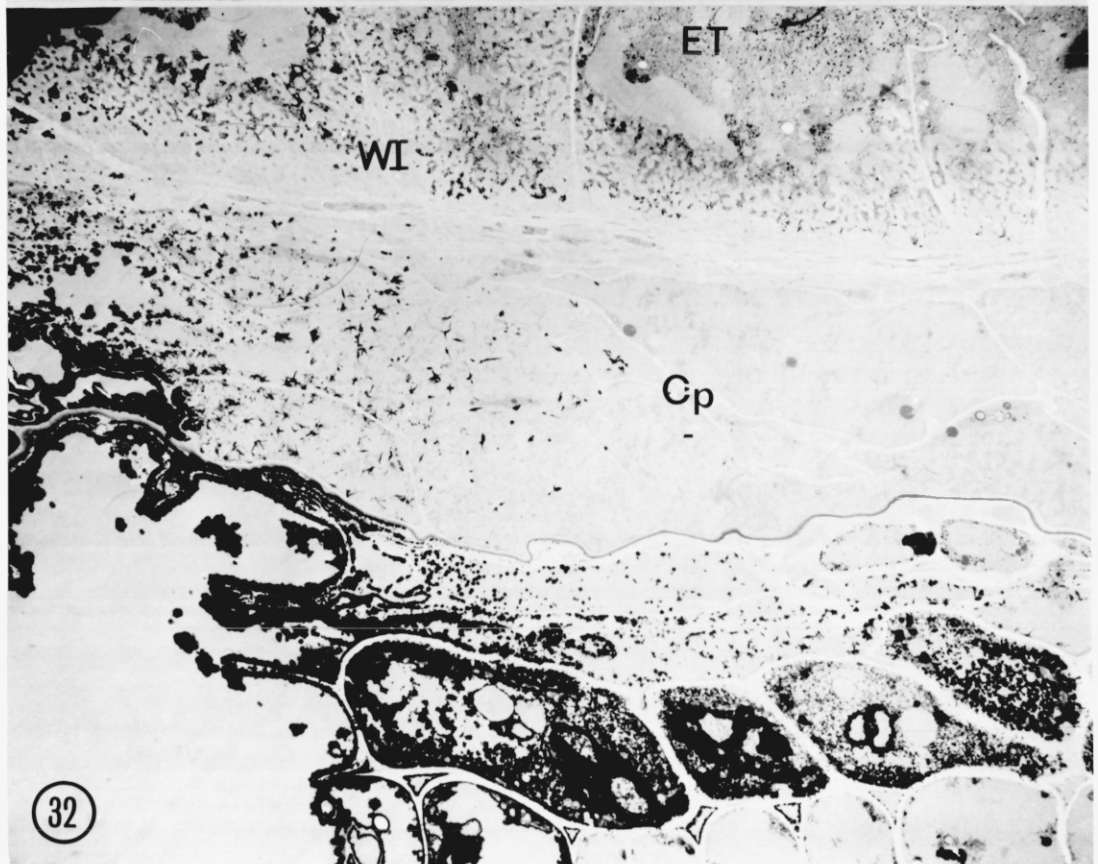
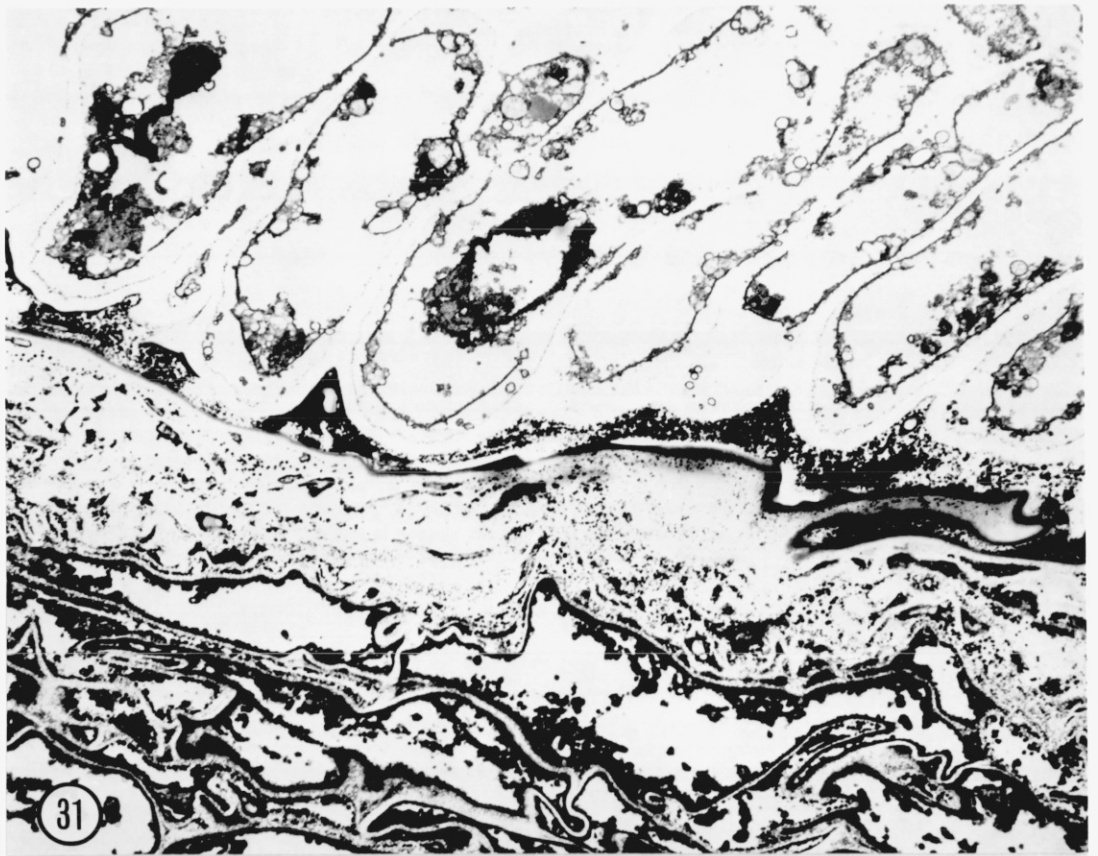


PLATE 3:33

A scanning electron micrograph of a longitudinal fracture through a mature millet seed.

Endosperm (En), scutellum(Sc),
radicle (Rd), coleorhiza (Cr),
epicotyl (e), coleoptile (Ct),

The chalazal pad (Cp) and hilum region (Hr) become crushed by the enlarged embryo and endosperm.

Magnification X 80



FIGURE 3:1

An illustration showing the types of tissues found within an unpollinated millet ovary. The embryo-sac (EmS) contains an egg cell (Ec), a central cell (Cc) and many antipodal cells (An). The embryo-sac is surrounded by the nucellus (N) the inner (II) and outer integuments (OI) and the ovary wall (OW) which on one side has developed as the hilar region, or hilum (Hr). A pollen tube would enter the embryo-sac via the micropyle (Mp).

Pd - Pedicel.

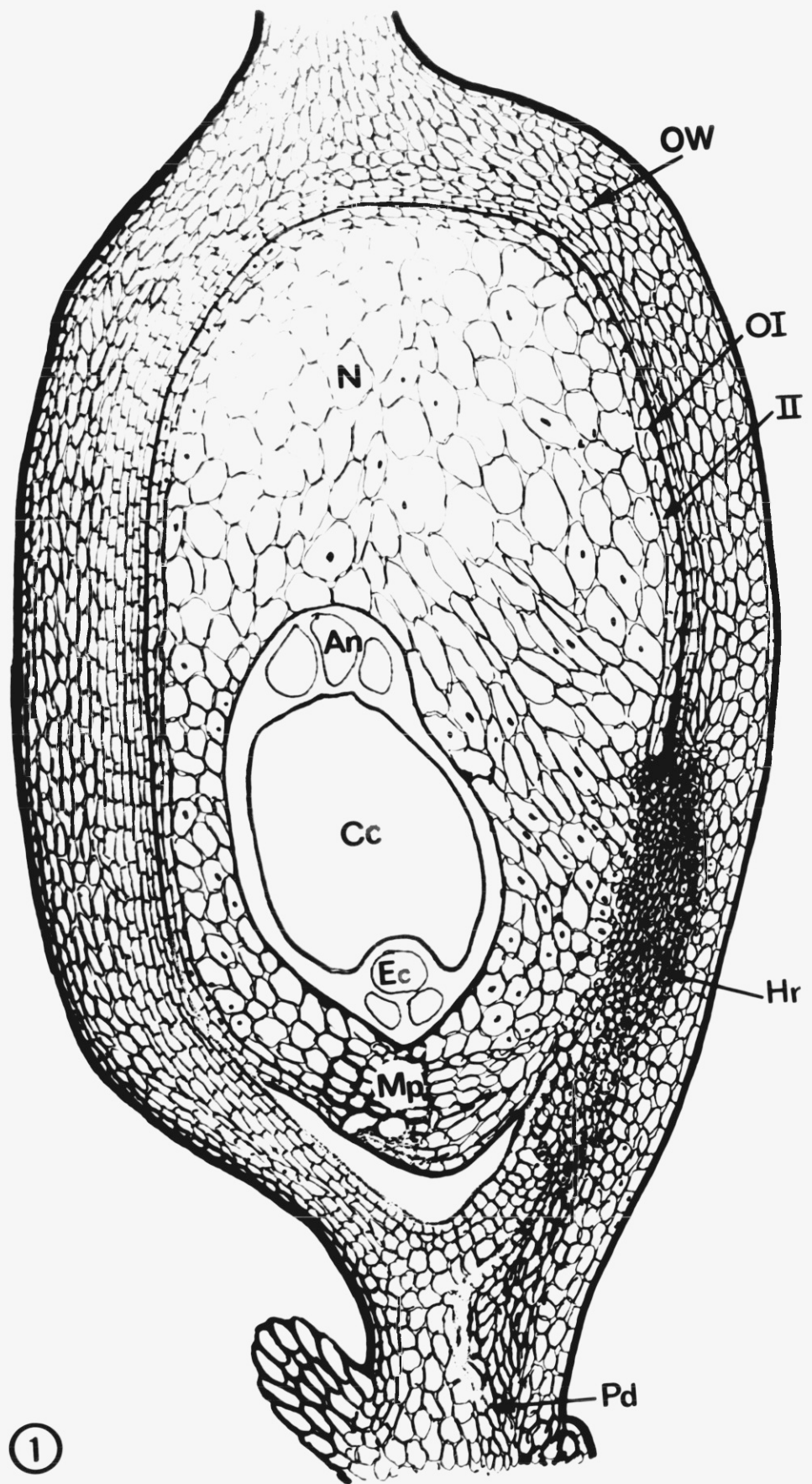
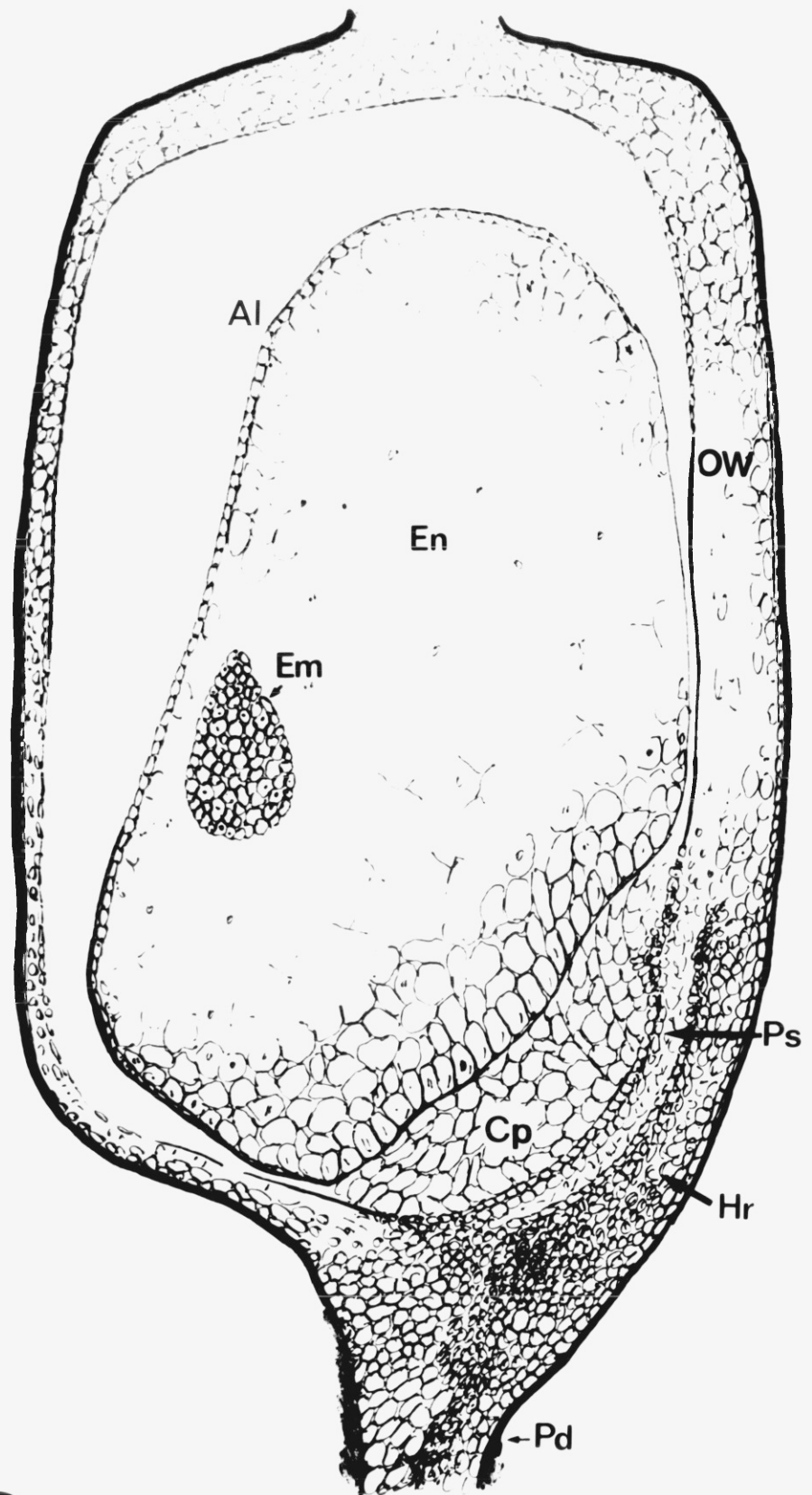


FIGURE 3:2

A diagram showing a longitudinal section through a millet ovary approximately 7 days post pollination.

The embryo (Em) has formed from the fertilized egg cell and grows within the developing endosperm (En). Some of the endosperm cells have differentiated to form the aleurone layer (Al) which surrounds the whole of the endosperm except in the region adjacent to the chalzal pad (Cp) where the endosperm transfer cells (ET) develop. The pigment strand (Ps) is a band of small darkly staining cells which lie between the chalazal pad and the vascular tissues within the hilum (Hr). Vascular tissues, except xylem tracheids, are continuous with those in the pedicel (Pd).

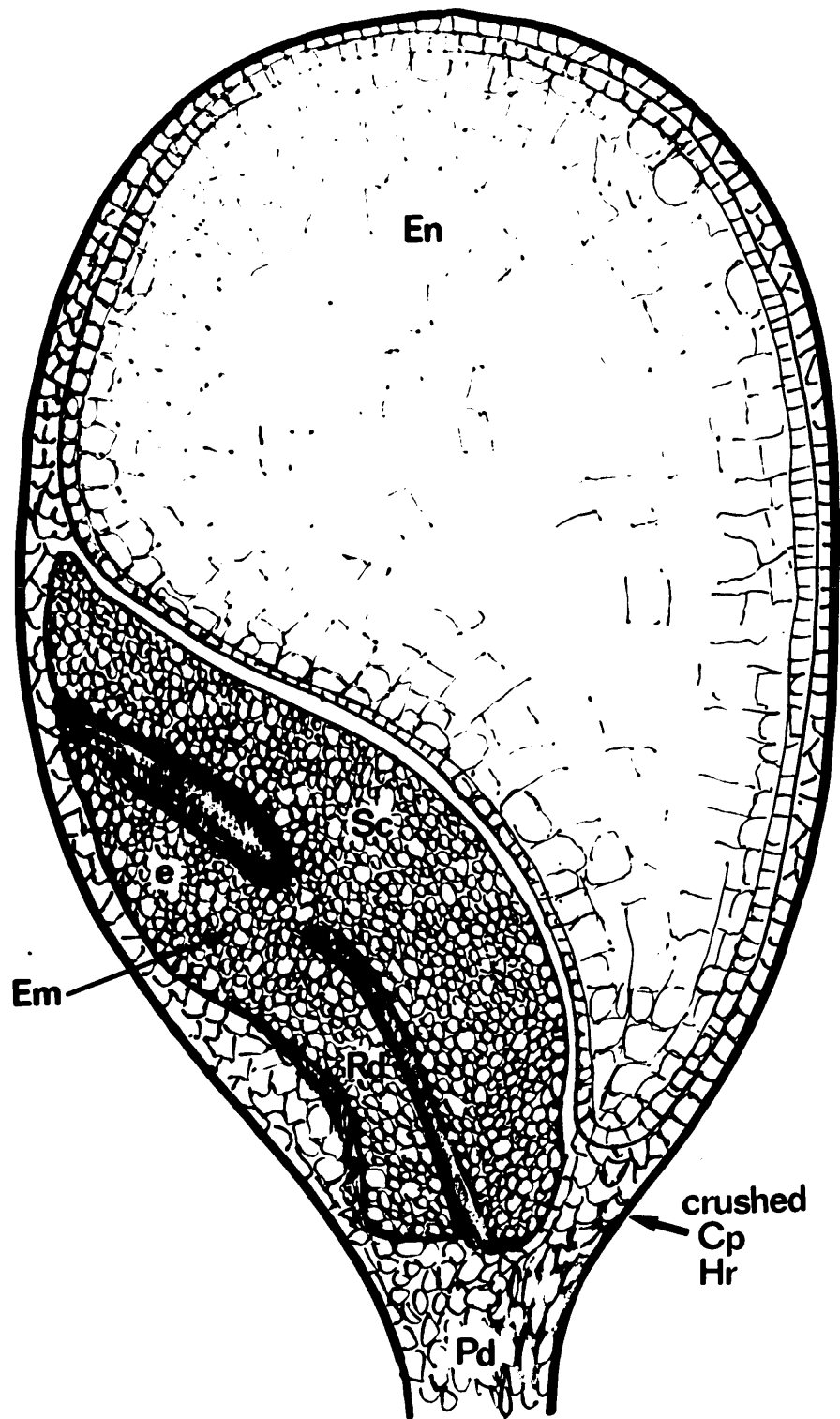


②

FIGURE 3:3

A diagram showing a longitudinal section through a mature millet caryopsis.

The embryo (Em) has differentiated to form the scutellum (Sc), epicoty (e) and radicle (Rd). The endosperm (En) occupies the remaining area and the placenta-chalazal tissues [Hilum region (Hr), chalazal pad (Cp)] are no longer visible having been crushed by the enlarging embryo



SECTION FOUR

THE COLONISATION OF MILLET BY *TOLYPOSPORIUM PENICILLARIAE*.

4.1. INTRODUCTION

The infection of millet by *T. penicillariae* has been previously investigated by Bhatt (1946), Subba Rao et al (1983) and Thakur et al (1983). An extensive study on the mode of infection on millet was carried out by Bhatt (1946). He concluded that infection occurs only through the flower prior to pollination, an observation born out by Thakur et al (1983) who also demonstrated that plants pollinated after infection with *T. penicillariae* showed significantly reduced severity of the disease. Bhatt also observed that infection of the host was confined to the flower. After the stigma was penetrated, the hyphae grew and developed between the aleurone layer and the pericarp entering the base of the ovary where they branched profusely. Intercellular and intracellular hyphae invaded all the tissues of the ovary eventually gelatinising to form sporeballs (Bhatt, 1946).

The present study was undertaken to examine the ultrastructural relationship established between the invading hyphae and the host tissues. Samples were taken 5, 10 and 14 days post inoculation (d.p.i.) (section 2.2.1) and processed for light microscopy (Section 2.4) and transmission and scanning electron microscopy (see sections 2.3 and 2.6.2.).

4.2. RESULTS

4.2.1. ENVIRONMENTAL CONDITIONS NECESSARY TO ESTABLISH CONSISTENT INFECTION.

Consistent infections were obtained when inoculated inflorescences were maintained at high humidity. The temperature, inoculum source and time of inoculation with respect to flowering,

were subsequently varied so that the optimum conditions necessary for infection were established. All results were scored according to an arbitrary scale modified from Thakur et al (1982) (Figure 4:1).

Heavy infection was obtained when inflorescences were inoculated and maintained at high humidity in either a high (30-35°C) or low (20-22°C) temperature (Table 4:1). It was necessary to keep the plants shaded during the first twelve hours as direct sunlight was found to be detrimental causing the inflorescence to brown and wither so that neither infection nor seed set occurred (Table 4:1).

Sporidial infections were successful at all flowering stages up to full protogynae after which pollination or withering due to stigma age prevented infection (Table 4:2). Inoculation with sporeballs (teliospores) never gave rise to infection. These results were gathered with the help of Miss A. Webb, a third year undergraduate completing her final year project.

4.2.2. COLONISATION OF THE OVARY.

Infected millet ovaries 5 d.p.i. were larger than uninfected or pollinated ovaries of the same age. The stigma became brown and withered, as recorded by Bhatt (1946). When examined under the scanning electron microscope the stigma and style were observed to have collapsed (PLATE 4:1). The 0.5 - 0.6mm region just above the ovary had become constricted and the stylodia were covered with sporidia and lengths of hyphae which had been formed from the germinating sporidia (PLATE 4:2). Despite extensive searching penetration sites were not observed and the growth of hyphae on the surface did not extend beyond the base of the collapsed region of the style (the tip of the ovary) (PLATE 4:2).

The ovary wall, especially on the side where the hilum develops, was grossly enlarged giving the ovary a gall-like appearance (PLATE 4:3). The infected ovary was also distinguishable from the uninfected by the deep green colour in the outer epidermis (PLATE 4:4). Electron microscopy revealed that the cells of this outer layer contained many osmiophilic, membrane-bound bodies which were distributed throughout the cytoplasm and vacuoles of these cells (PLATES 4:5 and 4:6). By light microscopy these bodies stained metachromatically green with toluidine blue indicating the presence of lipids or polyphenolic compounds. (Feder and O'Brien 1968). This outer layer subsequently changed from the deep green colour to olive and finally brown during the maturation of the infected ovary (PLATE 4:23).

The first sign of infection, apart from the external symptoms of colour and size, was the appearance within the upper part of the ovary of hyphal aggregates in the gap between the integuments and the nucellar epidermis (PLATES 4:7 and 4:8). The gap enlarged forming a cavity as the nucellus collapsed and the ovary wall thickened (PLATE 4:9). The cells of the ovary wall were undergoing hypertrophy, their enlargement causing the ovary wall to extend on one side (PLATES 4:3 and 4:11). By this time hyphae also extended out from the ovary wall into the cavity (PLATE 4:10). When cut open with a razor blade this cavity was observed, with the naked eye, to be fluid-filled. The cells of the outer epidermis surrounding the hypertrophic region contained dark staining bodies as did some of the cells within the lateral region of the ovary wall (PLATE 4:11).

By 10 d.p.i. hyphae had colonised the intercellular spaces in the ovary wall, the remaining nucellus, the unpollinated embryo-sac and

the tissues of the hilum (PLATE 4:12). The ovary, when cut open, was now observed to be filled with waxy worm-like aggregates of hyphae which were difficult to section, often seemingly ripping out of the embedding medium when stained with uranyl and lead salts (PLATE 4:13). However when stained with PATCH-SP these unstained areas became electron dense indicating the presence of a polysaccharide material surrounding the hyphae (PLATE 4:14). The walls of these hyphae also appeared thick an observation confirmed when hyphal aggregates were dissected from fresh material, squashed and mounted in lacto-phenol cotton blue (0.1%v/v) and examined with the light microscope. They were observed to have very thick walls with only small amounts of cytoplasm located mainly at the hyphal tips.

Fractured infected ovaries, examined with the scanning electron microscope, showed that the embryo-sac still remained intact within the ovary and the hyphal aggregates were confined to the cavity (PLATE 4:12). The hyphae in the lower regions remained aggregated whilst those in the upper region were observed to be differentiating into small teliospore initials (PLATES 4:12, 4:15 and 4:16).

Phloem sieve tubes and associated parenchyma cells were present in the pericarp tissue at the base of the ovary but no differentiation of the pericarp tissues, to form the placenta-chalaza as found in pollinated ovaries, had occurred, (PLATE 4:17). Both xylem and phloem vascular tissues in the pedicel region remained intact and uncolonised (PLATE 4:18).

By 14 d.p.i. the aggregates of hyphae had developed to form groups of sporeballs filling the cavity and the remaining nucellus tissue (PLATES 4:19, 4:20 and 4:21). The hilum region remained intact and here the numerous hyphae were predominantly intracellular

(PLATES 4:30, 4:31 4:32 and 4:33). Hyphae did not penetrate beyond the hilum tissue into the pedicel of the ovary and within the hilum region spores were never formed.

As the infected ovary matured the sporeballs, enclosed by a now thin ovary wall, were closely compacted. Each sporeball, approximately 90-150 μ m x 50-60 μ m, consisted of many teliospores 6-7 μ m in diameter (PLATES 4:20 and 4:21). The sporeballs eventually become released when the spores within the ovary have matured and the infected ovary dries so that during harvest the ovary ruptures releasing the sporeballs (PLATES 4:22 and 4:23).

4.2.3. CYTOLOGY OF THE HOST AND FUNGUS.

The embryo-sac remained isolated from the ovary tissue by the thick cuticles, though these, as in unpollinated tissue, were not present in the hilum tissue (PLATE 4:24). Any remaining nucellar cells surrounding the embryo-sac were very large, highly vacuolated and each contained a few starch grains (PLATES 4:25 and 4:26). The hilum was composed of parenchyma cells, some of which contained starch grains, forming a row parallel to the phloem in the pericarp of the ovary (PLATES 4:27 and 4:28). Each cell within the hilum region contained large quantities of endoplasmic reticulum, golgi, many mitochondria and a large nucleus (PLATE 4:29).

Hyphae occupied both intercellular and intracellular positions though they were predominantly intercellular within the ovary wall, the nucellus and the embryo-sac and predominantly intracellular within the hilum region (PLATES 4:30, 4:31, 4:32 and 4:33). The intracellular hyphae were surrounded, *to* varying degrees, by a material which was electron dense when stained by uranyl and lead

salts, PACP or PATCH-SP (PLATES 4:32, 4:34, 4:35 and 4:36). The material surrounding these hyphae was more dense than the host cell wall when stained with uranyl/lead salts or PACP (PLATES 4:32, 4:34, 4:36, 4:37 and 4:38), but was of equal density when stained with the polysaccharide stain PATCH-SP (PLATES 4:35 and 4:39). In some cases the invaginated host plasmalemma was separated from the fungal wall by an additional electron transparent material (PLATES 4:36, 4:38 and 4:39).

The invaginated host plasma-membrane surrounding this complex was indistinguishable from that of the remaining host plasma-membrane when stained with PACP, PATCH-SP or uranyl and lead salts (PLATES 4:36 and 4:38). The tonoplast often stained with the PACP stain though not as intensely as the host plasma-membrane (PLATE 4:36). In some cases it was hard to distinguish the staining property of the invaginated host plasmalemma because of its close association with the densely staining matrix (PLATE 4:37).

Only a few long lengths of hyphae were observed and these showed that the intracellular hyphae passed from one cell to another (PLATES 4:36 and 4:40). Some had septa (PLATE 4:40) and only one hyphal compartment was observed to have two nuclei (PLATE 4:33), all the others were uninucleate. Within the cavity hyphal segments gave rise to spores (PLATES 4:41 and 4:42). These spores had very thick walls composed of at least two layers when stained with PATCH-SP (PLATE 4:42). A material surrounding the spores within the cavity stained intensely with PATCH-SP indicating the presence of polysaccharide materials around and within the forming sporeball.

PLATE 4:1

Scanning electron micrograph showing a millet ovary 5 days after infection with *T. penicillariae* sporidia. The stigma (st) and fused stylodia (sl) are covered in sporidia, some of which have germinated to form various lengths of hyphae. The ovary (O) does not have sporidia or hyphae on its surface. A region of the style, approximately 0.5 - 0.6mm above the ovary has collapsed (Co).

Magnification X 40

PLATE 4:2

Scanning electron micrograph showing the base of the collapsed region (Co) of the style covered in sporidia (sp - arrowed) and hyphae (h). Only a few sporidia and hyphae are found on the style surface beyond the collapsed region.

Magnification X 660

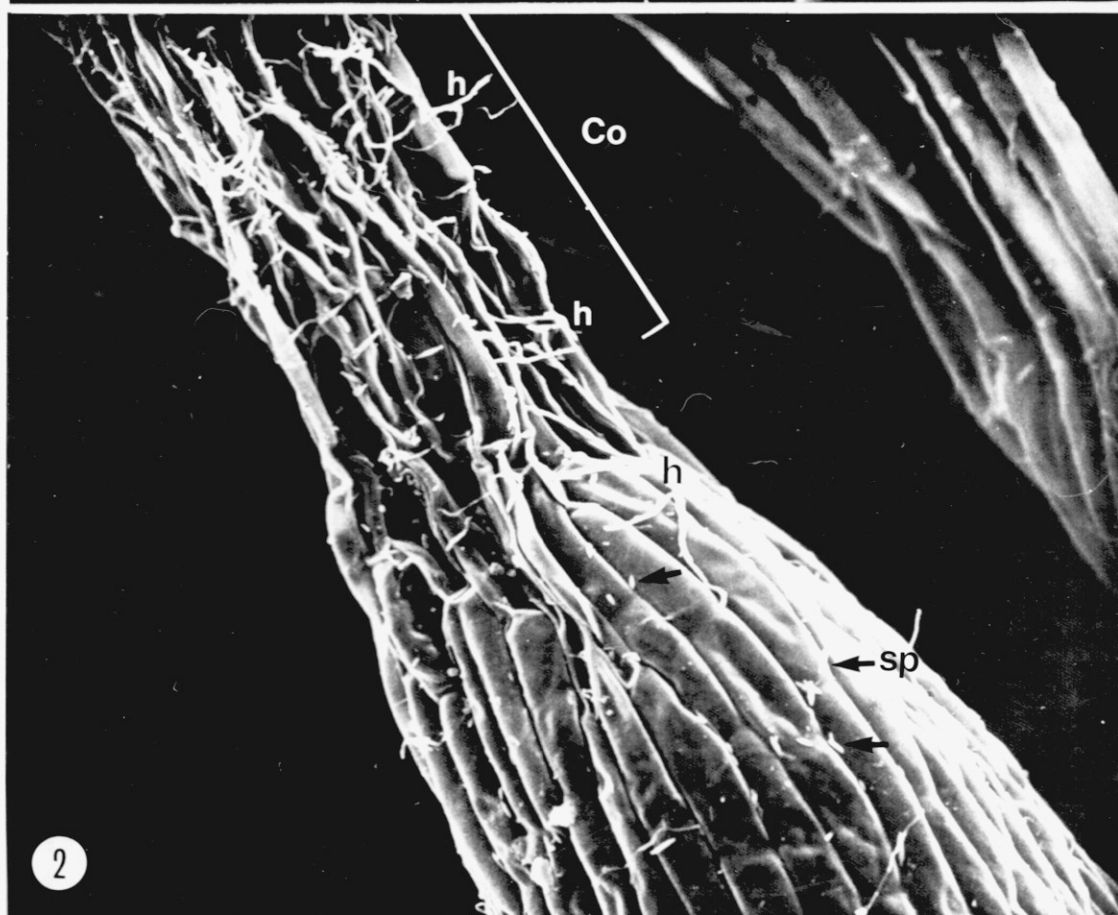


PLATE 4:3

Scanning electron micrograph showing a longitudinal fracture through a millet ovary 7 days post inoculation. The ovary wall (OW) on one side has grossly enlarged, the nucellus and embryo-sac (EmS) remain and fungal hyphae (h) occupy a cavity (C) in the upper portion of the ovary (right).

Magnification X 70

PLATE 4:4

A small millet inflorescence of the hybrid BJ104 showing the green colour exhibited by ovaries 5-7 days after infection with *Tolyposporium penicillariae*.

Magnification X 0.4

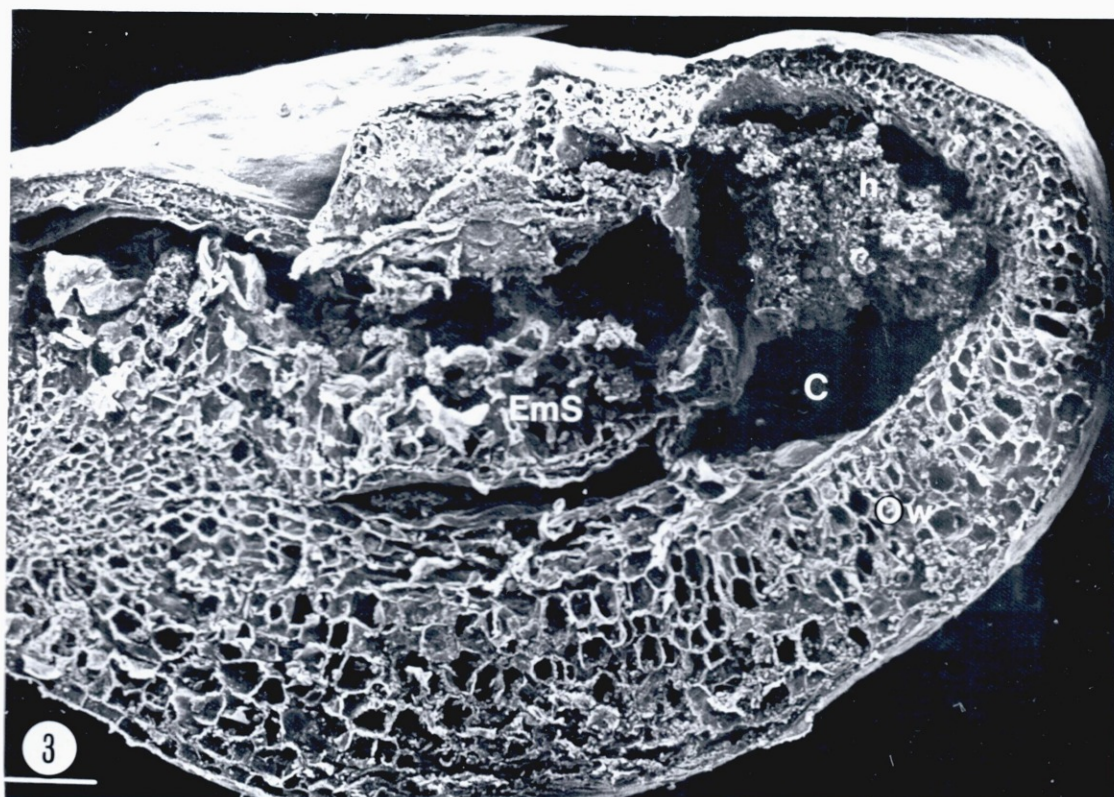


PLATE 4:5

A light micrograph showing part of the enlarged ovary wall (OW) in infected tissue. Metachromatically green staining bodies (b) are visible in the outer epidermis (OE).

Magnification X 380

Staining: Toluidine blue

PLATE 4:6

An electron micrograph showing the bodies which stained metachromatically in Plate 4:5. These osmiophilic bodies (b) in the outer epidermis of the ovary wall are all membrane-bound.

Magnification X 5300

Staining: Uranyl acetate and lead citrate

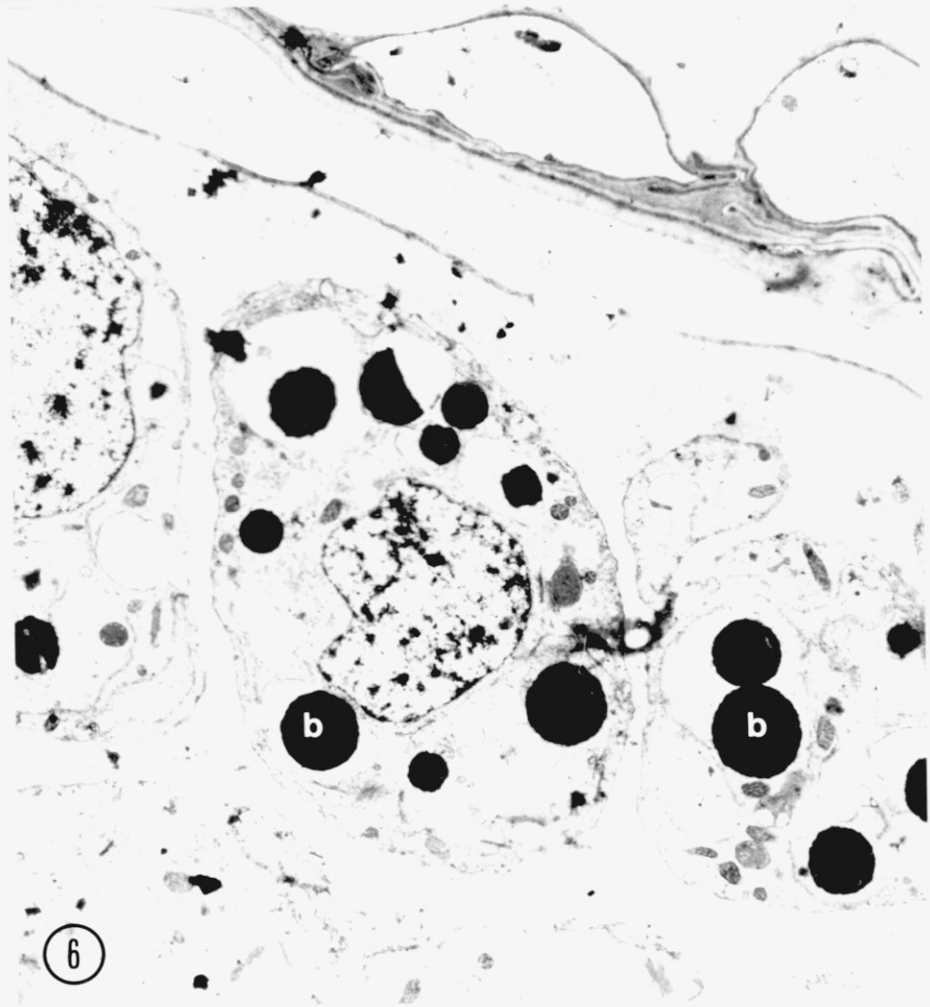
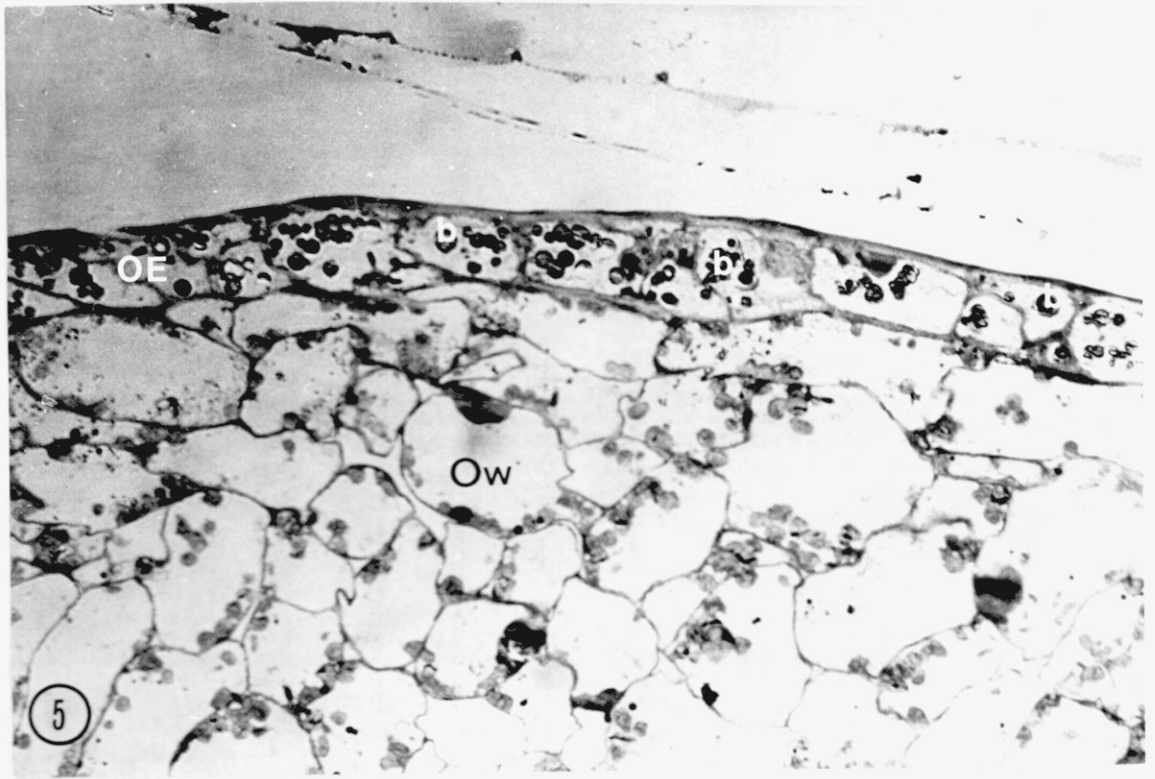


PLATE 4:7

A light micrograph showing part of a millet ovary 5 days post infection. Hyphal aggregates (h) are present between the inner (II) and outer integuments (OI) and the inner epidermis of the ovary wall (OW). Some cells of the ovary wall are hypertrophic, their enlargement causing the gall-like appearance of the infected ovary.

Magnification X 470

Staining: Toluidine blue

PLATE 4:8

A scanning electron micrograph of a millet ovary 5 days post infection. Hyphal aggregates (h) are present between the inner integument (II) and the ovary wall tissues (OW).

Magnification X 660

PLATE 4:9

A scanning electron micrograph of a millet ovary 5-7 days post infection. Hyphal aggregates (h) grow within a cavity (C) formed by the extension of the ovary wall (OW) from the nucellus (N).

Magnification X 300

PLATE 4:10

Scanning electron micrograph showing part of Plate 4:9. Hyphae (h) extend from the inner surface of the ovary wall (OW) into the cavity (C).

Magnification X 1680

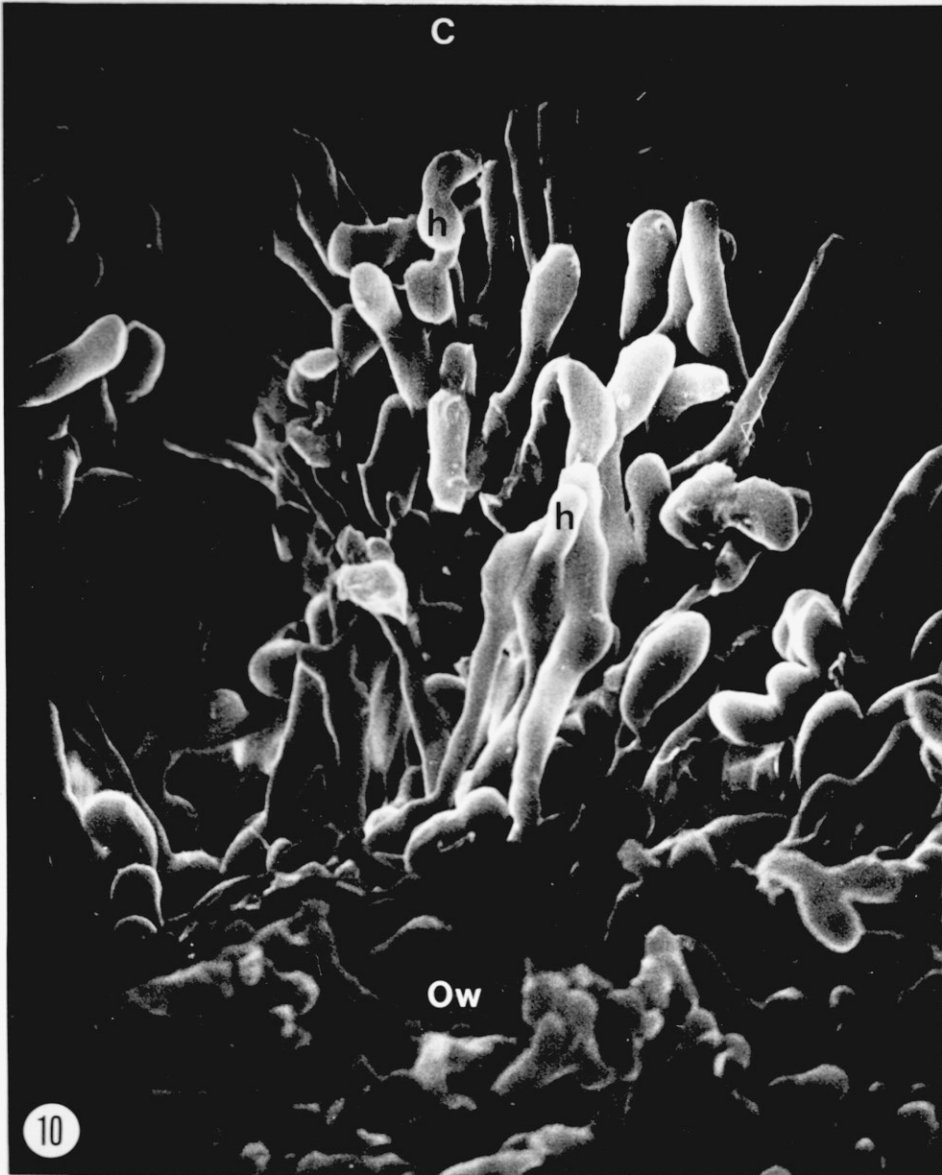
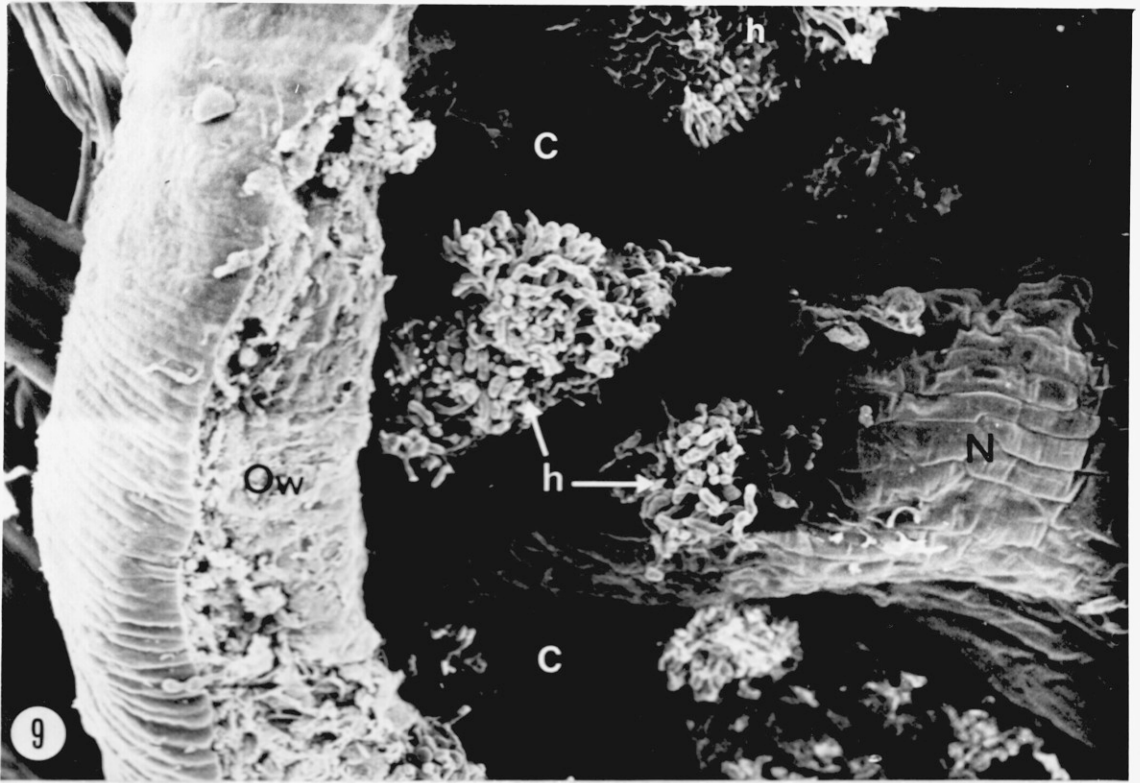


PLATE 4:11

A light micrograph showing a transverse section through an infected millet ovary 7 days post infection. Fungal hyphae (h) occupy the cavity (C) and small areas of the nucellus (N). The hypertrophic cells of the ovary wall (OW) are easily distinguishable and it is only the cells of the outer epidermis surrounding these hypertrophic cells which contain the dark staining bodies (b).

Magnification X 250

Staining Toluidine blue

PLATE 4:12

A scanning electron micrograph of a fractured millet ovary 10 days after infection by *T. penicillariae*. Fungal hyphae have colonised the intercellular spaces of the ovary wall (OW), the nucellus (N) and embryo-sac (EmS) and the cells of the hilar region (Hr). Aggregates of hyphae (h) are present in the cavity (C) and in the upper portion of the cavity they are differentiating into spores (s). The stigma and style have now detached from the ovary but the part below the collapsed region remains attached to the tip of the ovary (arrow).

Magnification X 50

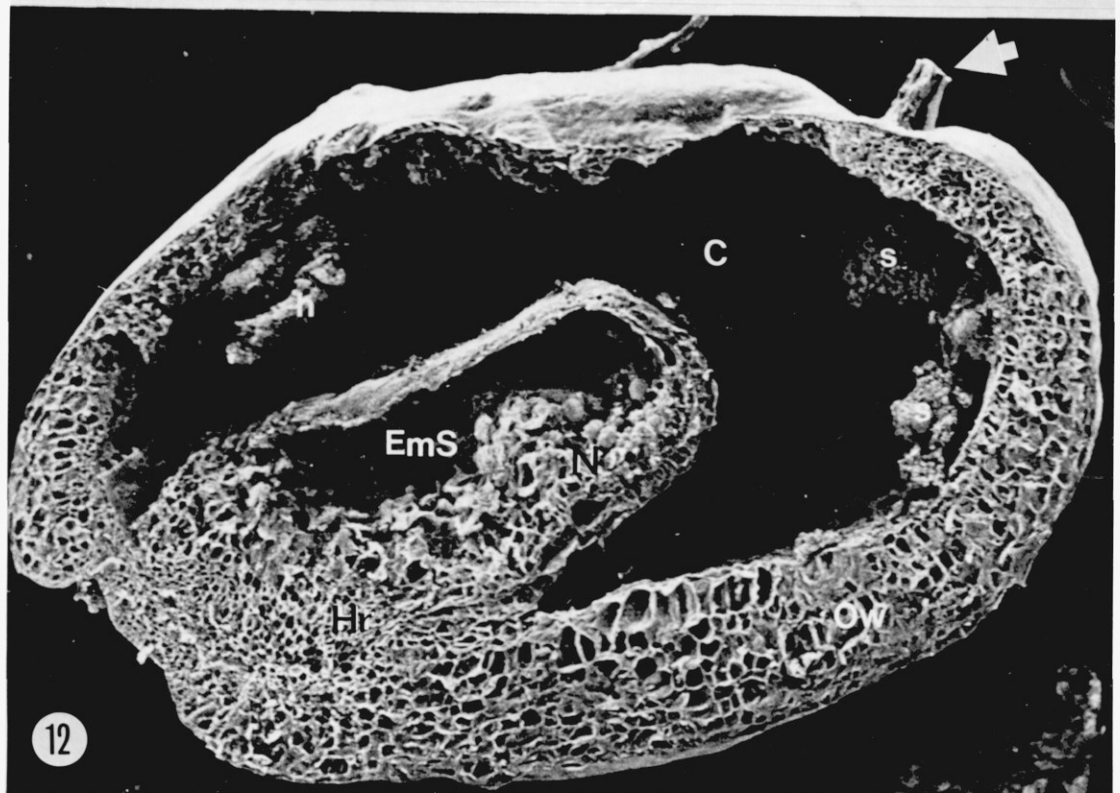
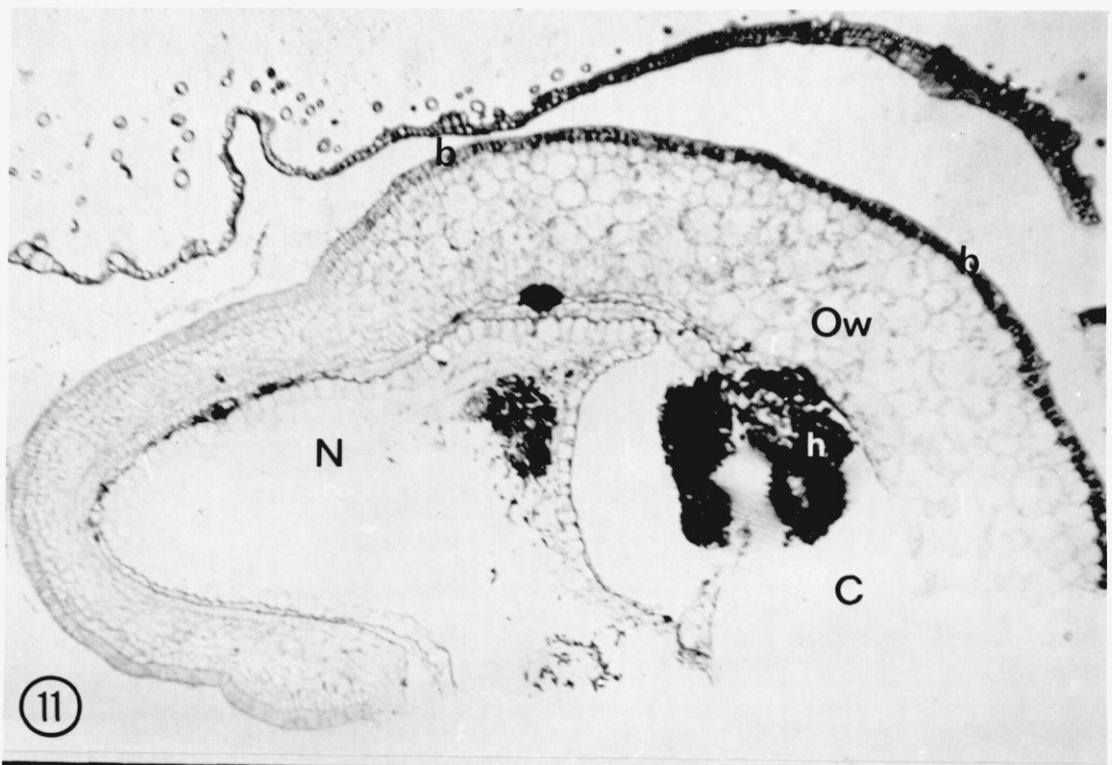


PLATE 4:13

An electron micrograph showing hyphal aggregates (h) within the cavity of the enlarged ovary. Areas appear to have torn out of the embedding medium when thin sectioned.

Magnification X 2230

Staining:uranyl acetate and lead citrate

PLATE 4:14

An electron micrograph showing hyphal aggregates in the ovarian cavity treated with a stain specific for polysaccharides (PATCH-SP). This reveals an electron dense (Ed) material, where in Plate 4:13 there seemed to be a space. The walls of the hyphal aggregates (h) and spore initials (si) are also heavily stained.

Magnification X 6100

Staining PATCH-SP

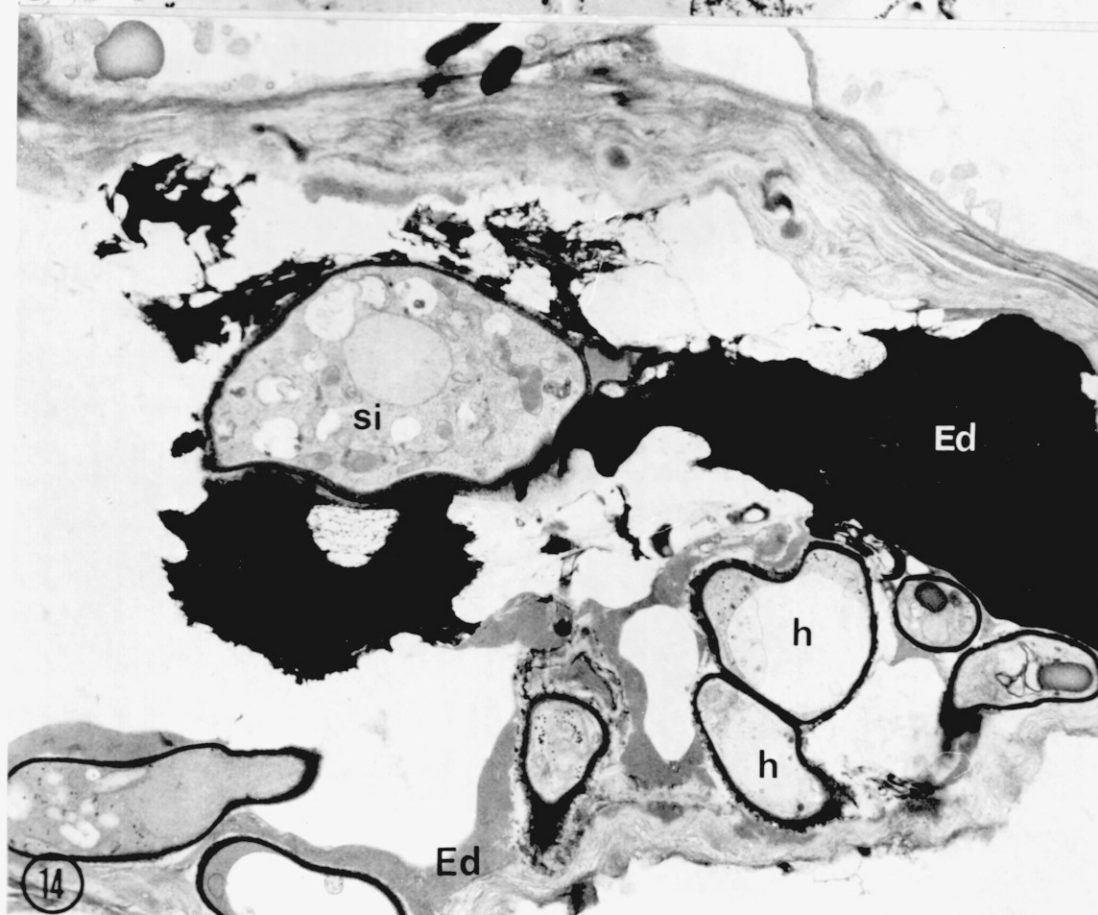
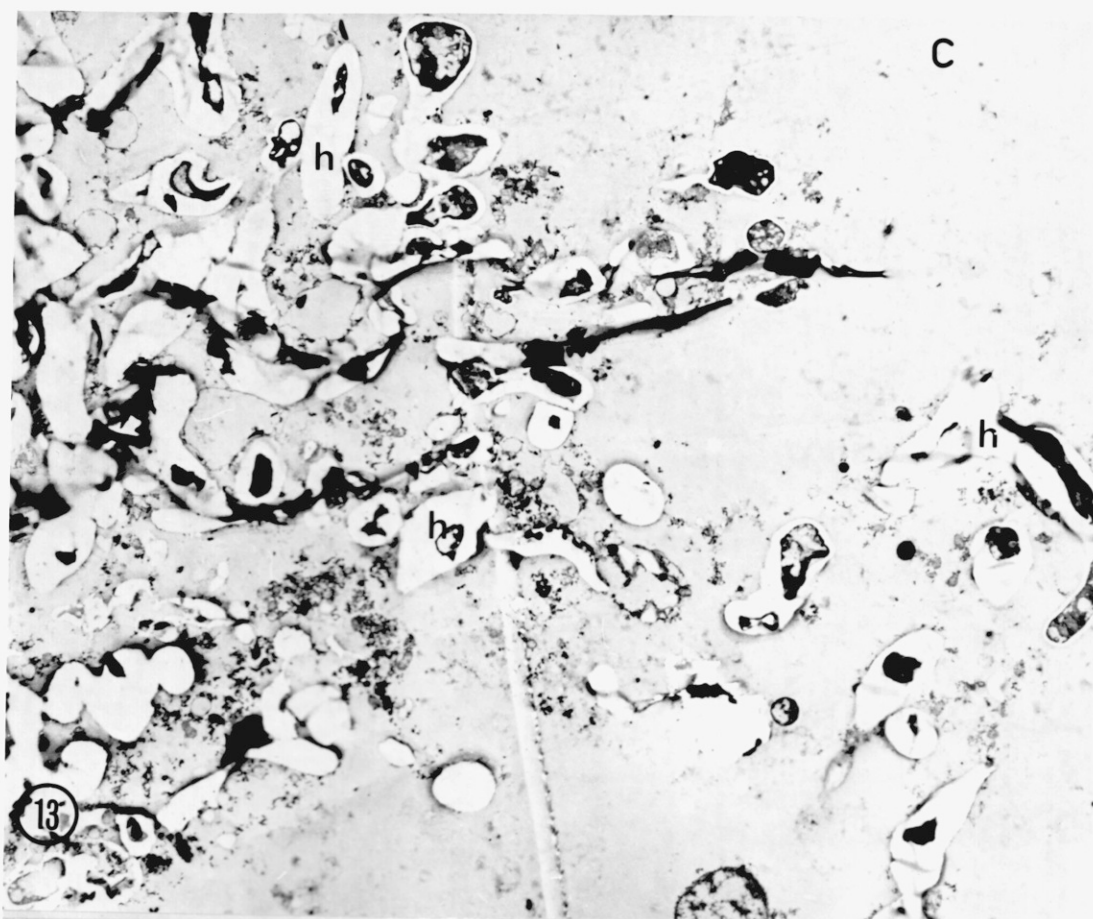


PLATE 4:15

A scanning electron micrograph of the upper portion of the cavity (C) showing the hyphal aggregates differentiating to form spores (s).

[Part of Plate 4:12]

Magnification X 130

PLATE 4:16

A scanning electron micrograph of spores (s) approximately 6-7 μm in diameter, developing within the ovarian cavity. They remain aggregated so that sporeballs (SB) are produced at maturity.

Magnification X 550

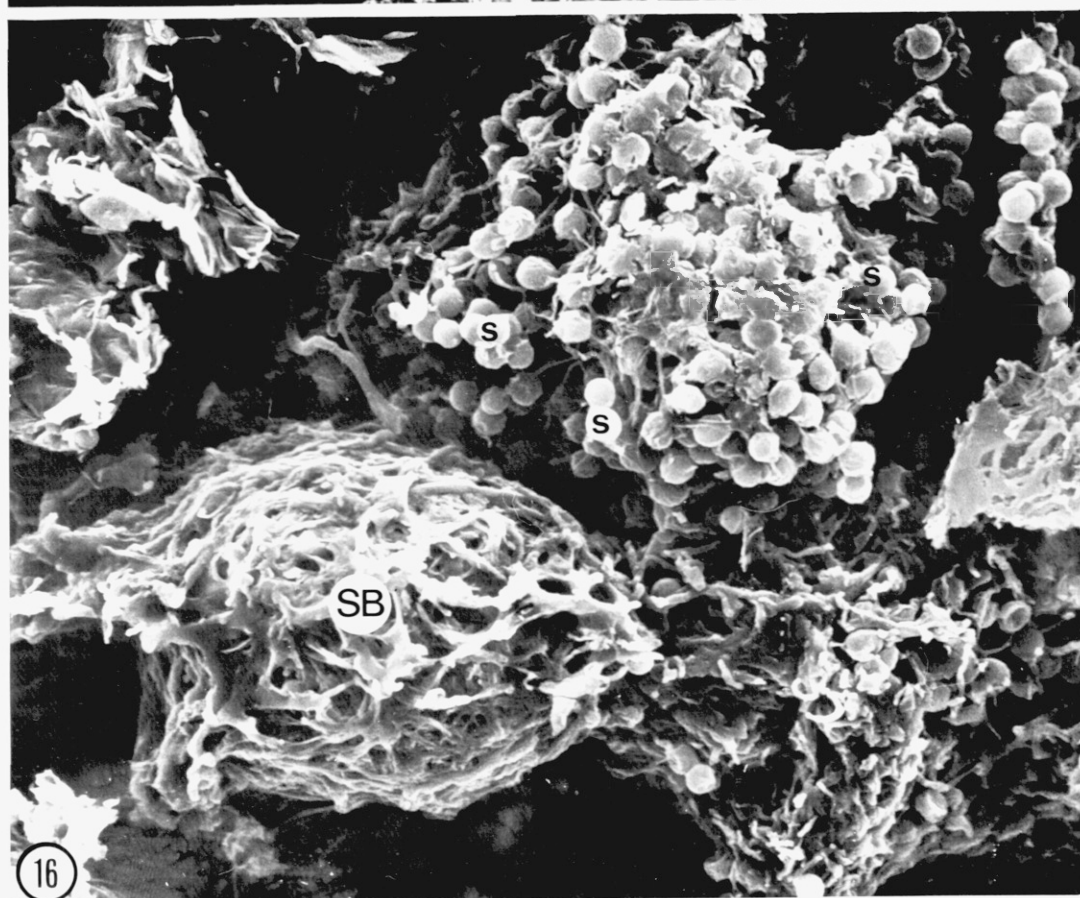


PLATE 4:17

A light micrograph of a longitudinal section through the base of an infected millet ovary fixed 10 days after inoculation. Phloem sieve tubes (Ph) and associated parenchyma cells are present in the hilum (Hr) at the base of the ovary and in the pedicel (Pd). Hyphal aggregates (h) are beginning to form between the ovary wall (OW) and the nucellus (N).

Magnification X 250

Staining Toluidine blue

PLATE 4:18

An electron micrograph showing both xylem tracheids (Xy) and phloem sieve tubes (Ph) in the pedicel tissue of the infected millet ovary. These tissues are never occupied by fungal hyphae.

Magnification X 2330

Staining PATCH-SP

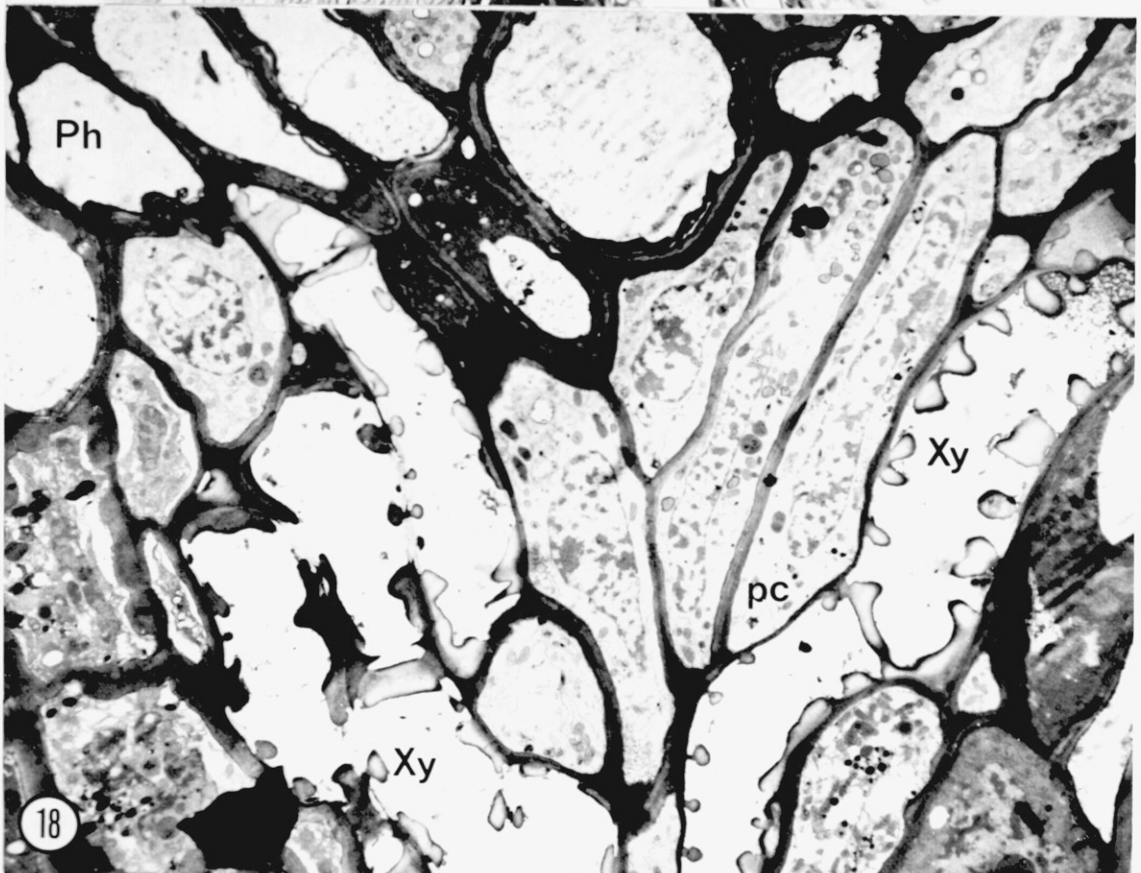
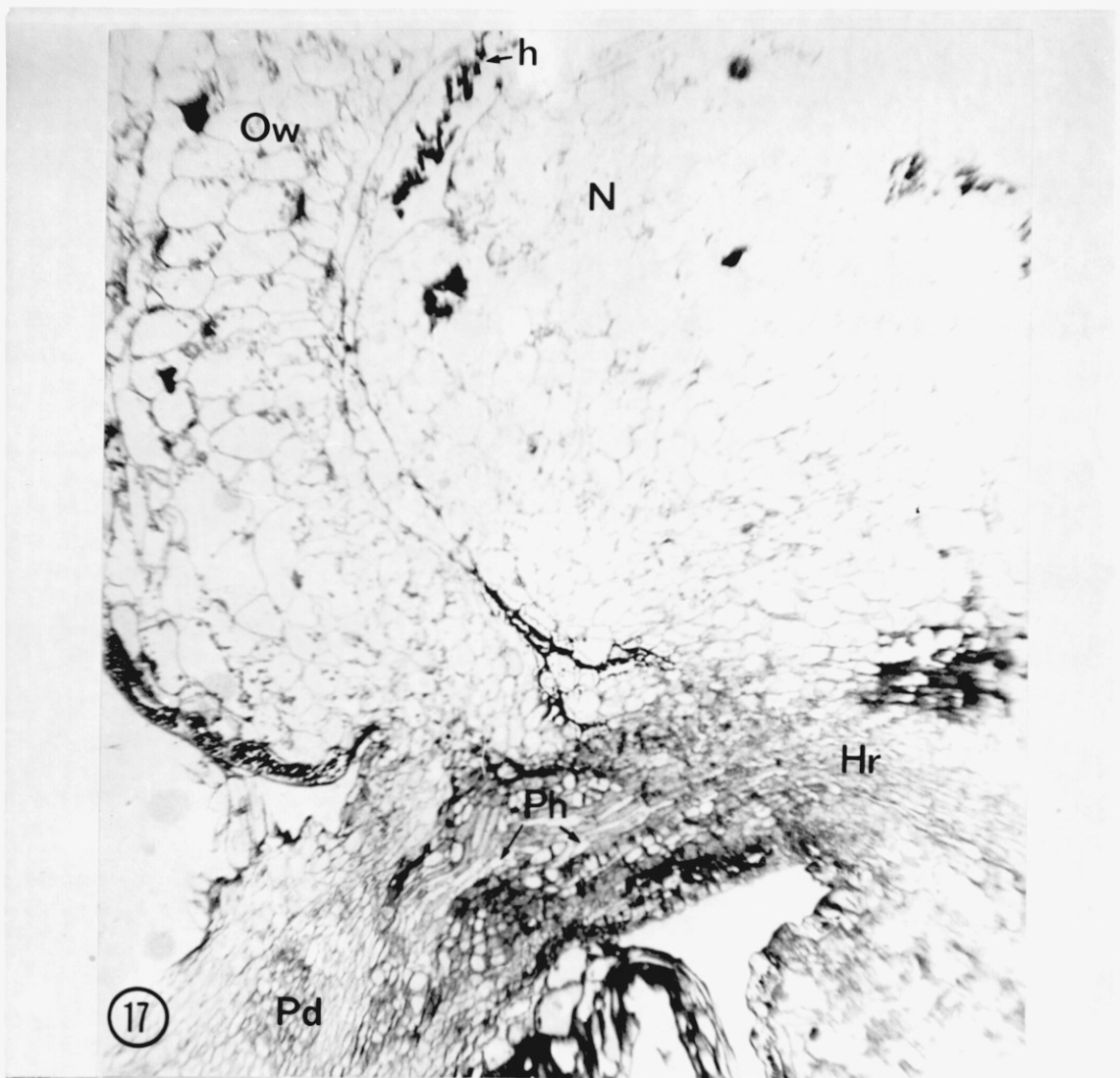


PLATE 4:19

A scanning electron micrograph showing a longitudinal fracture through an infected millet ovary 14 days after inoculation with *T. penicillariae*. The hyphal aggregates within the cavity have differentiated to form groups of sporeballs (SB). The ovary wall (OW) is now thinner than previously (compare Plate 4:12). The nucellus (N) and embryo-sac (EmS) have also been colonised and spores have developed.

Hilum (Hr) ; pedicel (Pd)

Magnification X 40

PLATE 4:20

Scanning electron micrograph showing interlocking sporeballs (SB) which are approximately 90-150µm by 50-60µm and composed of many teliospores.

Magnification X 80

PLATE 4:21

Scanning electron micrograph showing individual sporeballs are composed of teliospores (T) each approximately 6-7µm in diameter.

Magnification X 750

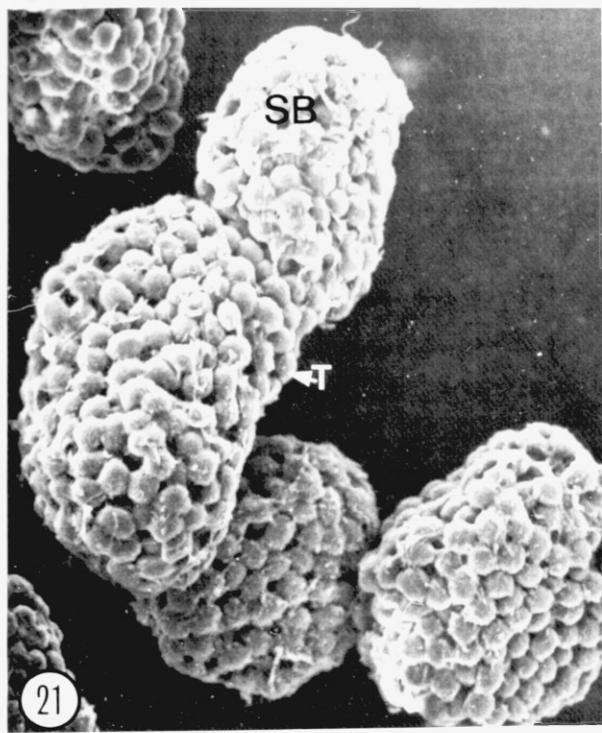
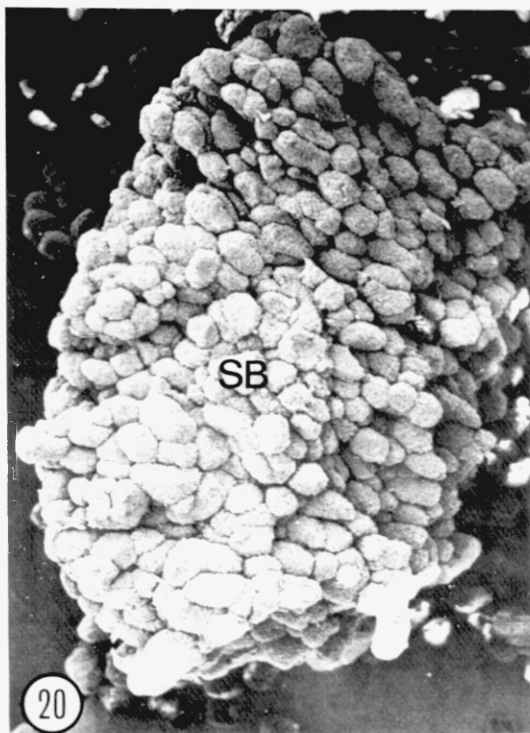
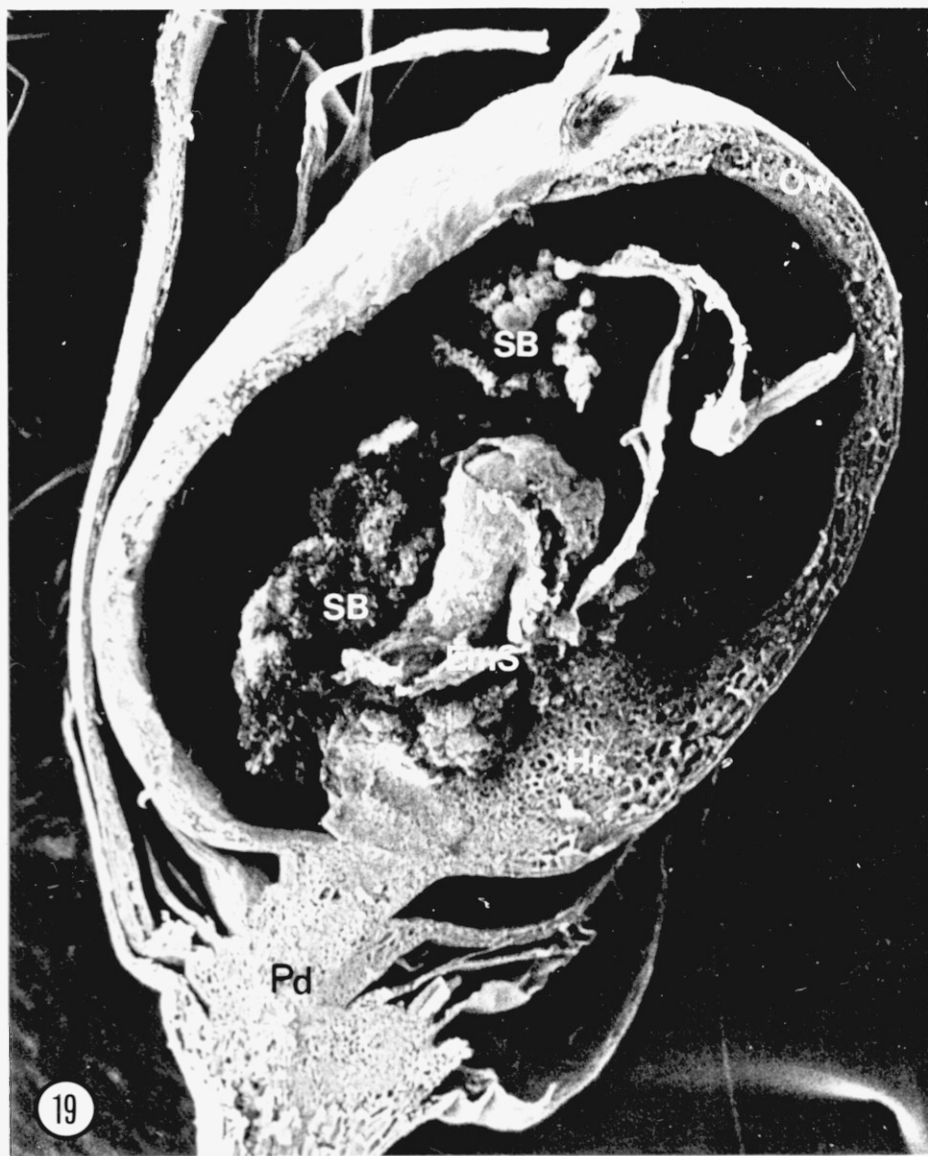


PLATE 4:22

An inflorescence of millet showing the brown colouration of the mature *T. penicillariae*-infected millet ovaries.

Magnification X 0.4

PLATE 4:23

A scanning electron micrograph showing the sporeballs (SB) released from a ruptured ovary.

(OW) ovary wall

Magnification X 20

PLATE 4:24

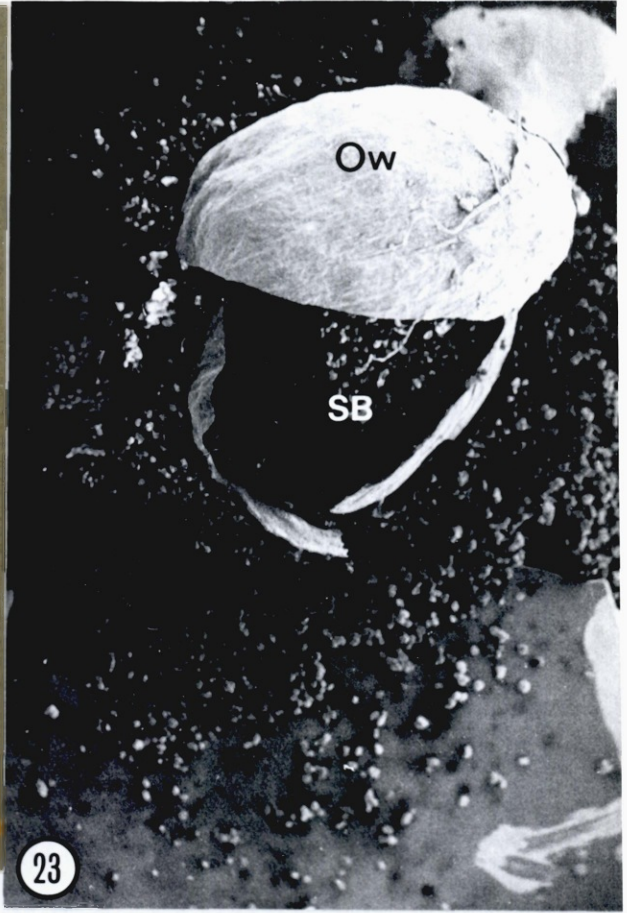
An electron micrograph showing that fungal hyphae (h) first colonise the region between the nucellus (N) and the ovary wall (OW), and bounded by the cuticles (cu) which line the integuments (I).

Magnification X 6600

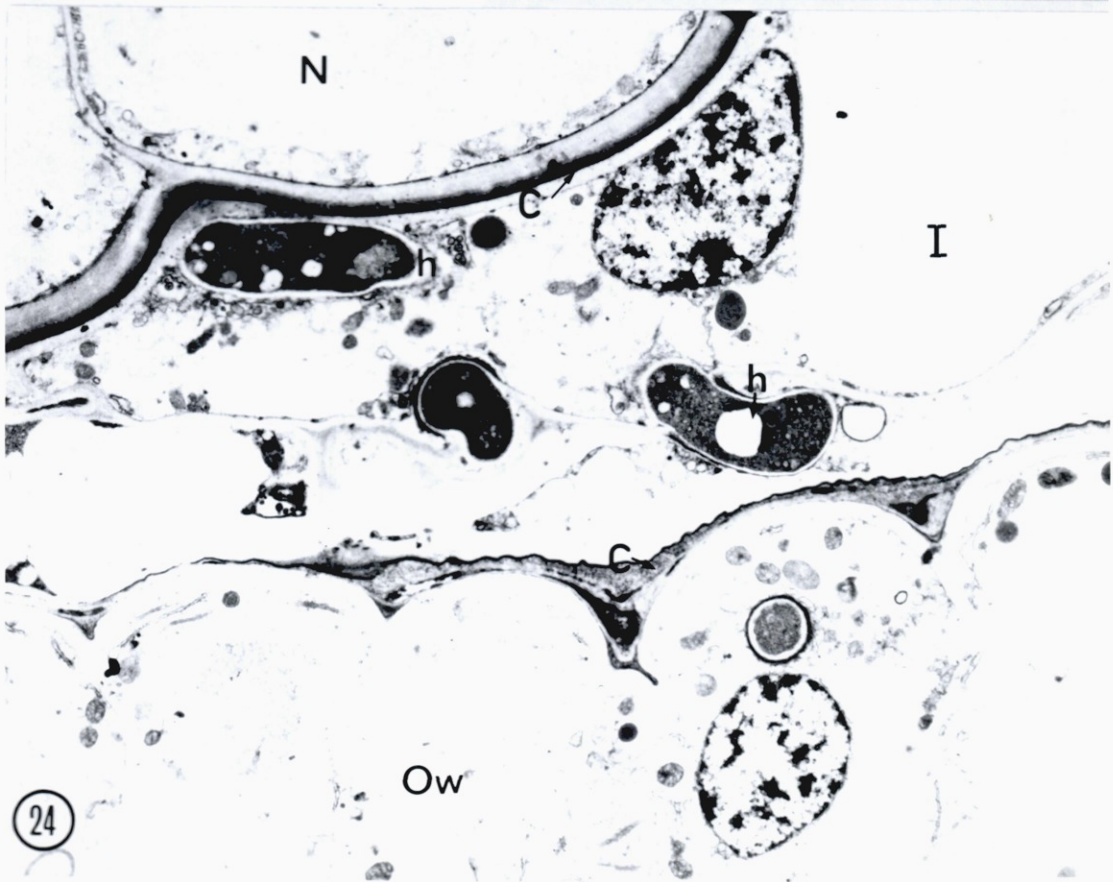
Staining: Uranyl acetate and lead citrate



22



23



24

PLATES 4:25 and 4:26

Electron micrographs taken from ovaries 10 days after infection. The nucellar cells (N) are partly disorganised and some membranes are recognisable. Intracellular hyphae (Ih) are surrounded by a plasma membrane (pm) indistinguishable from the host plasma membrane and by both electron transparent (Et) and electron dense (Ed) materials.

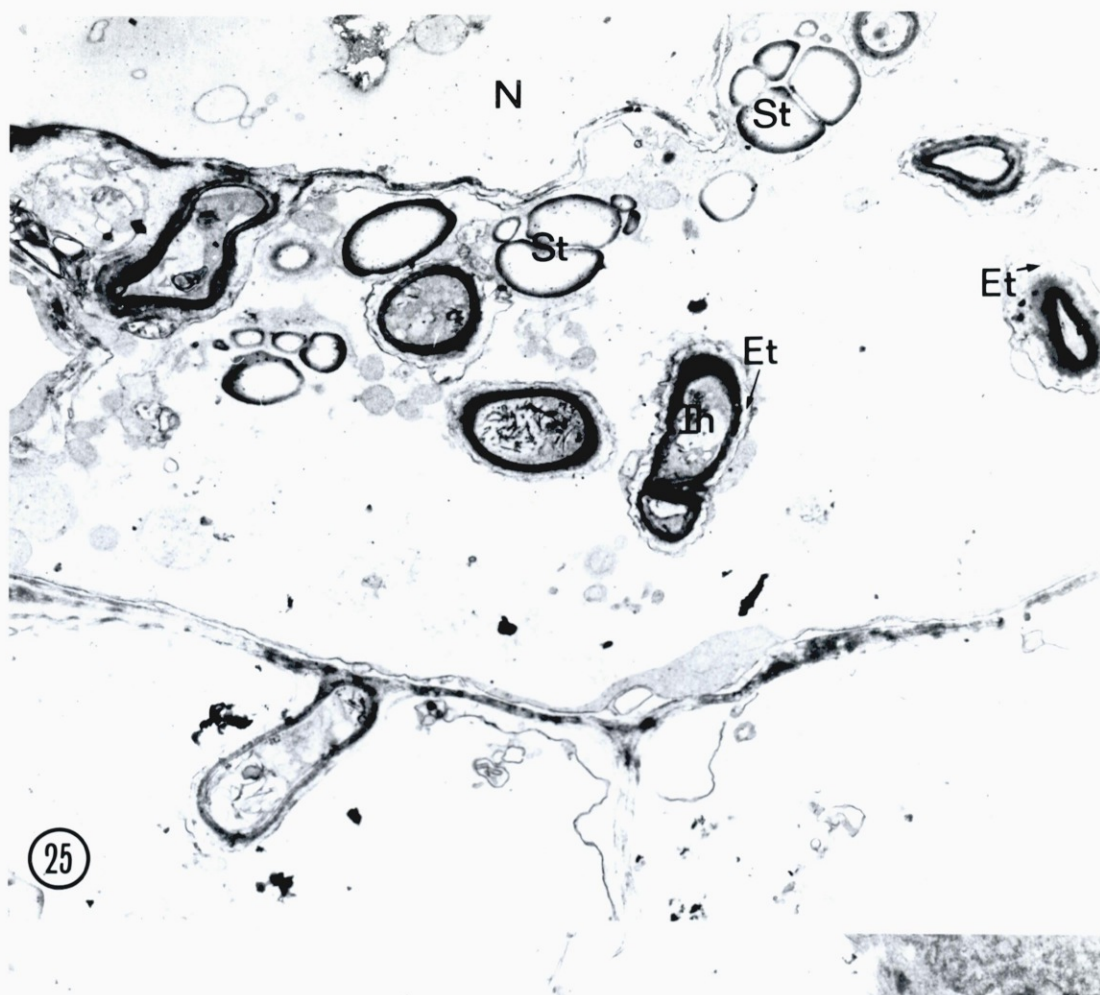
HW - Host wall St - Starch grain

Plate 4:25 Magnification X 7150

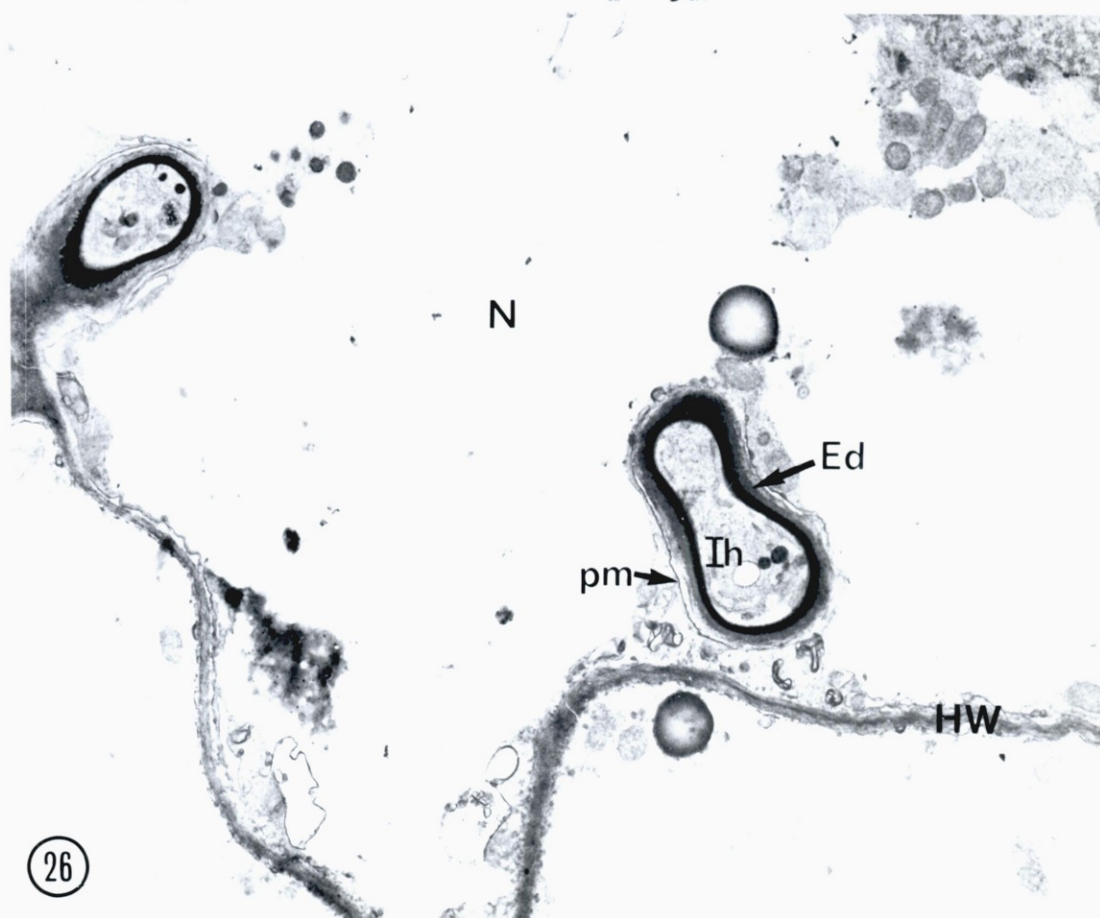
Staining: PACP

Plate 4:26 Magnification X 9300

Staining: PATCH-SP



25



26

PLATES 4:27 and 4:28

Electron micrographs of millet ovaries 10 days after infection. Host cells within the hilum region are smaller than those in the nucellus (N). They have smaller vacuoles, larger nuclei and many organelles within their cytoplasm. Several rows of cells parallel to the phloem in the pericarp contain many starch grains (st) which are electron dense due to their reaction with the polysaccharide stain. Hyphae occupy both intercellular (h) and intracellular (lh) positions within the hilum.

Plate 4:27 Magnification X 2330
 Staining: PATCH-SP

Plate 4:28 Magnification X 4920
 Staining: PATCH-SP

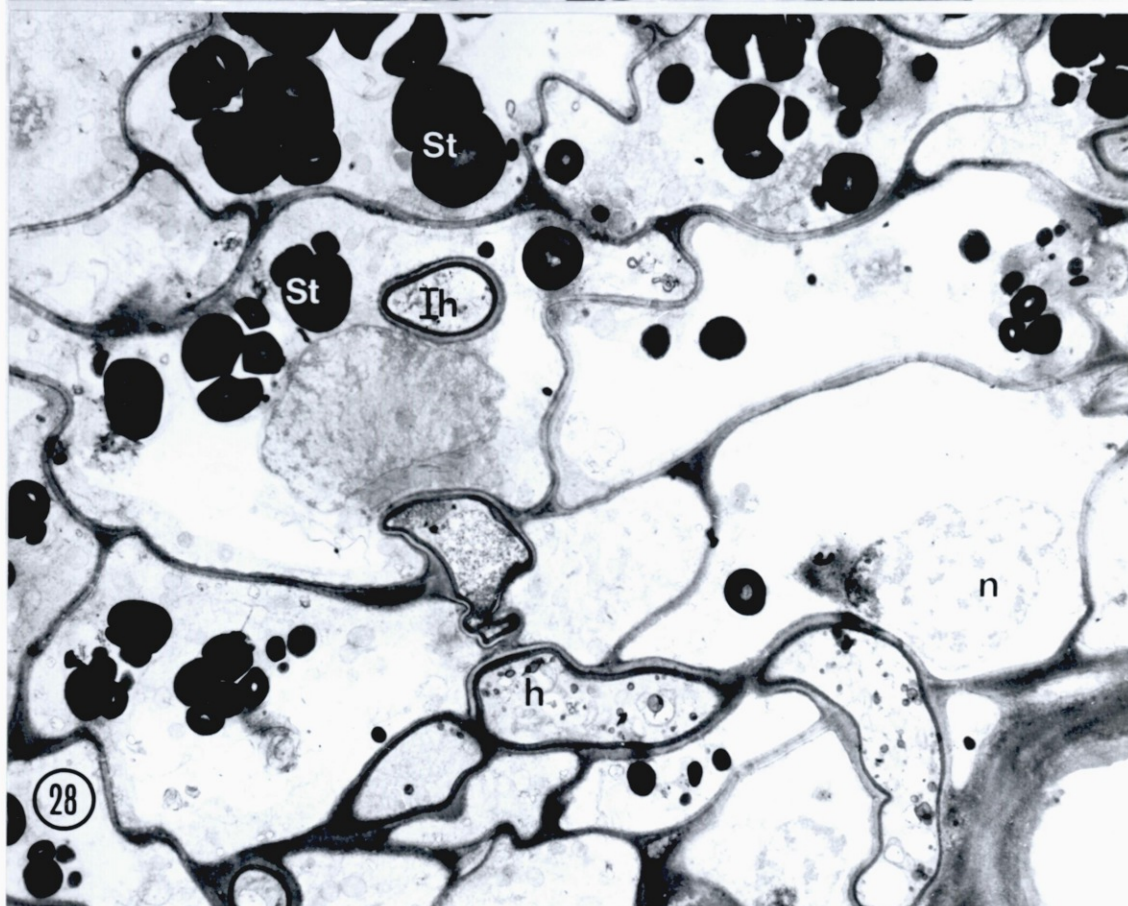
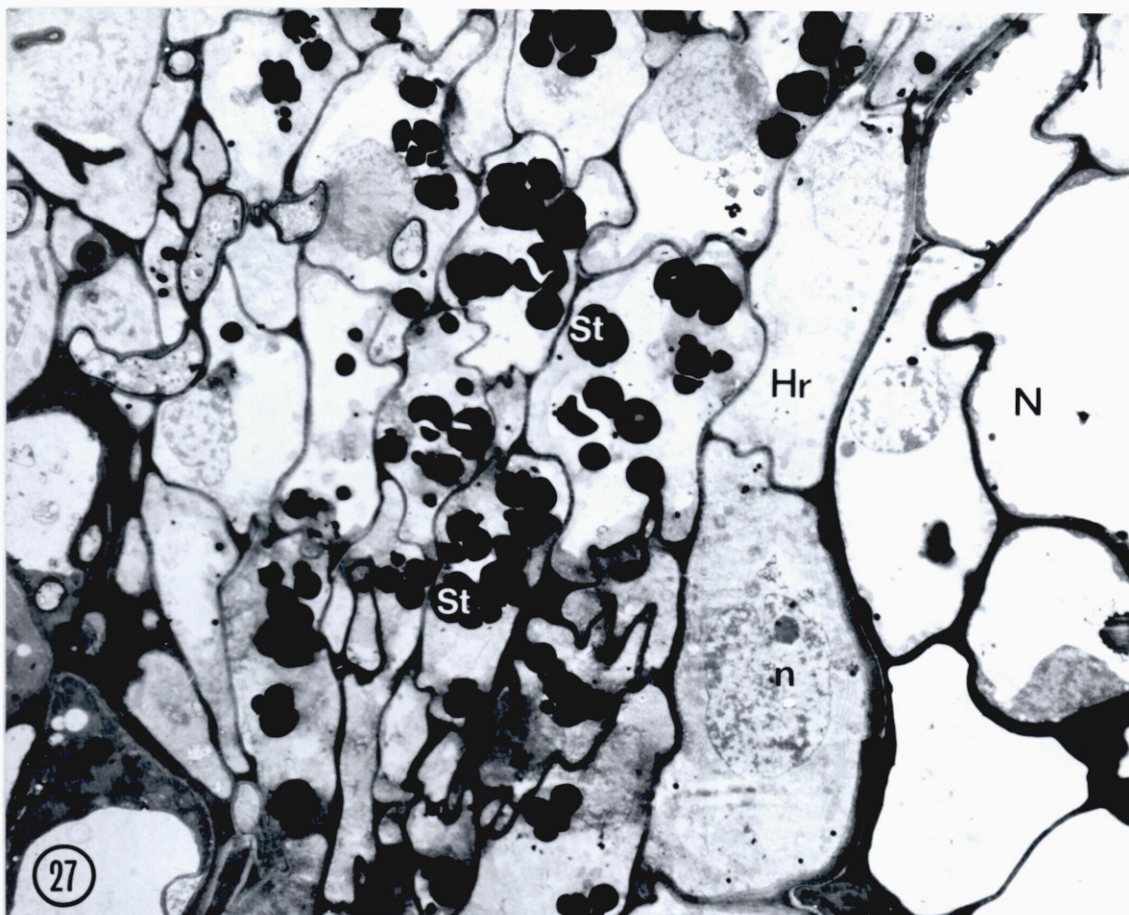
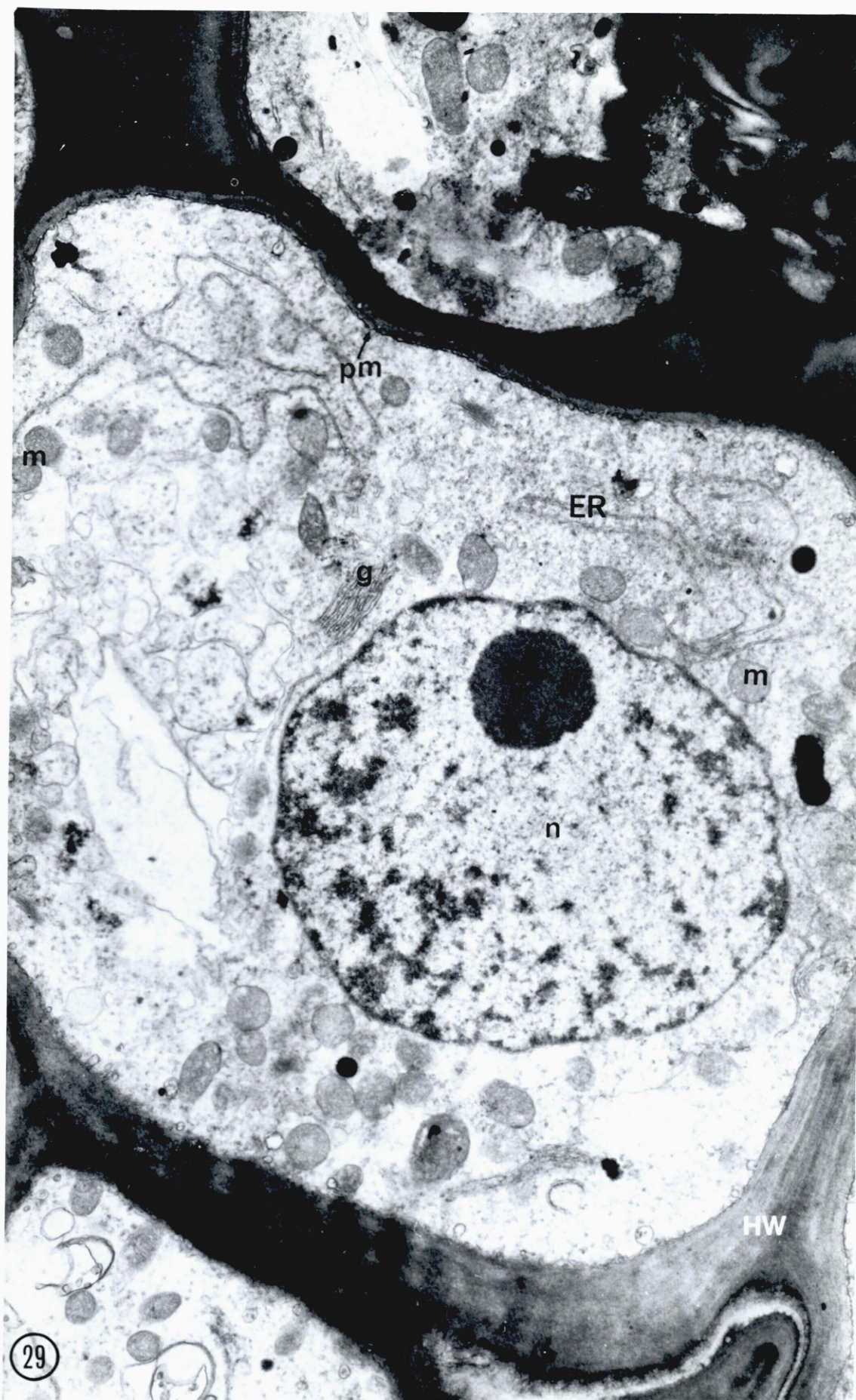


PLATE 4:29

Electron micrograph of an uninfected cell within the hilum region which contains large quantities of endoplasmic reticulum (ER), many mitochondria (m), golgi apparatus (g) and a large nucleus (n).

Magnification X 10900

Staining: PATCH-SP



PLATES 4:30 and 4:31

Electron micrographs of infected host cells within the hilum region 10 days after infection. Fungal hyphae are predominantly intracellular (Ih) in cells associated with phloem sieve tubes (Ph) (Plate 4:30) and other cells associated with the vascular tissue (Plate 4:31).

Plate 4:30

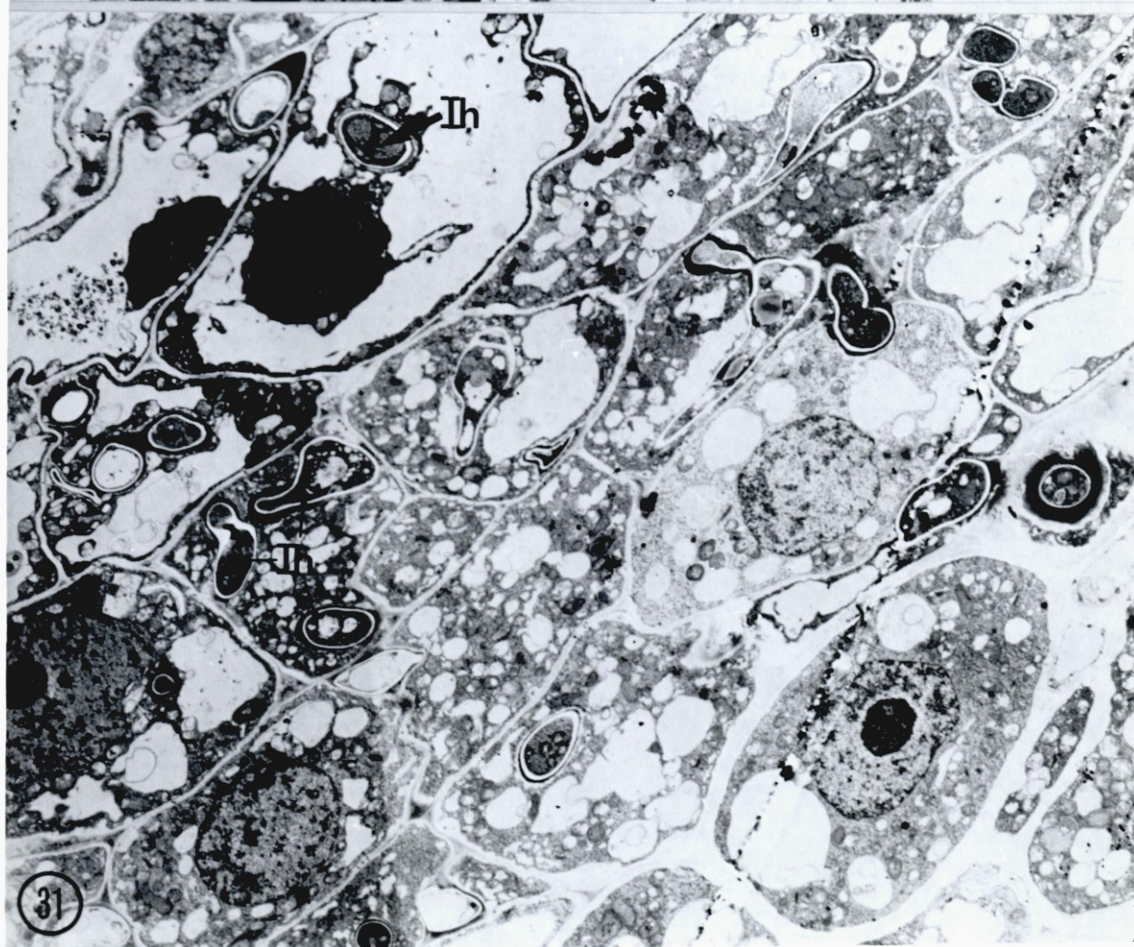
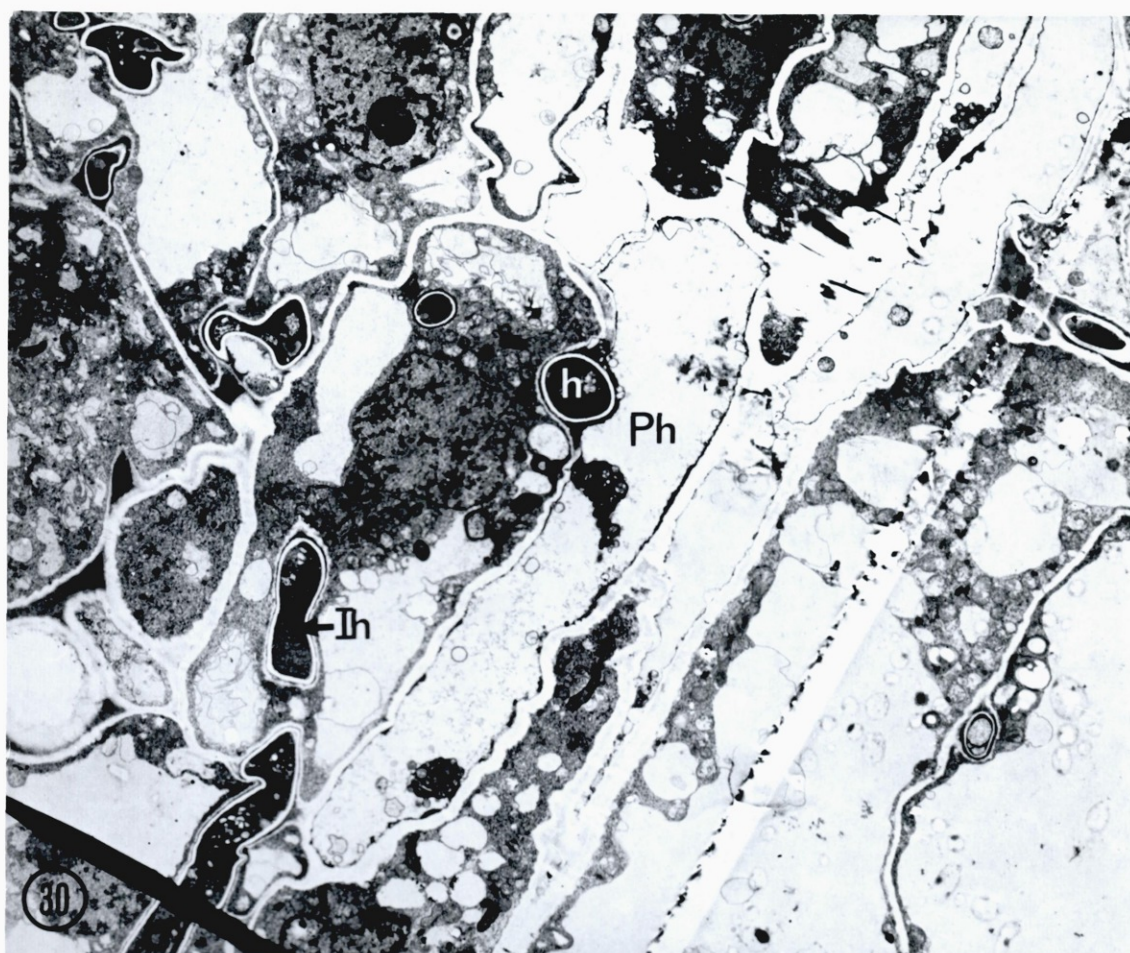
Magnification X 3200

Staining: Uranyl acetate and lead citrate

Plate 4:31

Magnification X 3000

Staining: Uranyl acetate and lead citrate



PLATES 4:32 and 4:33

Electron micrographs of infected host cells within the hilum region 10 days after infection. A small group of xylem tracheids (Xy) occur at the junction between the hilum and the pedicel (Plate 4:32). Intracellular hyphae (Ih) in the cells around the tracheids are abundant but no further penetration towards the pedicel vascular tissue was observed. Intracellular hyphae are surrounded by an osmiophilic material of various thicknesses. A few hyphal segments contained two nuclei (Fn) (Plate 4:33).

Plate 4:32	Magnification X 3200
	Staining:Uranyl acetate and lead citrate
Plate 4:33	Magnification X 4900
	Staining:Uranyl acetate and lead citrate

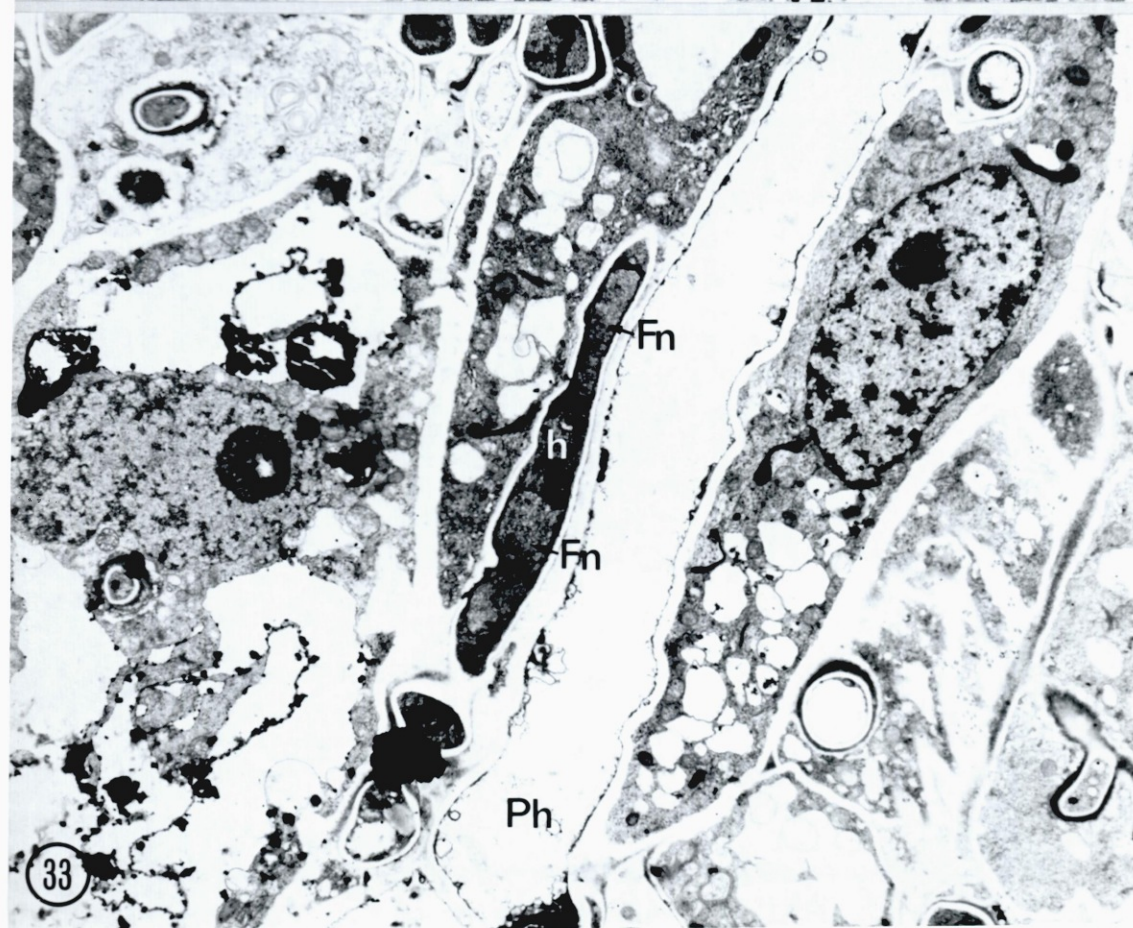
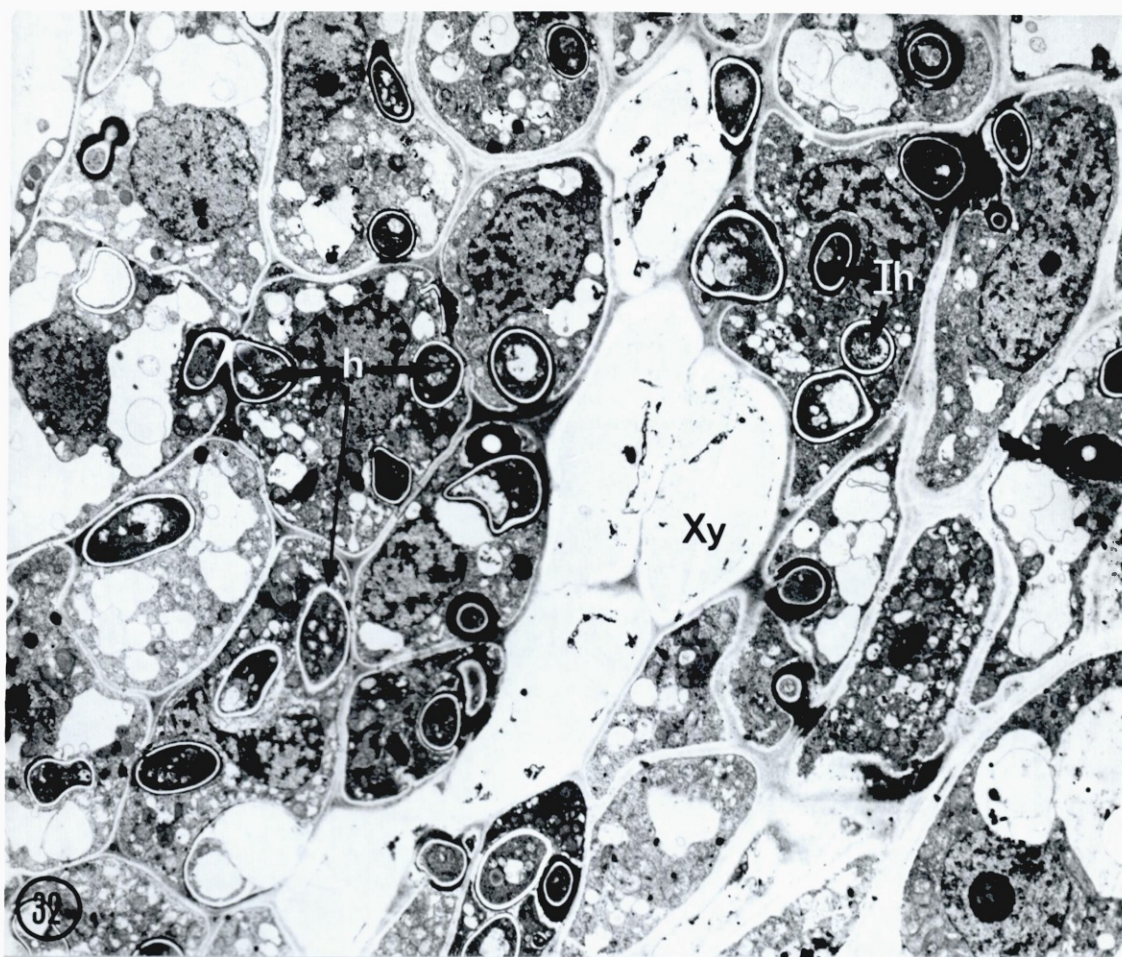


PLATE 4:34

Electron micrograph showing intracellular hyphae (Ih) within cells of the hilum region. Each is surrounded by an electron dense material (Ed) of various thickness, uniform in texture and more electron dense than the host cell wall. A material of similar staining properties is sometimes observed at the intercellular junctions between 3 host cells.

Magnification X 6100

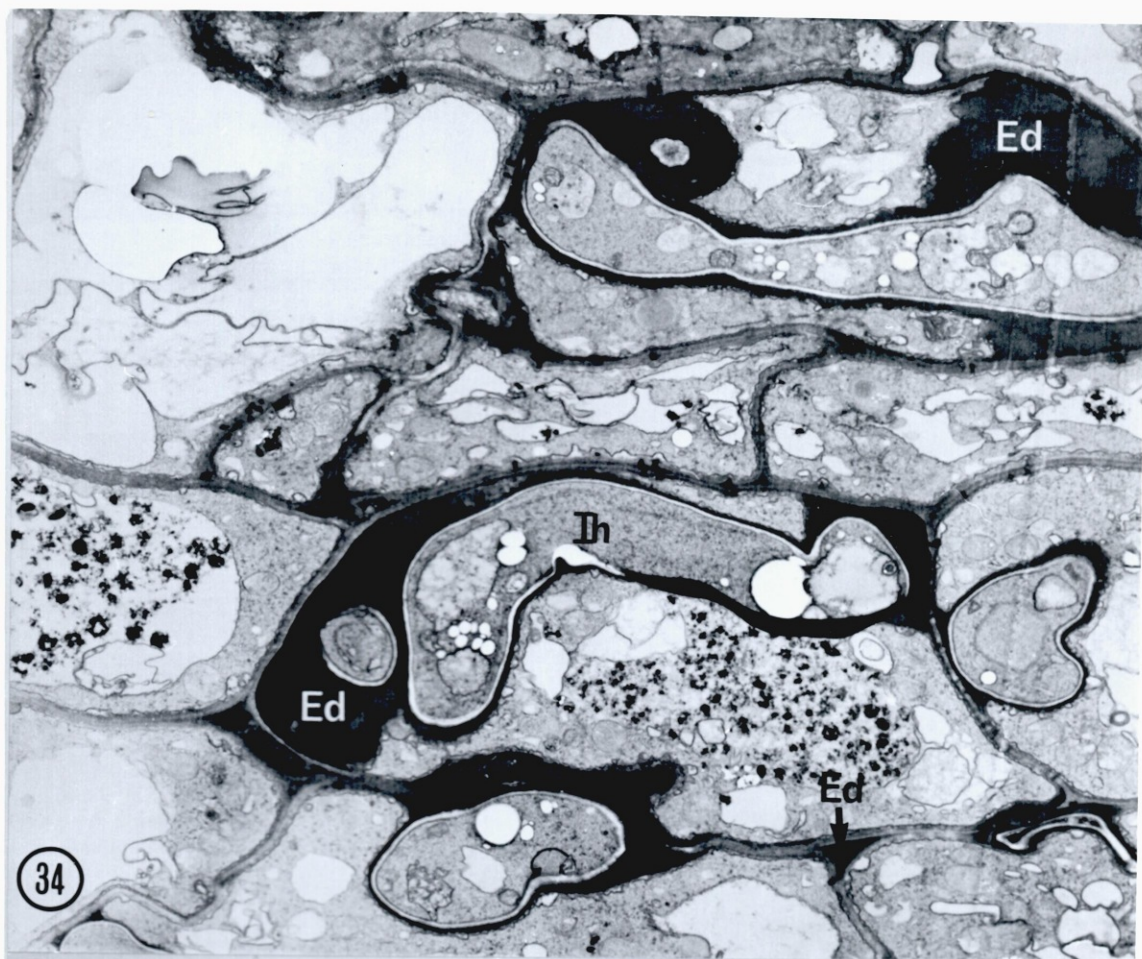
Staining: PACP

PLATE 4:35

Electron micrograph showing both intercellular (h) and intracellular hyphae (Ih) within the hilum region 10 days post infection. The fungal walls stain more intensely than the host cell wall (HW) and a wall-like material (wm) surrounds the intracellular hyphae, though it is much thinner than in Plate 4:34 and stains similarly to the host cell wall (HW). Note the darker staining band of the middle lamella (ml) region between host cell walls.

Magnification X 7150

Staining: PATCH-SP



PLATES 4:36, 4:37 and 4:38

Electron micrographs of intracellular hyphae within the hilum region of a millet ovary 10 days post infection with *T. penicillariae* taken from sections stained with PACP.

PLATE 4:36

An intracellular hypha (Ih) passing from one cell to another and covered in each cell by the host plasma membrane. The invaginated plasma-membrane is indistinguishable from that lining the host cell wall. Some other membranes within the cell also stained.

Magnification X 11200

PLATE 4:37

Intracellular hypha (Ih) surrounded only by the electron dense material (Ed). In such cases it is difficult to distinguish the membrane (pm) surrounding the hypha except where small particles protrude from the matrix.

Magnification X 16500

PLATE 4:38

The intracellular (Ih) fungal wall is surrounded by both an electron dense (Ed) and an electron transparent (Et) material, the latter staining similarly to the host cell wall (HW). The plasma membrane (pm) surrounding the hypha stains intensely.

Magnification X 15900

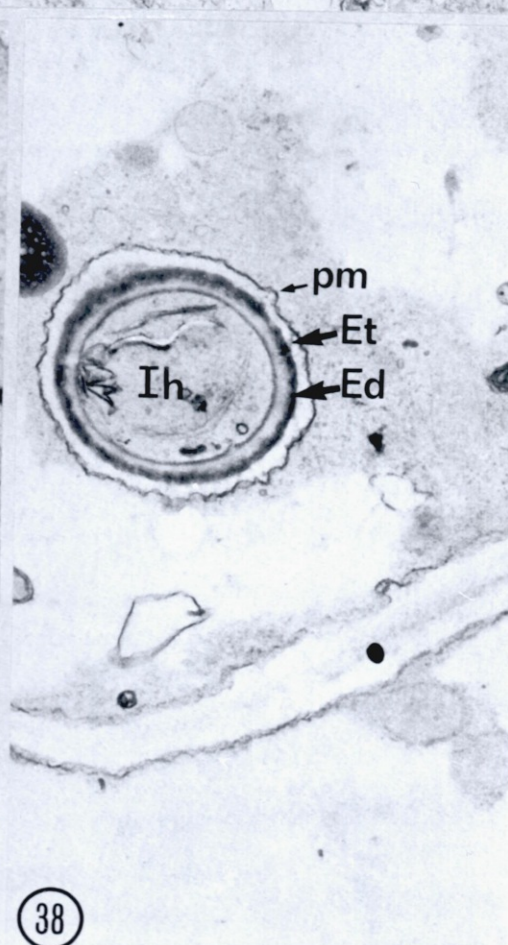
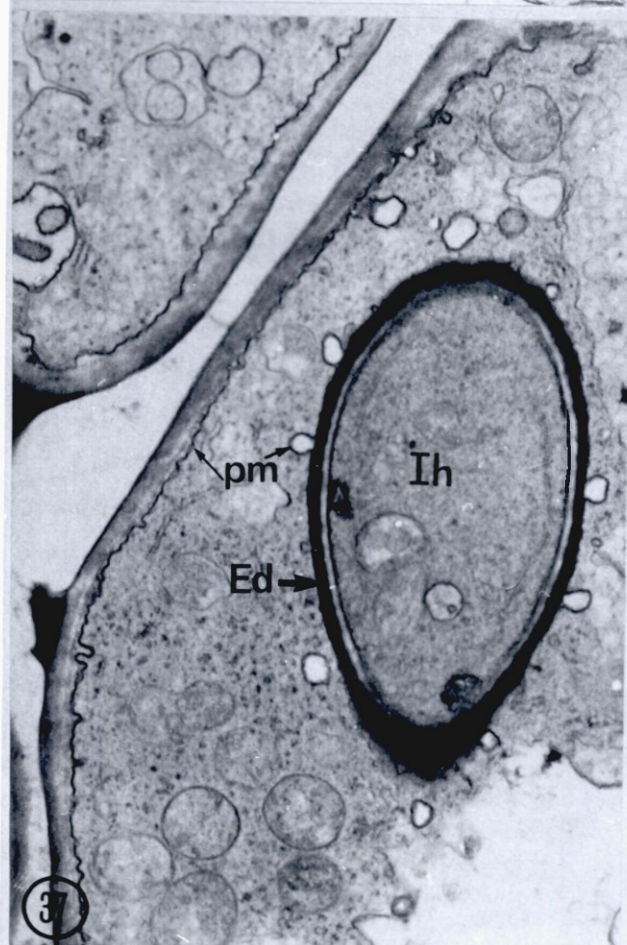
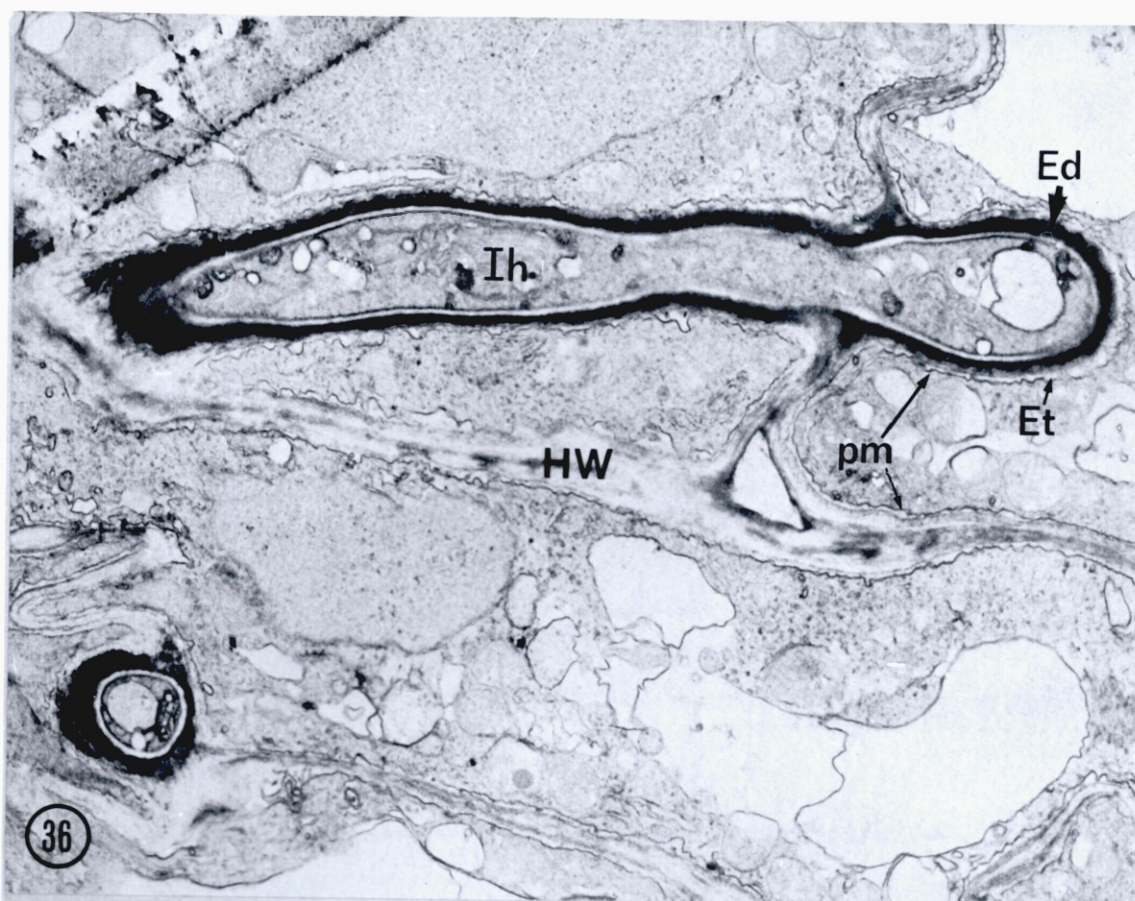


PLATE 4:39

Electron micrograph showing intracellular hyphae (Ih) covered by an electron dense (Ed) matrix which stains similarly to the host cell wall (HW). Occasionally an additional transparent material (Et) is laid down between the invaginated host plasma-membrane and the cell wall-like matrix material (Ed).

Magnification X 9400

Staining: PATCH-SP

PLATE 4:40

Electron micrograph showing a simple septa (sp) in an intracellular hypha (Ih).

Magnification X 9400

Staining:Uranyl acetate and lead citrate

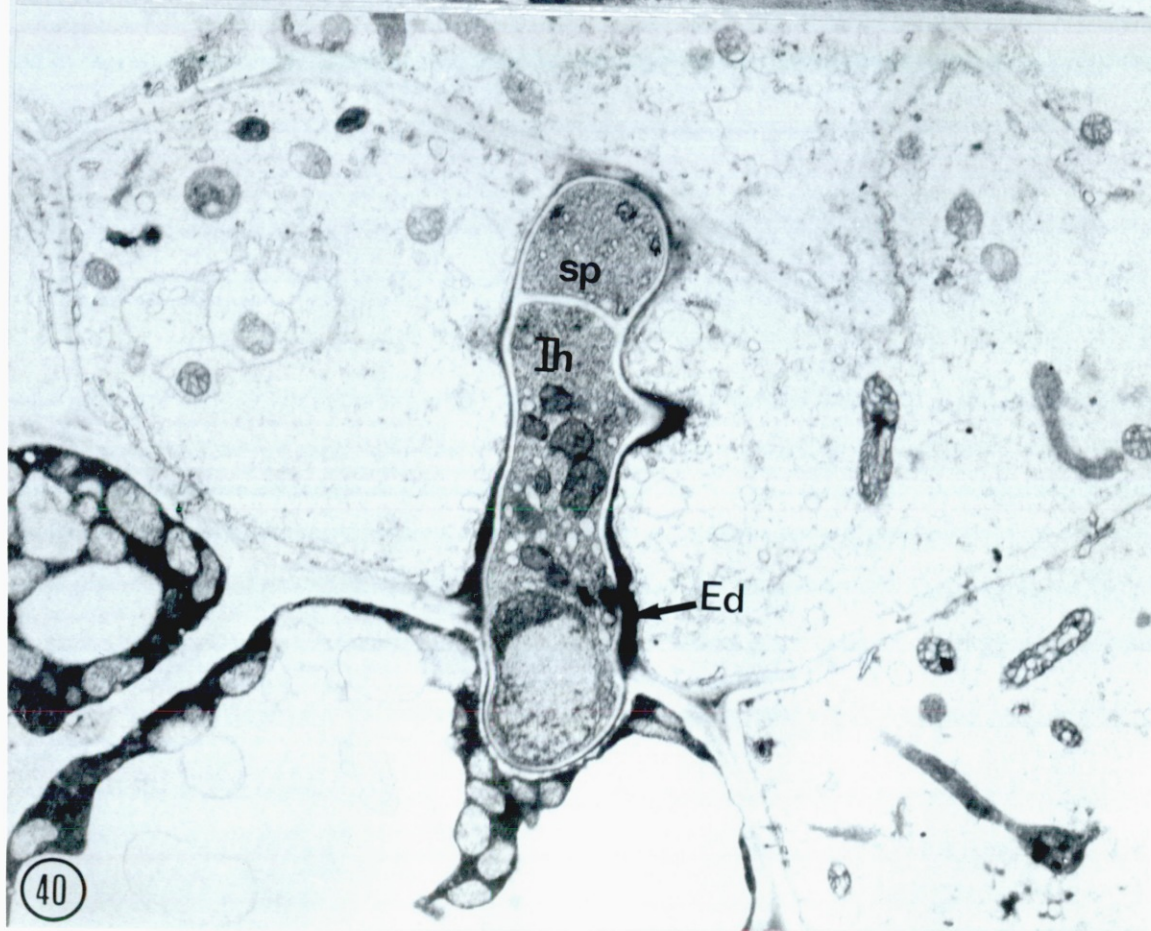
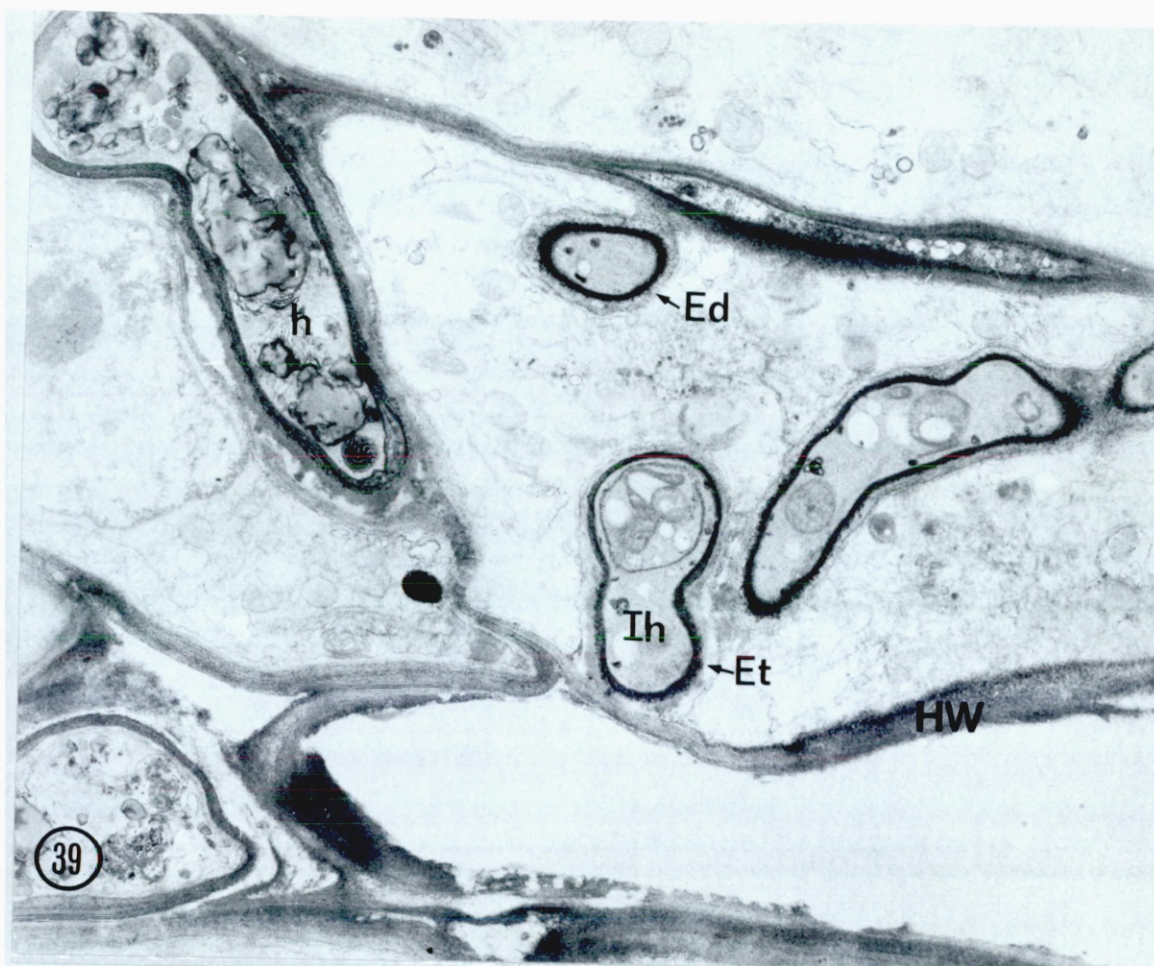


PLATE 4:41

Electron micrograph showing spore initials (si) among the hyphae of an aggregate, (h) within the cavity (C). The whole hyphal aggregate is enveloped in a material which stains intensely with PATCH-SP (Plate 4:42).

Magnification X 2230

Staining: Uranyl acetate and lead citrate

PLATE 4:42

Electron micrograph showing a section of developing teliospores. The spores (s) have thick walls composed of at least two layers, the outer being more electron dense than the inner. Each spore contains a nucleus (Fn) and many lipid bodies (Lb), the nucleus being approximately 1.3 times larger than the nuclei observed in hyphal compartments. The material surrounding both spores and hyphae stains intensely.

Magnification X 6300

Staining: PATCH-SP

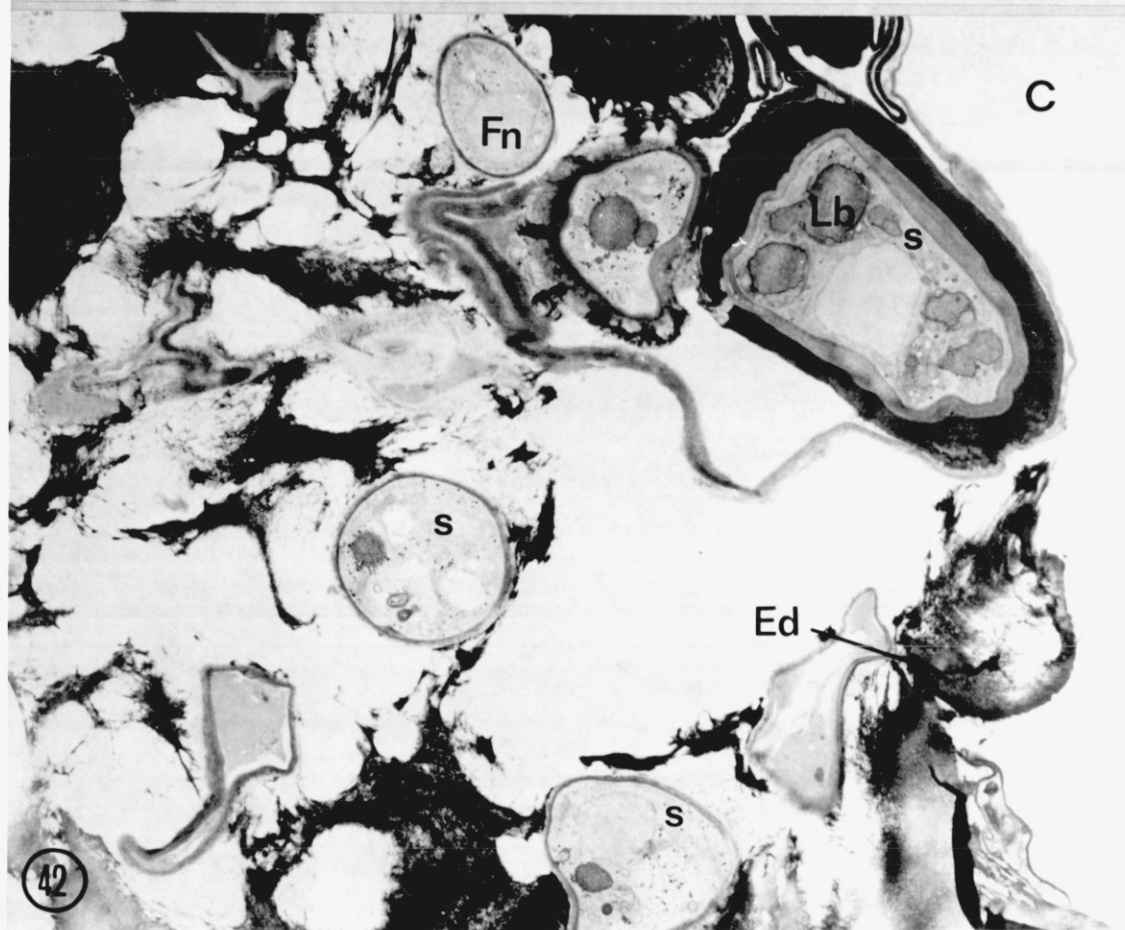
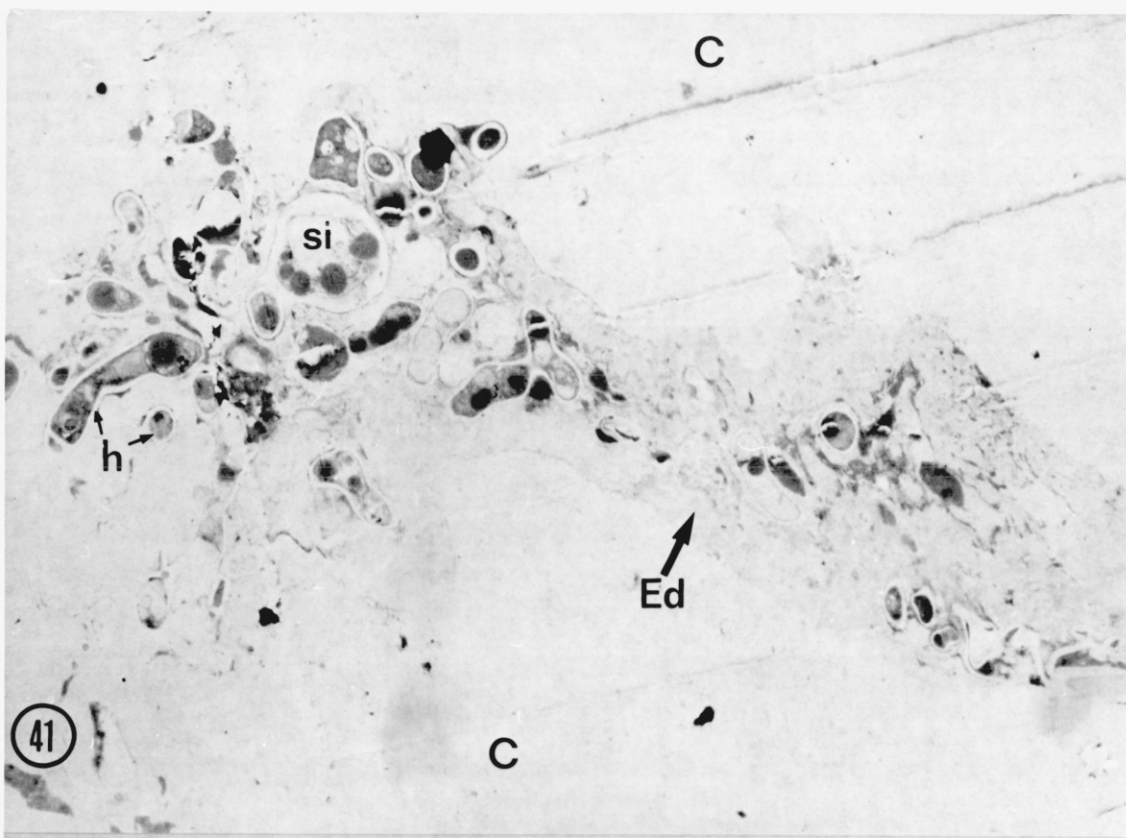


TABLE 4:1

TABLE TO SHOW THE EFFECT OF TEMPERATURE ON THE INFECTION
OF MILLET BY *TOLYPOSPORIUM PENICILLARIAE*.

Temp. at 95-100%		Infection	Seed set	Browning
Humidity		Score	Score	Score
Shaded		9	<1	<1
for 12	21°C	5	0	5
hours		4	4	2
Shaded		8	<1	1
for 12	30-35°C	6	1	3
hours		6	0	4
		7	0	3
In direct	21°C	2	1	7
Sunlight		3	1	6
In direct	30-35°C	<1	0	>9
Sunlight		0	<1	>9
		0	0	10

The score number is taken from the arbitrary scale FIGURE 4:1

Browning is where neither infection or seed set occurs.

TABLE 4:2
TABLE TO SHOW THE EFFECT OF FLOWERING ON THE INFECTION
OF MILLET BY *TOLYPOSPORIUM PENICILLARIAE*.

Time of inoculation	Full protogynae	Half of the stigma emerged	Inflorescence emerging from boot
Score			
Seed set score	4	71	21
Infection score	4	8	10

The score number is taken from the arbitrary scale FIGURE 4:1

FIGURE 4:1

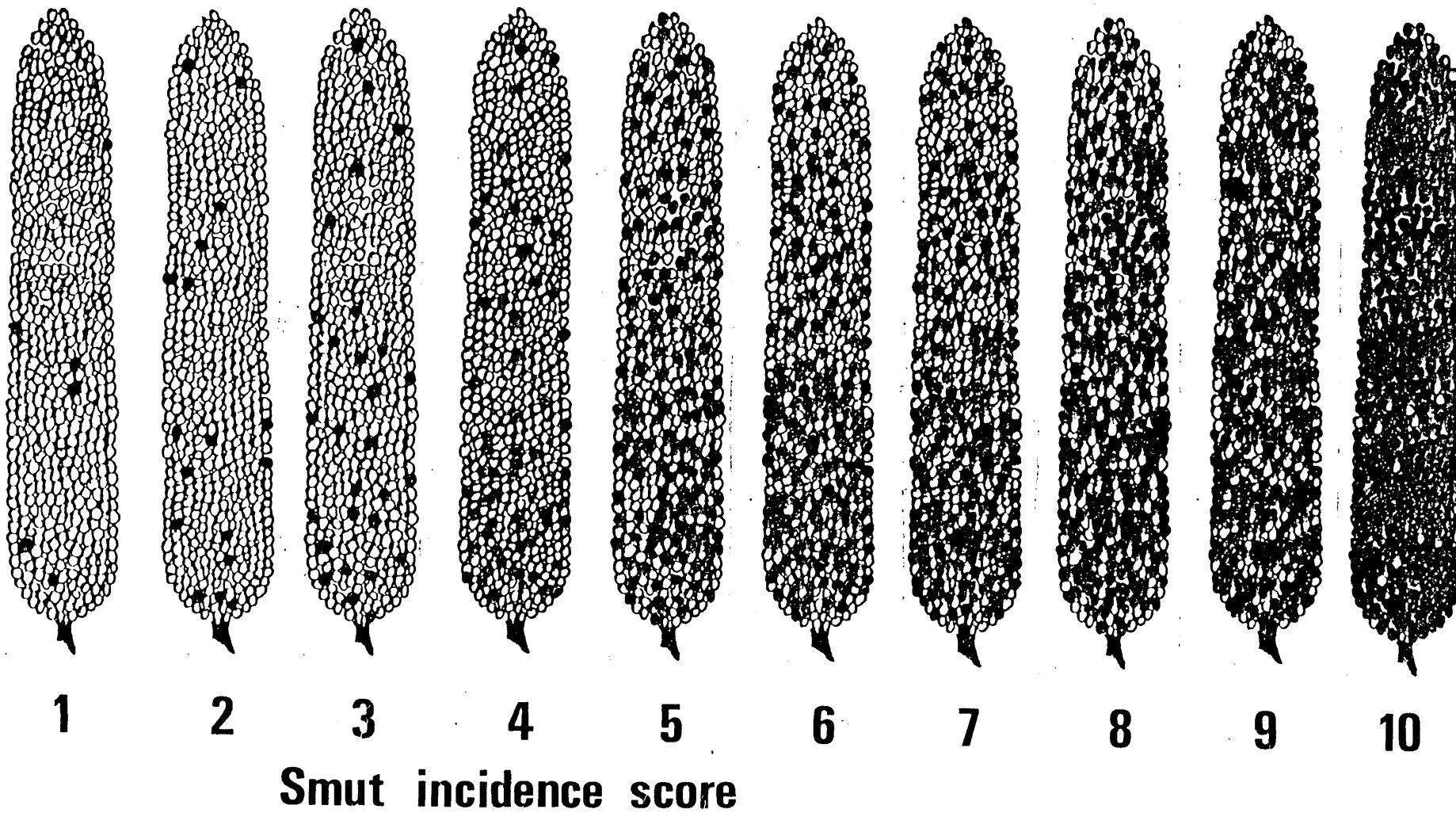
PEARL MILLET SMUT SEVERITY ASSESSMENT KEY

Standard drawings used to aid estimation of smut
severity in pearl millet inflorescences.

Based on the assessment key used by Thakur et al, 1982

KEY : Smutted floret ●

PEARL MILLET SMUT SEVERITY ASSESSMENT KEY



SECTION FIVE

THE COLONISATION OF MILLET BY *CLAVICEPS FUSIFORMIS*.

5.1. INTRODUCTION.

Recent studies have described the early stages of infection of millet by *C. fusiformis* and its significance in future plant breeding programmes for the selection of resistant millet varieties (Willingale et al ,1986). Conidia lying on the stigmatic surface germinate, penetrate and grow down the style into the ovary, mimicking pollen-tubes by taking the same route. On entering the top of the ovary the fungal hyphae continue their pathway through the abaxial wall tissue towards the hilum region where the main vascular tract is located (Willingale and Mantle, 1987a).

The present investigation has concentrated on the subsequent colonisation of the host tissues after infection had been initiated. Particular attention is given to the interface which is established between the developing fungal stroma and the remaining host cells immediately beneath and within those cells of the pedicel.

Samples of infected millet taken 4,7,10 and 15 days post inoculation (d.p.i), see section 2.2.1., were processed and examined by light and transmission electron microscopy (see sections 2.3. and 2.4.).

5.2. RESULTS.

5.2.1. COLONISATION OF THE OVARY.

Four d.p.i. hyphal branches were observed within the abaxial wall of the ovary, penetrating intercellularly towards the base of the ovary. Initial colonisation of the ovule occurred in the region of the micropyle (Willingale and Mantle, 1986) and it continued outward and upward forming a stroma which displaced the embryo-sac and

nucellus into the upper portion of the ovary, still visible in ovaries 7.d.p.i. (PLATE 5:1). By 7 d.p.i. hyphae had become established at the base of the ovary forming a narrow interface between the fungal stroma, which had developed above, and the underlying host cells at the upper end of the pedicel. The ovary was now totally enclosed by hyphae proliferating between the outer epidermal cells of the ovary wall and the lemma and palea of the protective tissues (PLATE 5:1). Further penetration beyond the stromal interface was not apparent unless median sections were taken through the pedicel (PLATE 5:2). Hyphae, termed vascular associated hyphae, grew in a root-like configuration associating with the host vascular tissue within the pedicel. Their darkly stained cytoplasm made them distinct from the surrounding host cells (PLATE 5:2).

By 15 d.p.i. the developing stroma had enlarged 2 to 3-fold in both length and diameter. Host cells were no longer visible within any part of the ovary except in the basal region where the pedicel and a few cells of the ovary wall remained intact (PLATE 5:3). The sphacelial tissue of the stroma at the base of the ovary, termed the generative zone because hyphae form here, matures acropetally (Luttrell, 1980). Just above this zone the sphacelial tissue had differentiated forming compacted hyphae which were larger in diameter, bulbous and contained many lipid bodies within their cytoplasm. This is the sclerotial tissue. Sphacelial tissue in the upper portion of the stroma had formed conidial pockets (PLATES 5:3 and 5:4). Macro and micro-conidia were produced within these pockets and although not evident from the micrographs, examination by phase-contrast light microscopy of honey-dew exuded from the top of the floret confirmed the presence of the two types. Secretion of honey-

dew started approximately 7 d.p.i. and continued for about a further 7 days, the exact period depending upon the enviromental conditions. Vascular associated hyphae were still visible within the core of the pedicel (PLATE 5:5) and by this stage the neighbouring host cells contained large spherical bodies which stained a deep purple or sometimes metachromatically lime-green with toluidine blue. These bodies were not as evident in host cells associated with the vascular tissues or with the vascular associated hyphae within the core (PLATE 5:5).

5.2.2. CYTOLOGY OF THE HOST AND FUNGUS WITHIN THE PEDICEL.

Fungal hyphae within the pedicel were only associated with the phloem sieve tubes, xylem tracheids and their adjacent parenchyma cells (PLATE 5:6). These hyphae were predominantly intracellular and although in thin-section there appeared to be many hyphal segments within each host cell very few penetration sites were observed (PLATE 5:7). PLATE 5:8 shows how the segments arose by multiple branching.

Hyphae grew between the host cells pushing them apart. The middle lamella often showed signs of degradation around the hypha (PLATE 5:9) and sometimes the wall was distorted in a manner suggesting that penetration may follow (PLATE 5:10).

Where intercellular hyphae penetrated host parenchyma cells associated with the vascular tissue of the pedicel, the penetrating hypha was surrounded by both electron opaque and electron transparent layers between the hyphal wall and the host's invaginated plasma membrane (PLATES 5:12 and 5:13). The layer adjacent to the fungal wall was electron opaque and stained even more intensely with both PATCH-SP and PACP cytochemical stains than in uranyl acetate and lead

citrate stained preparations. This layer was similar to the matrical material between adjacent walls of the host and to the host's cell wall in density though more loose and open in structure (PLATES 5:12, 5:14 and 5:11). The overlying electron transparent layer appeared as a tapering collar and was similar in texture to papillae, collars and encasements associated with fungal haustoria (Aist, 1976) (PLATES 5:11 and 5:12). The material of the displaced host wall referred to above and shown in PLATE 5:9 also resembled that of the collar. Unlike the distinct extrahaustorial membrane surrounding haustoria the invaginated host plasma-membrane surrounding the penetrating hypha was indistinguishable in preparations stained with uranyl and lead salts, PATCH-SP or PACP from that surrounding the remaining host cytoplasm and in uninfected host cells (PLATES 5:12, 5:14 and 5:16). The PACP stain was variable sometimes giving no reaction around intracellular hyphae (PLATE 5:15). Once penetrated the hyphae colonised the periphery of the host cell (PLATE 5:13) before filling the cell so that the vacuolar volume was almost completely eliminated (PLATE 5:7). Some penetrated cells showed signs of disruption but the plasma membrane and nucleus remained intact (PLATE 5:14). Beyond the region of host wall penetration the walls of intracellular hyphae were only surrounded by the fibrillar electron opaque layer and an undifferentiated invaginated host plasma membrane (PLATES 5:13 and 5:16). Intracellular hyphae which penetrated neighbouring host cells were not associated with additional wall layers other than the opaque fibrillar layer (PLATE 5:14).

The cytoplasm of the vascular associated hyphae was less dense and contained fewer lipid bodies than those within the generative zone, the developing sclerotial tissue and the remaining sphacelium.

The dikaryotic nature of this *Claviceps* species was not as evident as in *C. purpurea* on rye (Luttrell, 1980) or wheat (Shaw, 1980) but a few vascular associated hyphal compartments were observed to contain two nuclei (PLATE 5:14). The nuclear status of the hyphal tissues above the pedicel region was not examined. Woronin bodies were visible within hyphae near septa (PLATES 5:11 and 5:12). Intracellular hyphae penetrating into a neighbouring cell were observed to possess a septa and to be slightly constricted at the junction between the two host cells (PLATES 5:8 and 5:14).

All remaining host cells within the pedicel which were not invaded by vascular associated hyphae or directly associated with phloem sieve-tubes or xylem vessels contained spherical, osmiophilic bodies (PLATES 5:3, 5:5 and 5:6). These bodies were contained in vesicles (PLATE 5:17) and accumulated within the host vacuole (PLATE 5:18). Eventually they coalesced forming one large mass with a dense outer region and bubble-like contents (PLATES 5:19 and 5:20).

Phloem sieve tubes at the top of the pedicel and the remaining pericarp fluoresced brightly when thick sections stained with aniline blue (see section 2.5) were examined by fluorescence microscopy (PLATES 5:21 and 5:22). No fluorescence was observed in association with the intracellular vascular associated hyphae (PLATE 5:21). The osmiophilic bodies within the host cells were not autofluorescent (PLATE 5:21) and phloem sieve tubes further into the pedicel did not fluoresce.

Localisation of ATPase activity within the fungal stroma and the host pedicel proved to be unsuccessful (see section 2.3.3.). Although lead was deposited within the tissue the intensity and location was related more to the penetration of the substrate medium

than could be ascribed to ATPase activity at these sites . No ATPase or general phosphatase activity was observed within the compacted hyphae of the sclerotial tissue or at the interface (PLATE 5:24). Further into the sphacelial tissue a general phosphatase activity was located around the hyphae within the honey-dew (PLATE 5:23). No precipitates were observed within the core of the pedicel tissue (PLATES 5:25 and 5:26) though heavy precipitates were located between the cells in the outer few layers of the pedicel. Lead precipitates were only found within cells when they had been broken open and their contents disrupted.

PLATE 5:1

A light micrograph showing a longitudinal section through a millet ovary 7 days after infection with *C. fusiformis*. The nucellus (N) is displaced into the upper part of the ovary. Hyphae (h) have proliferated between the ovary wall (OW) and the protective tissues (Pt). A stromal interface (I) is formed between the fungal stroma (St) in contact with the host cells in the upper portion of the pedicel (Pd).

Magnification X 300

Staining: Toluidine blue

①

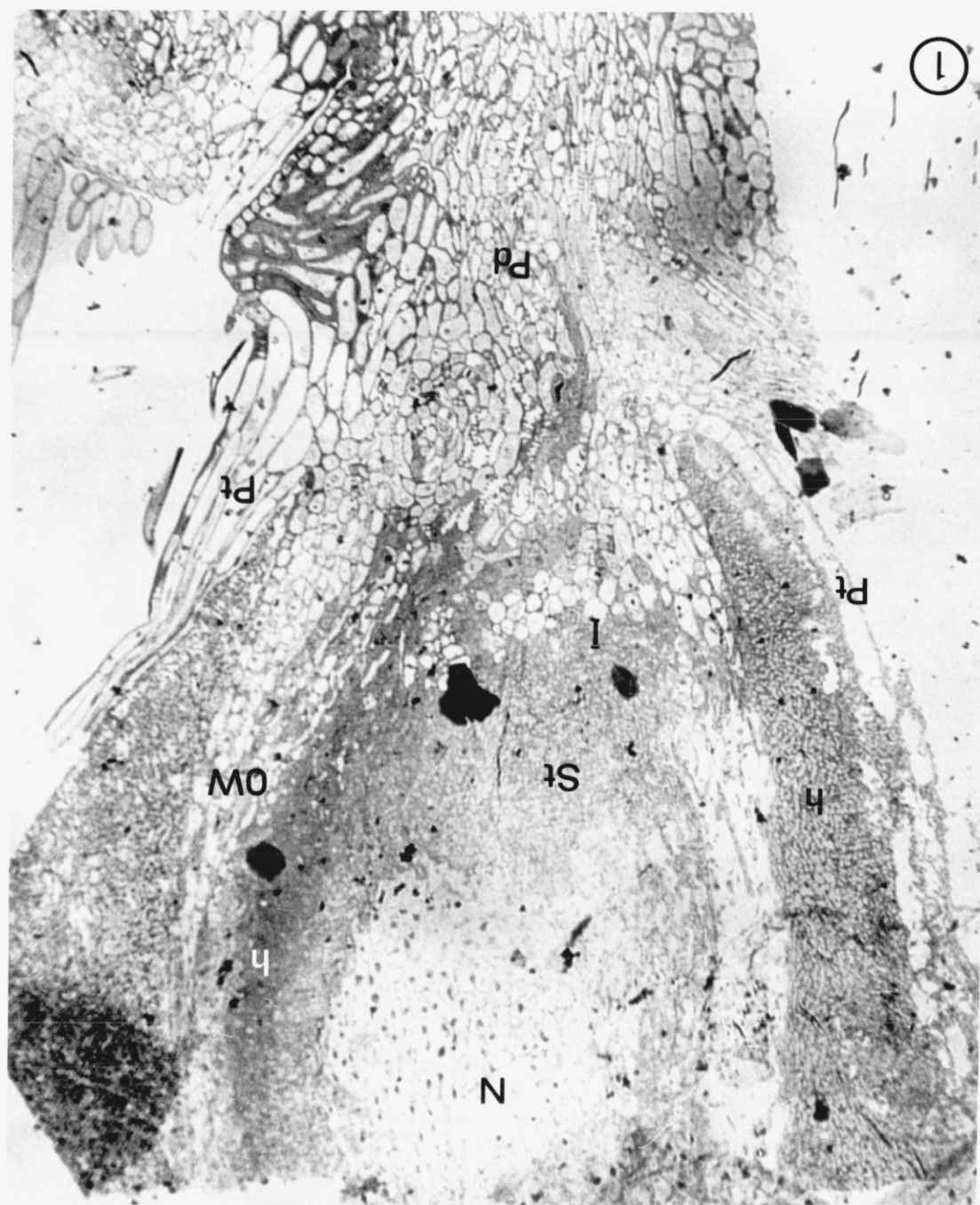


PLATE 5:2

A higher magnification from Plate 5:1 showing the region within the pedicel just below the stromal interface (I). The penetration of hyphae (h) beyond the stromal interface (I) is observed in this median section. These hyphae, termed vascular associated hyphae (Vh), penetrate between and within living cells associated with xylem tracheids (Xy) and phloem sieve tubes (Ph).

Magnification X 1450

Staining: Toluidine blue

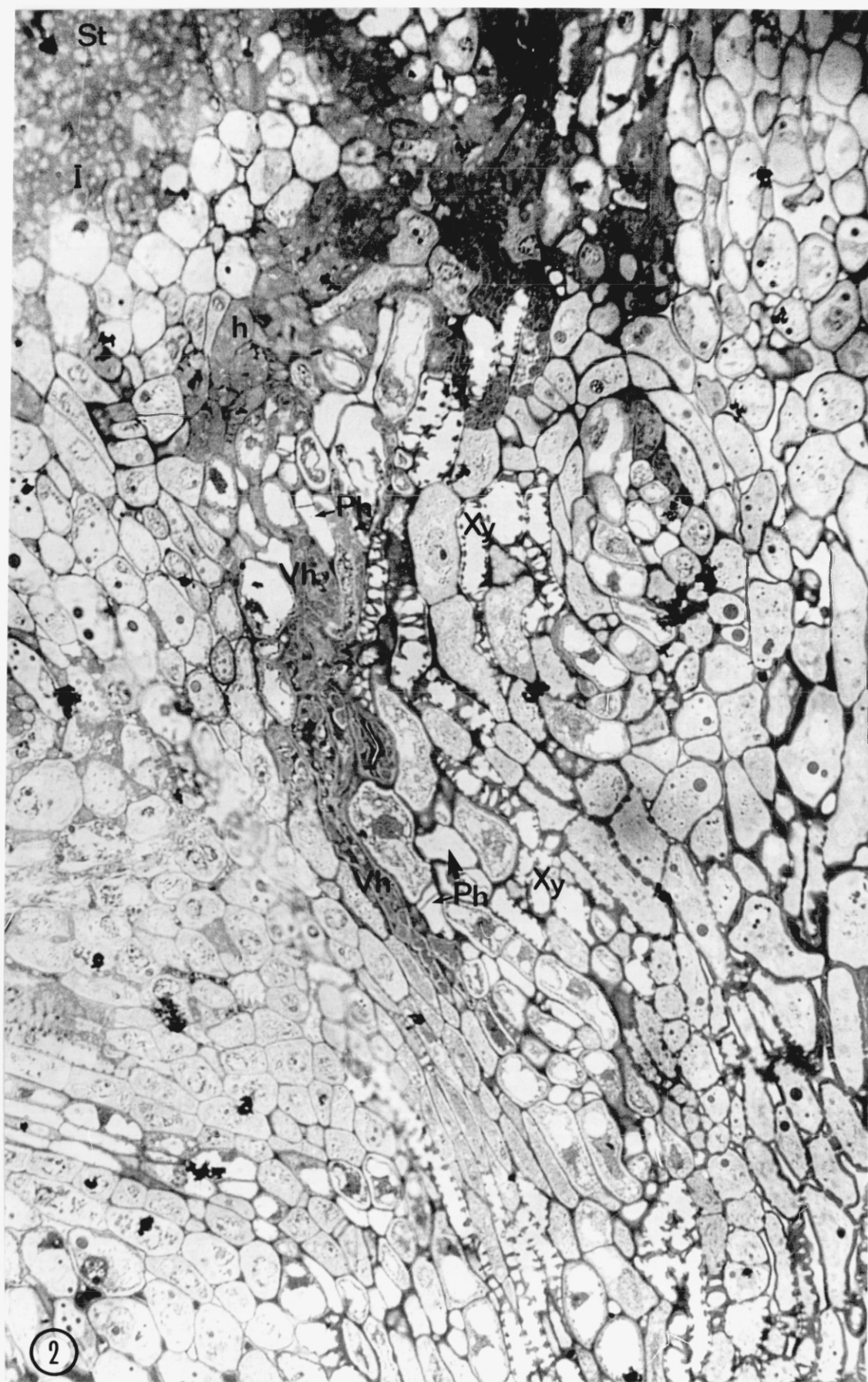


PLATE 5:3

A montage of light micrographs showing a longitudinal section through a millet ovary 15 days after infection . The fungal stroma, initially sphacelial tissue (Sp), has begun to differentiate just above the stromal interface (I) to form the sclerotial tissue (Sc). Conidial pockets (Co) occupy the upper region of the sphacelium. All host cells, except for those of the pedicel (Pd) below the stromal interface (I), have been replaced by the developing fungus.

Magnification X 195

Staining: Toluidine blue



PLATE 5:4

Light micrograph showing a conidial pocket (Co) from Plate 5:3. Macro- and micro-conidia are produced from cells making up the wall of the pocket in the sphacelial hyphae (Sp).

Magnification X 980

Staining: Toluidine blue

PLATE 5:5

Light micrograph showing the pedicel region in Plate 5:3. Vascular associated hyphae (Vh) are visible near xylem tracheids (Xy) and phloem sieve tubes (Ph). Host cells surrounding the core of vascular tissues in the pedicel contain darkly staining, sometimes metachromatically green staining, bodies (b).

Magnification X 385

Staining: Toluidine blue

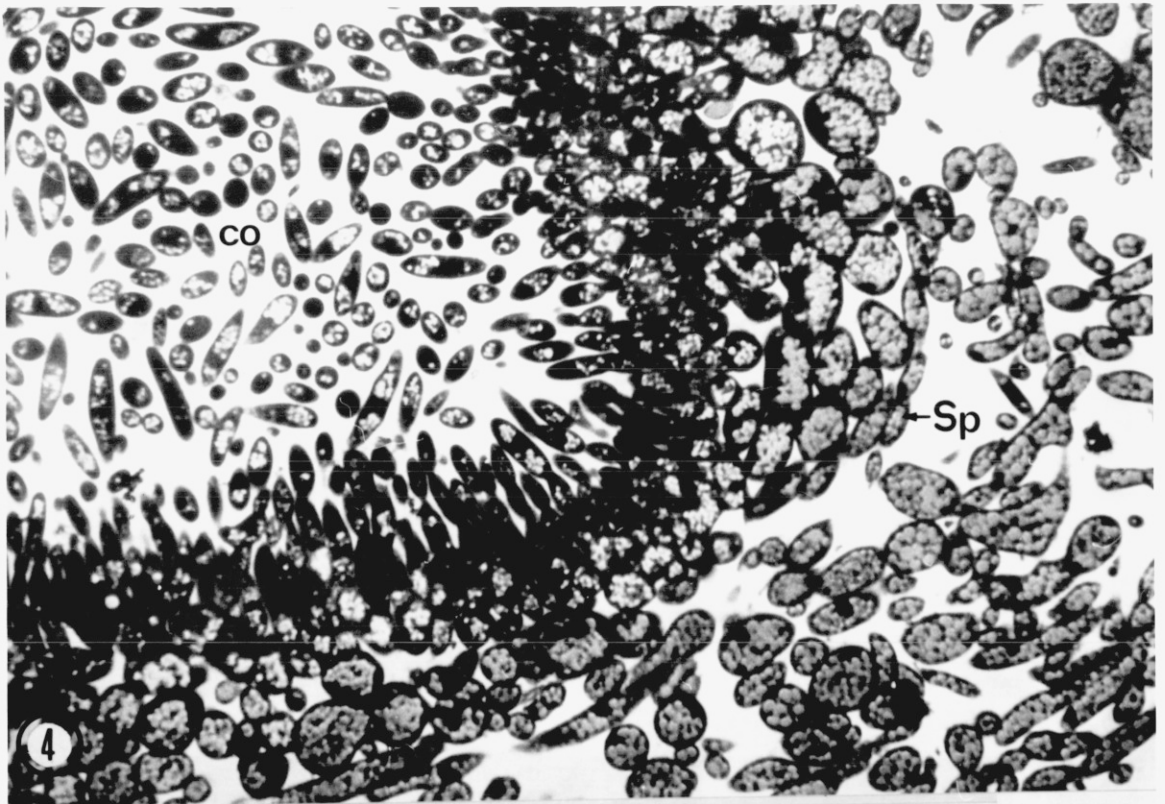


PLATE 5:6

Electron micrographs showing the penetration of hyphae (Vh) beyond the stromal interface (I) into the pedicel (Pd) of millet 10 days post inoculation. These hyphae occupy both intercellular (Vh) and intracellular (VIh) positions adjacent to phloem sieve tubes (Ph) and xylem tracheids (Xy). Osmiophilic bodies (b) are observed in cells surrounding the core of vascular tissues.

Magnification X 700

Staining: Uranyl acetate and lead citrate



PLATES 5:7 and 5:8

Electron micrographs of the pedicel of an infected millet
ovary 10 days post inoculation.

PLATE 5:7

In the pedicel immediately below the stromal
interface most of the host cells are infected and
completely filled with fungal hyphae (Ih).

Magnification X 3220

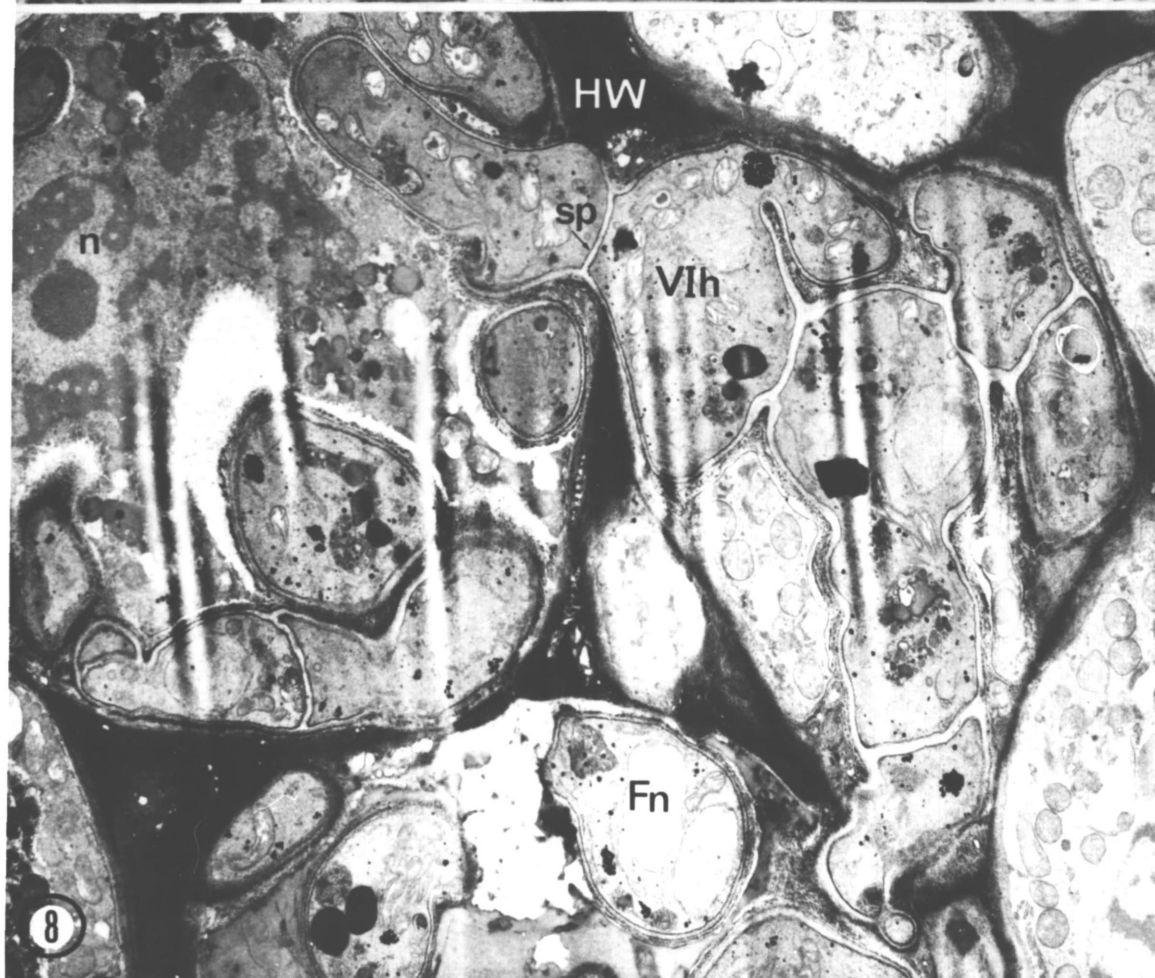
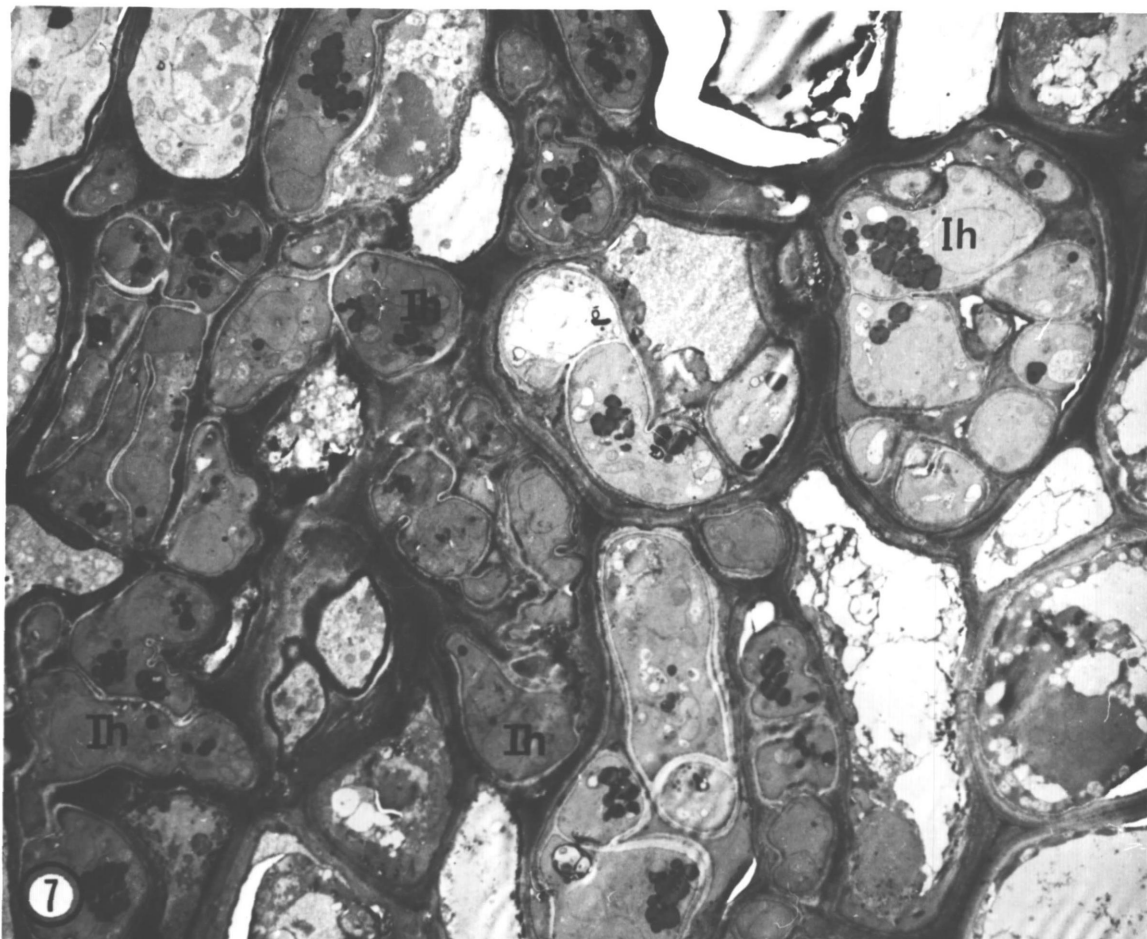
Staining:Uranyl acetate and lead citrate

PLATE 5:8

The hyphal segments arise from multiple branching
of the intracellular hypha (VIh). A cell-to-cell
penetration is visible and a septum (sp) occurs at
the point of entry.

Magnification X 8100

Staining:Uranyl acetate and lead citrate



PLATES 5:9 and 5:10

Electron micrographs taken from a lower region in the pedicel of an infected millet ovary 7 days post inoculation.

PLATE 5:9

Intercellular hyphae (Vh) separate the walls of neighbouring host cells. The middle lamella (ml) often shows signs of degradation.

Magnification X 18000

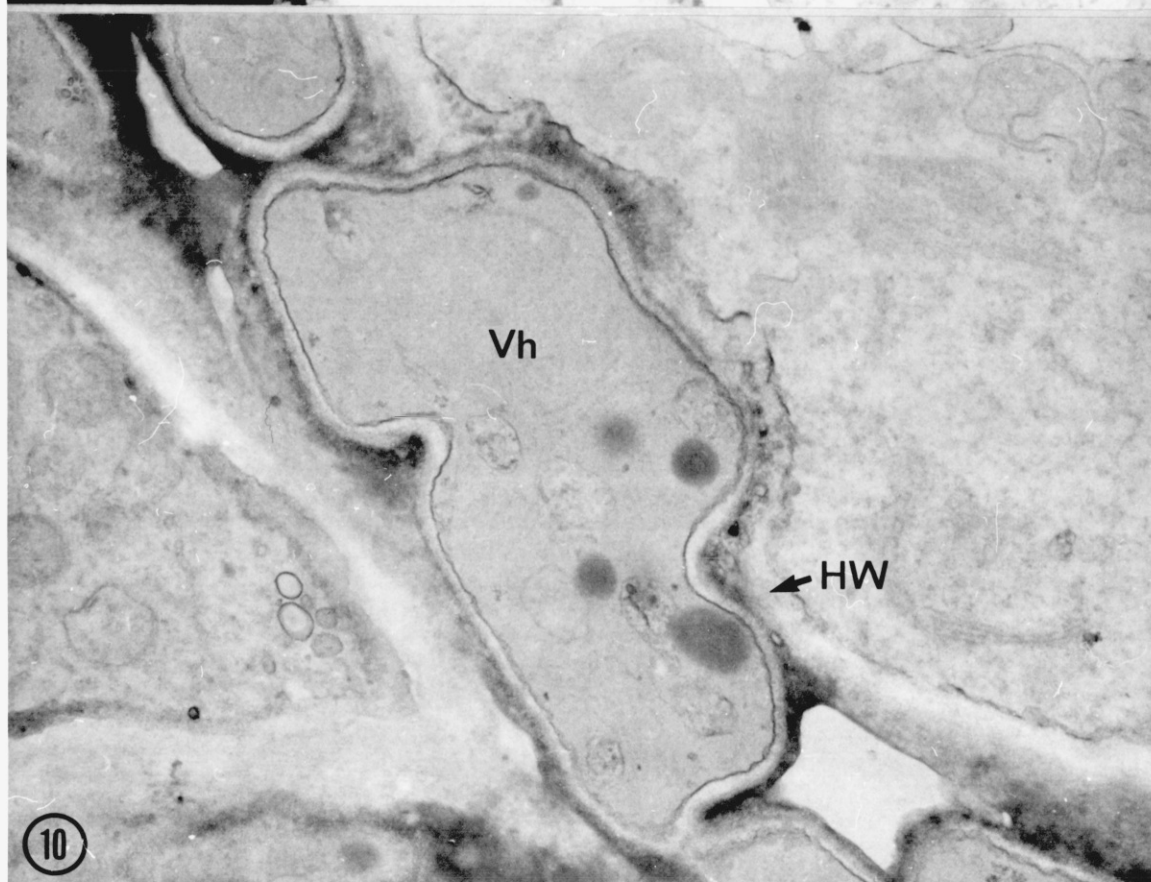
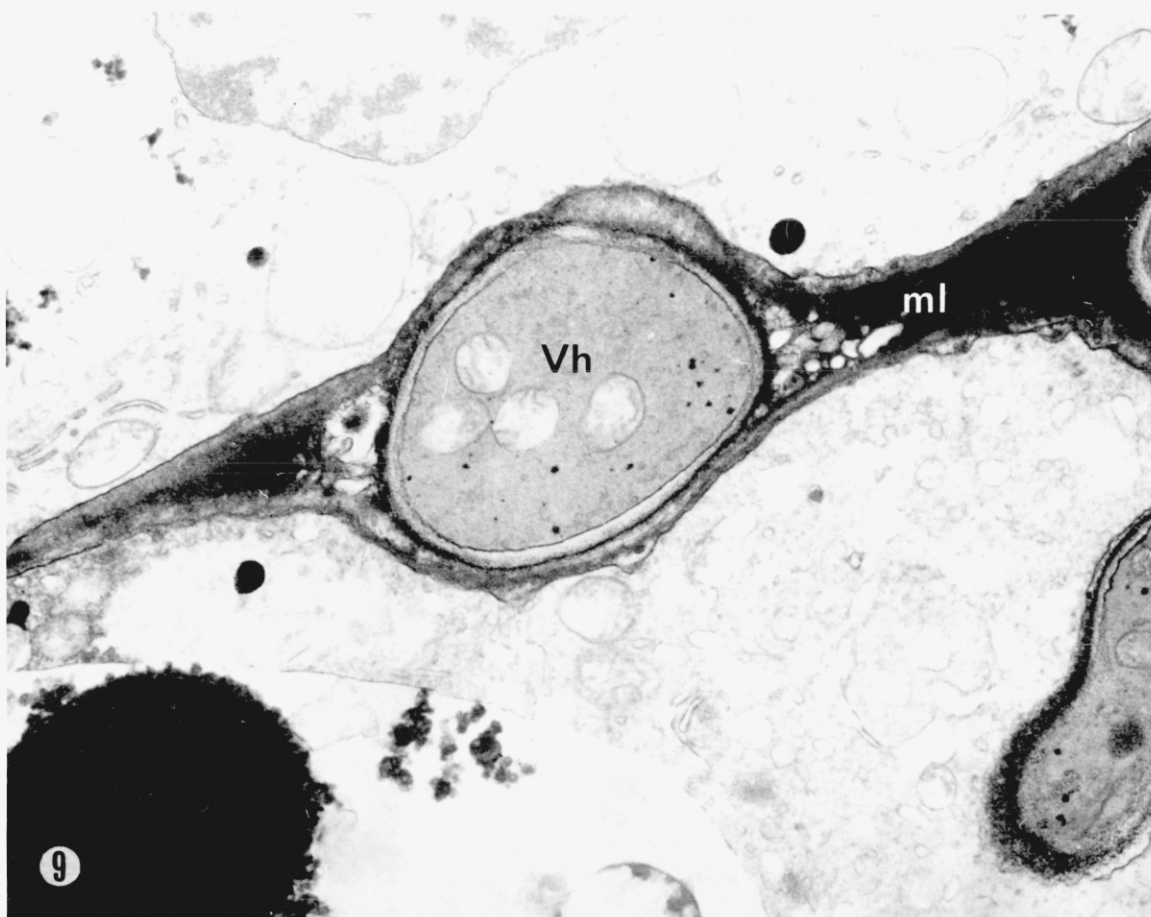
Staining: Uranyl acetate and lead citrate

PLATE 5:10

Intercellular hyphae (Vh) displace the middle lamella (ml) and host wall to an extent suggesting that degradation is occurring and that penetration of that cell may follow.

Magnification X 16500

Staining: PACP



PLATES 5:11 and 5:12

Electron micrographs showing serial sections, which are stained differently, taken near the top of the pedicel of an infected millet ovary 7 days post inoculation.

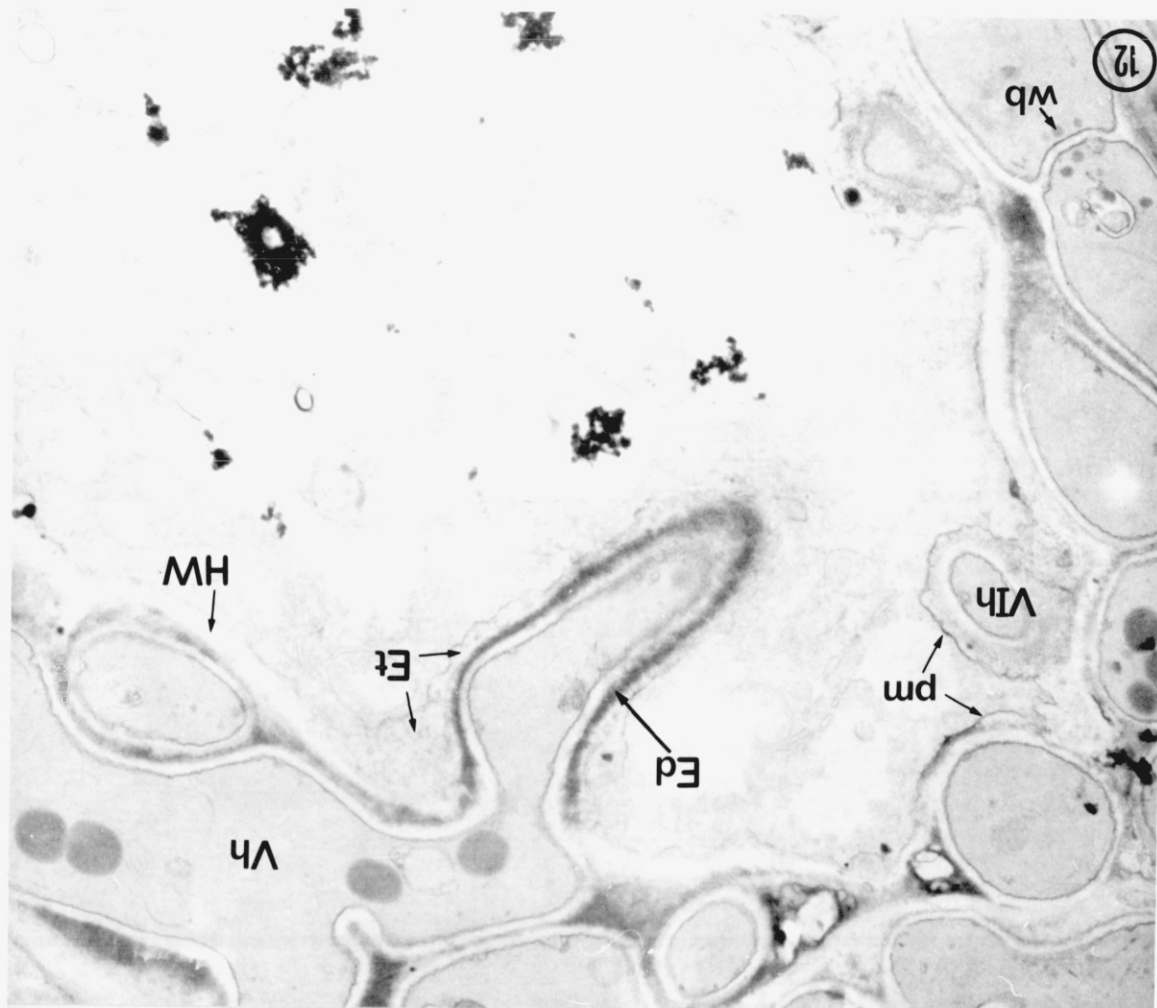
The intercellular hypha (Vh) is constricted slightly as it penetrates the host cell wall. This penetration is associated with the formation of both electron dense material (Ed) adjacent to the fungal wall and an electron transparent material (Et) adjacent to the invaginated host plasma membrane. The deposition site and texture of the electron transparent material (Et) is similar to papillae associated with penetrations by haustoria. The electron dense material (Ed) stains more intensely than the host cell wall (HW) in both staining preparative procedures and is similar to the material between the host cells and surrounding the intercellular hyphae (Vh). The plasma membrane surrounding the penetrating hypha stains intensely, as does the rest of the host plasma membrane, when stained with either the PACP (PLATE 5:11) or the PATCH-SP (PLATE 5:12) procedures. Woronin bodies (Wb) are observed near septa (sp) in some fungal hyphae.

PLATE 5:11 Magnification X 10200

 Staining: PATCH-SP

PLATE 5:12 Magnification X 10200

 Staining: PACP



PLATES 5:13 and 5:14

Electron micrographs showing hyphae further into the pedicel of an infected millet ovary 10 d.p.i..

PLATE 5:13

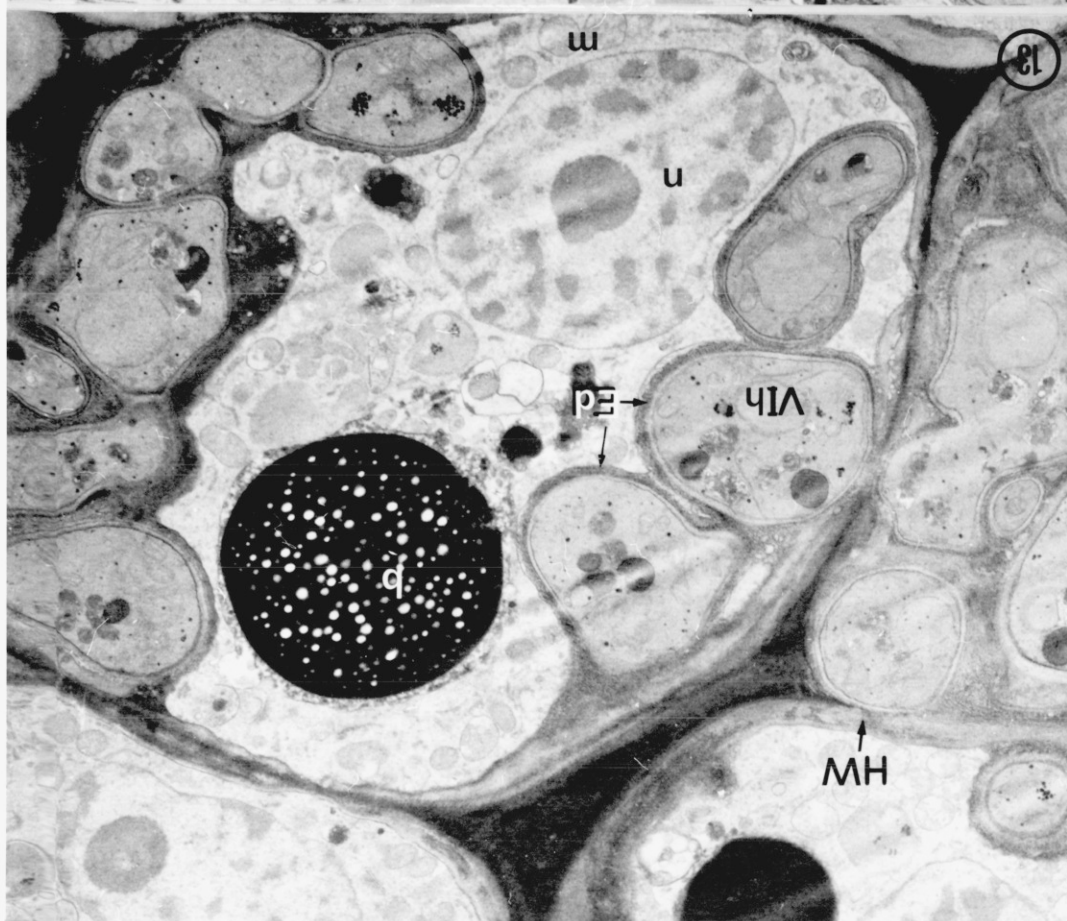
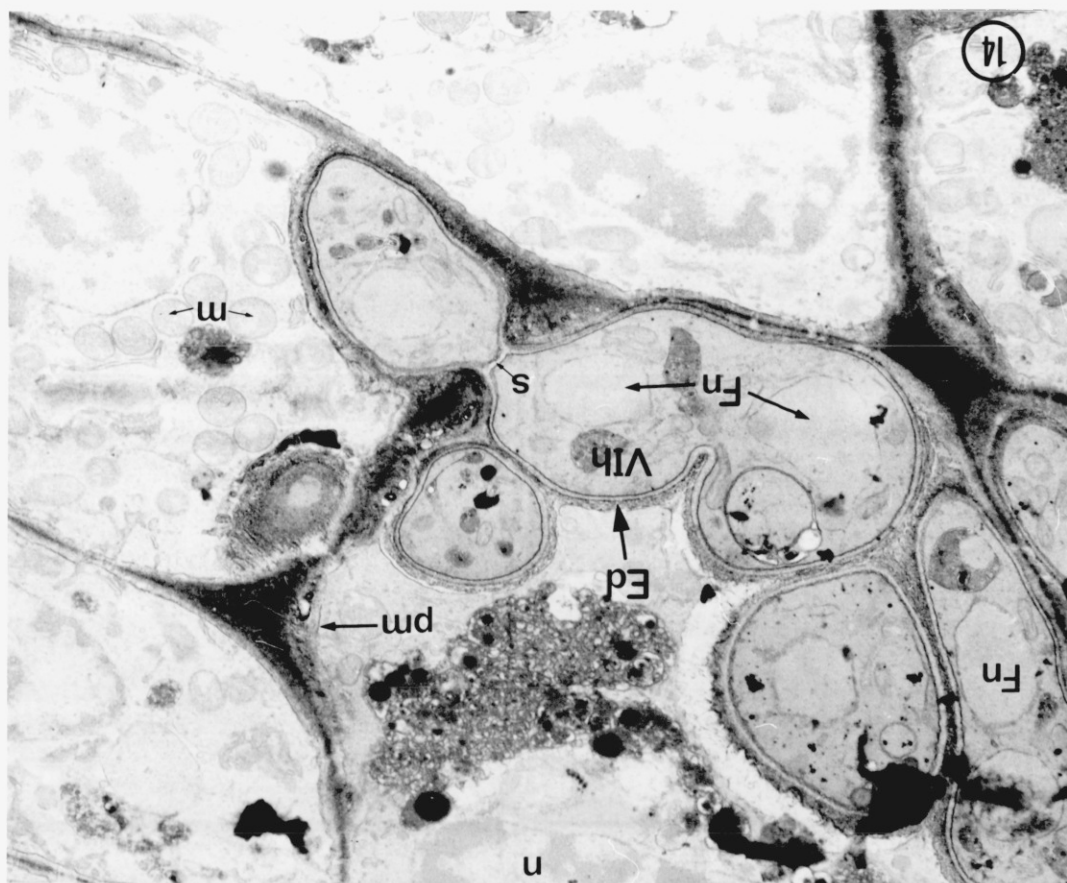
Within the host cell the intracellular hypha (VIh) first colonises the periphery of the host cell. These hyphal segments are only surrounded by the electron dense material (Ed) which stains similarly to the host cell wall (HW), though it is looser in structure and has a fibrillar texture. Host organelles, nucleus (n), mitochondria (m) and vesicles (ve) remain visible and in some cells large osmiophilic bodies (b) fill the vacuoles. There are no signs of the disorganisation of membranes or organelles.

Magnification X 7150 Staining: PATCH-SP

PLATE 5:14

A septum(sp) forms where the intracellular hypha (VIh) is constricted at the penetration site. Penetration of the new cell is only associated with the fibrillar electron dense material (Ed) and there is no sign of the electron transparent material. The cell from which the fungus is leaving is showing signs of disruption. Two fungal nuclei (Fn) are observed in some hyphal segments. Nucleus (n), mitochondria (m), plasma membrane (pm).

Magnification X 7900 Staining: PATCH-SP



PLATES 5:15 and 5:16

Electron micrographs taken from the pedicel region, near the lowest extent of fungal penetration, From an infected millet ovary 10 days post inoculation, both are stained with PACP.

PLATE 5:15

Intracellular hyphae (VIh) in cells adjacent to phloem sieve tubes (Ph). Sometimes the cytochemical stain, specific for plasma membranes (PACP), did not give a positive reaction with the invaginated membrane surrounding the intracellular hyphae and with other parts of the host plasma membrane.

Magnification X 7800

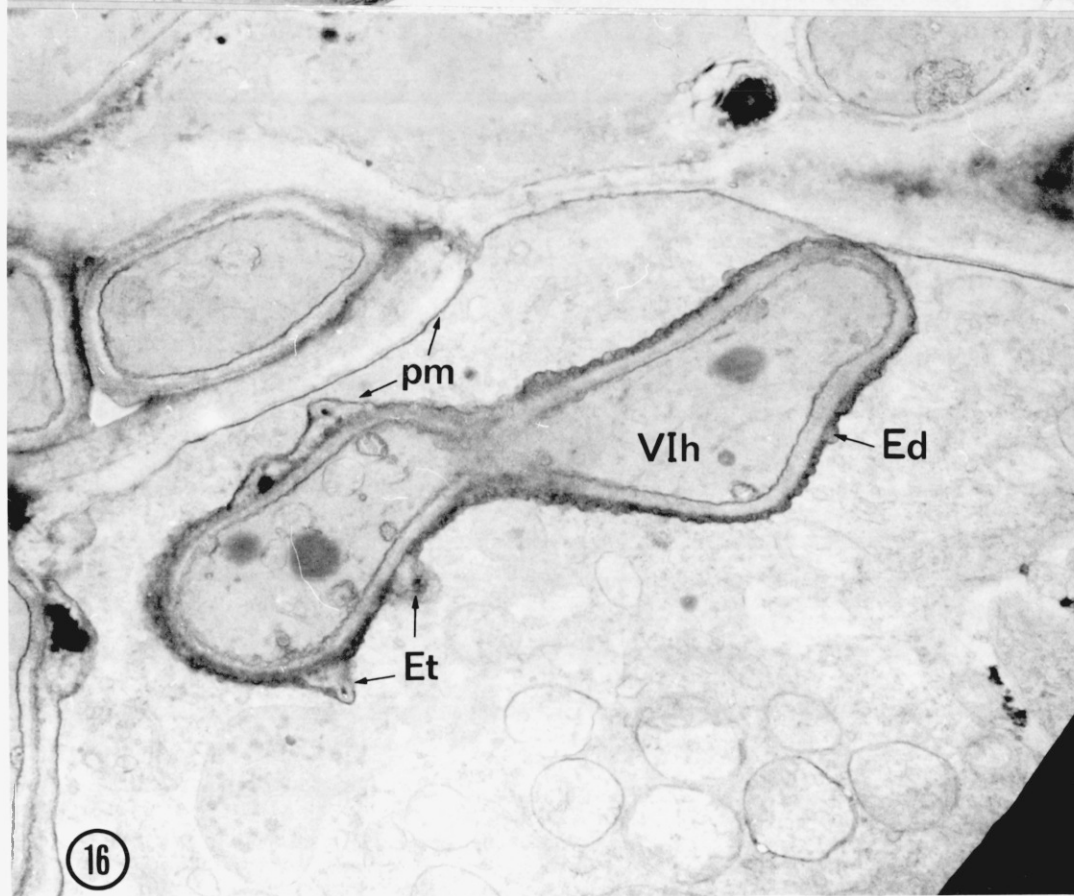
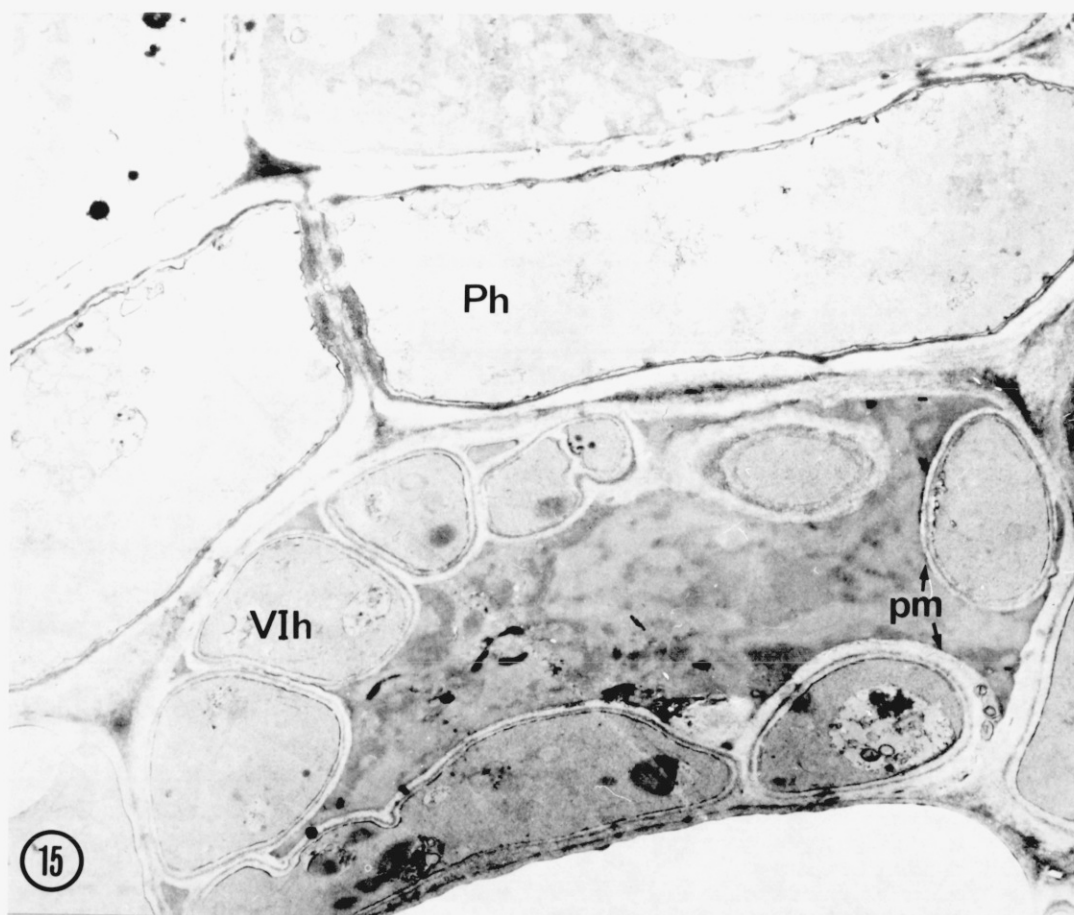
Staining: PACP

PLATE 5:16

The intracellular hypha (VIh) is surrounded by the electron dense material (Ed). The texture of the projections of electron transparent material (Et) suggest that part of the hypha is surrounded by collar-like material. In this section the invaginated host plasma membrane surrounding the hypha stains intensely (pm) as does the membrane surrounding the rest of the host cell and neighbouring cells.

Magnification X 16500

Staining: PACP



PLATES 5:17 and 5:18

Electron micrographs showing details of cells within the pedicel of an infected millet ovary 10 days post inoculation.

PLATE 5:17

Osmiophilic bodies (b) in the cells surrounding the core of vascular tissues in the pedicel region (See Plates 5:3, 5:5 and 5:6 for their distribution). They are contained within a single membrane which is probably formed from the endoplasmic reticulum (ER).

Magnification X 29550

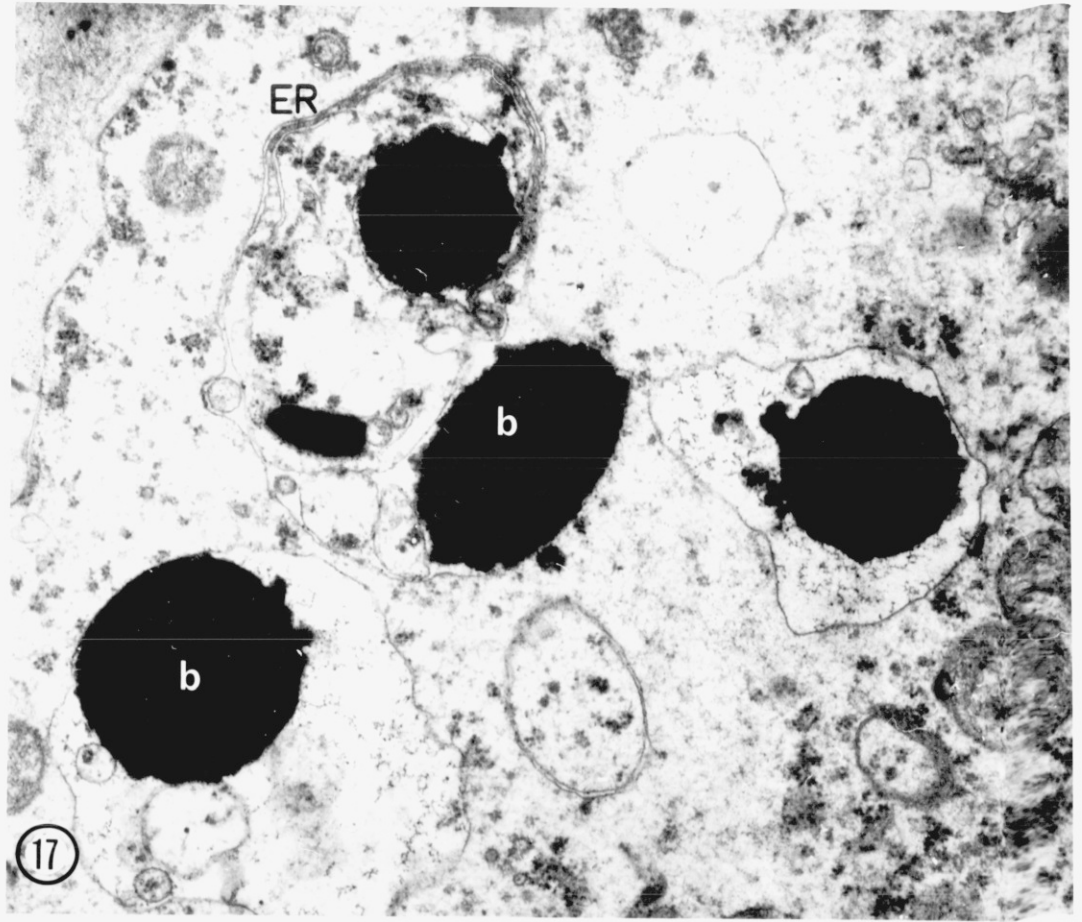
Staining: Uranyl acetate and lead citrate

PLATE 5:18

The osmiophilic bodies (b) have accumulated in the host vacuole (v). These bodies have an intensely dark outer layer surrounding the fragmented central region.

Magnification X 6530

Staining: PACP



PLATES 5:19 and 5:20

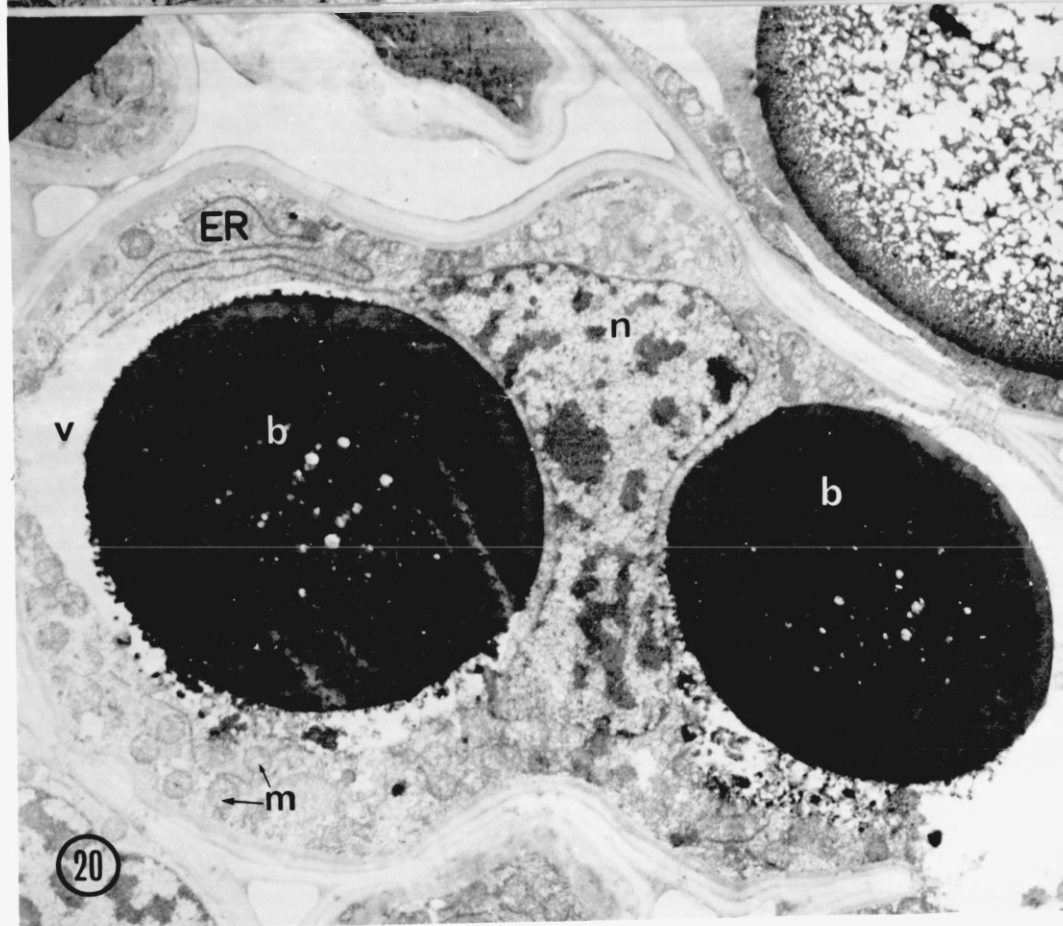
Electron micrographs showing details of cells within the pedicel of infected millet.

The osmiophilic bodies (b) are much larger than in previous micrographs and have probably coalesced to form one large mass within the host vacuole (v). Some intercellular hyphae (Vh) are observed in this tissue surrounding the core of vascular tissues.

Host vacuole(v), nucleus(n), mitochondria(m),
endoplasmic reticulum (ER)

PLATE 5:19 Magnification X 4250
Staining:Uranyl acetate and lead citrate

PLATE 5:20 Magnification X 1230
Staining:Uranyl acetate and lead citrate



PLATES 5:21 and 5:22

Light micrographs of thick sections stained with
aniline blue and viewed with UV light.

PLATE 5:21

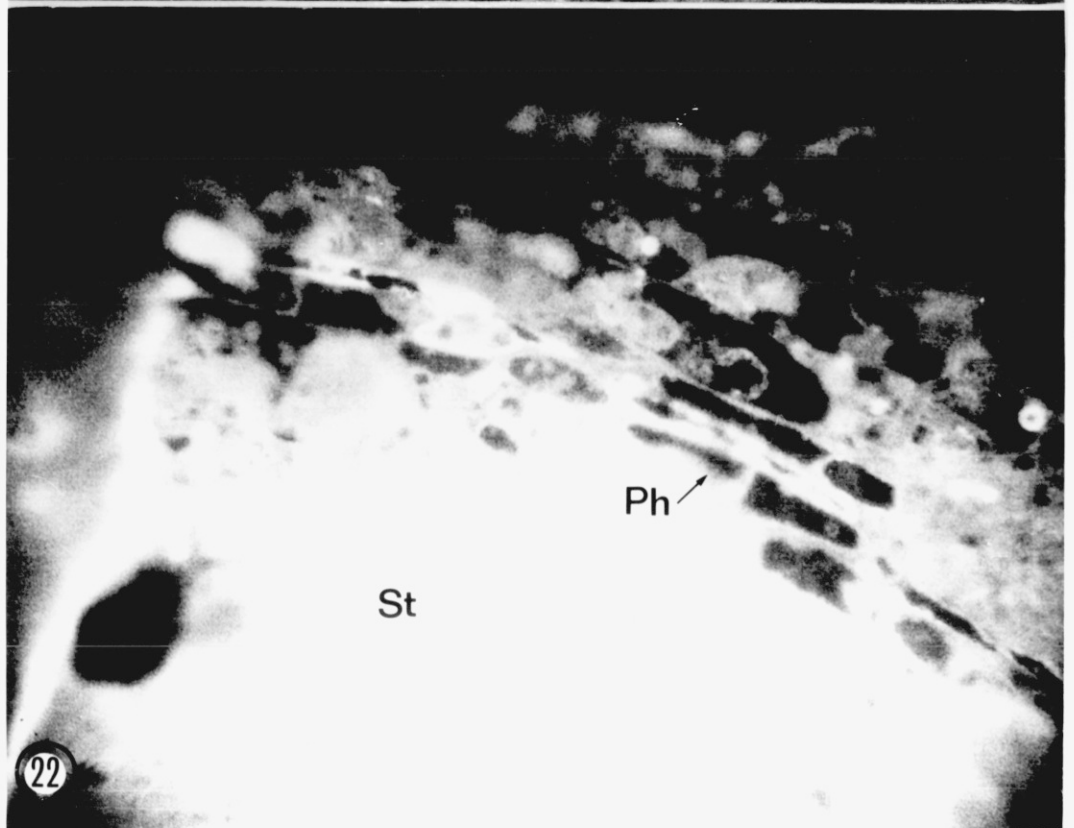
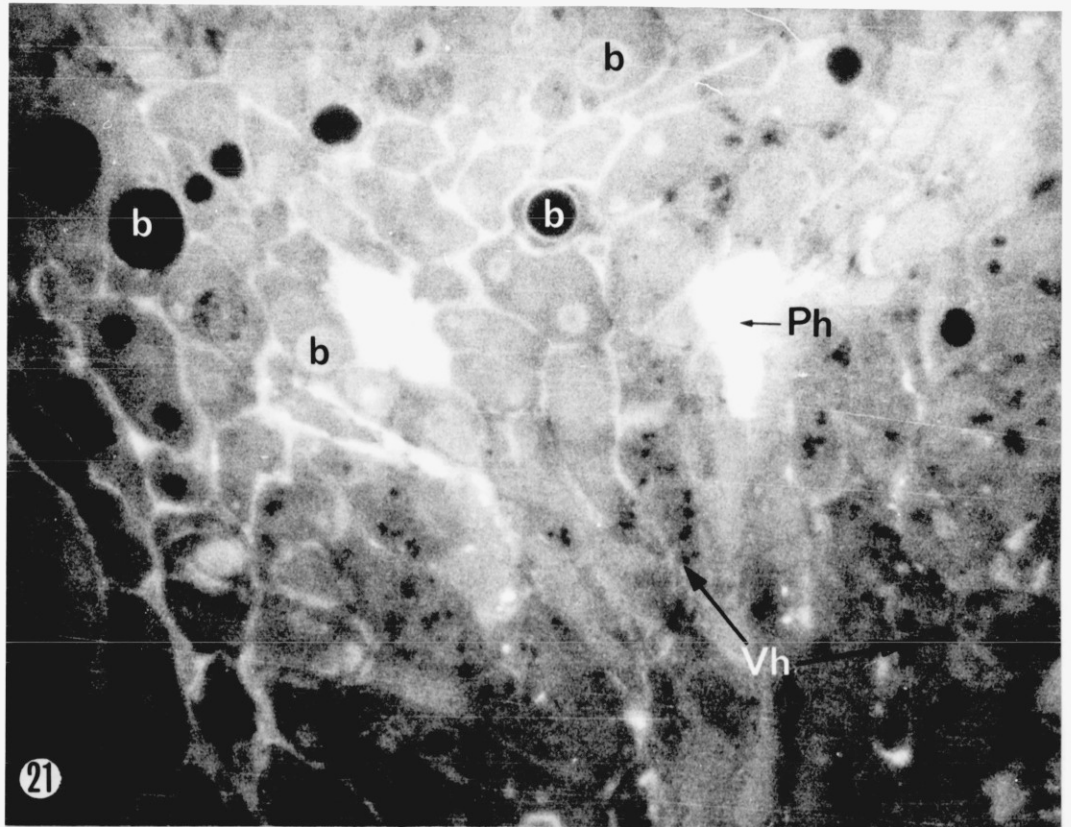
Section from the pedicel of an infected millet
ovary 10 days post inoculation. The only parts
which fluoresce brightly are some phloem sieve
tubes (Ph) at the top of the pedicel. No aniline
blue fluorescence is associated with the vascular
associated hyphae (Vh). The osmiophilic bodies
(b) are not fluorescent (nor autofluorescent)
though their degrees of osmiophilic staining
varied.

Magnification X 1230

PLATE 5:22

Section above the stromal interface where only
small patches of remaining phloem sieve tubes in
the ovary wall fluoresce when stained with aniline
blue. The brightness of the fungal stroma (St) is
due to folding of the section in the staining
medium.

Magnification X 1230



PLATES 5:23 and 5:24

Electron micrographs taken from infected millet ovaries 10 day post inoculation prepared for the localization of phosphatase activity.

PLATE 5:23

Lead deposits are localized within a material surrounding the hyphae (h) of the sphacelial tissue and in the hyphal cytoplasm but are absent from the lipid droplets. This reaction is due to general phosphatase activity.

Magnification X 5300

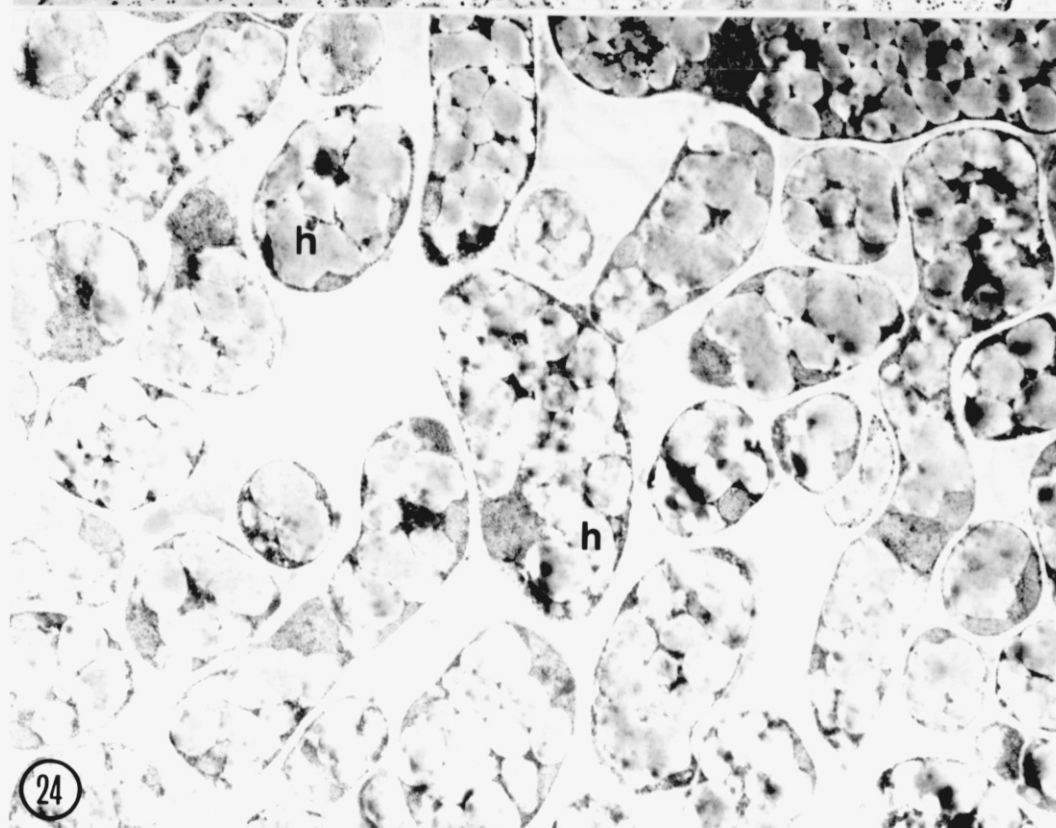
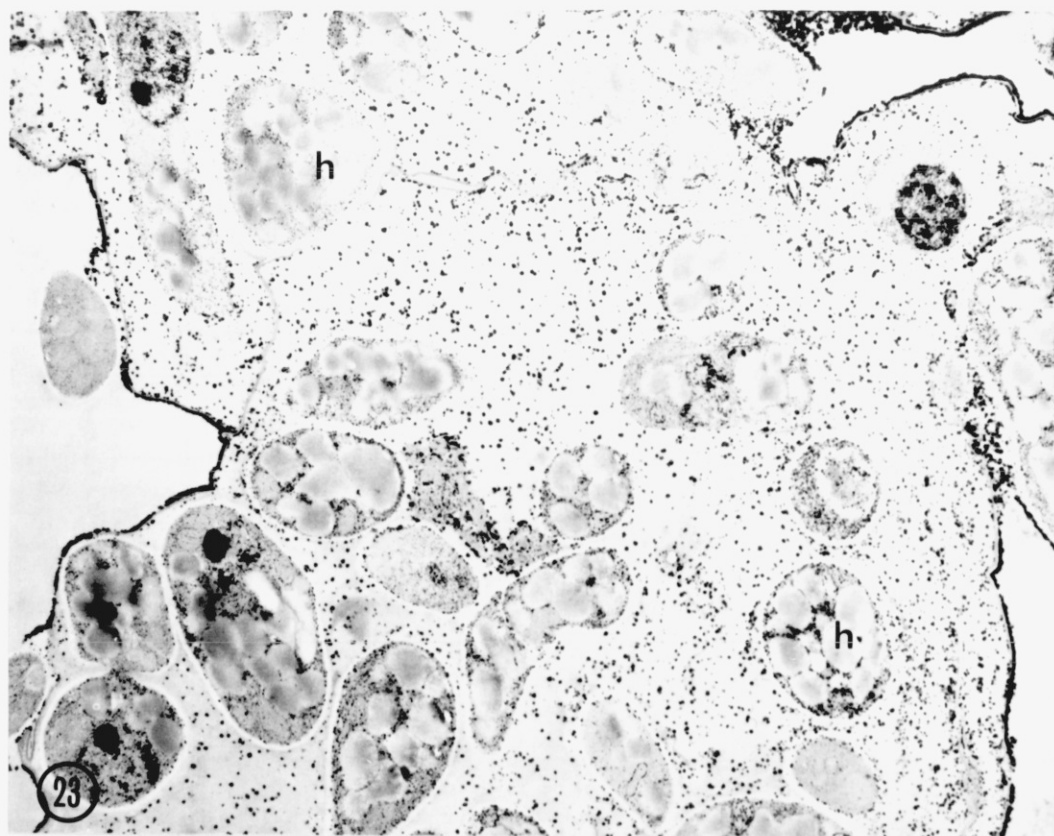
Staining: β -glycerophosphate substrate

PLATE 5:24

No deposits of lead are observed within or among the hyphae (h) of the sclerotial tissue. This is either due to the absence of activity of ATPase in this tissue or, as found with pollinated millet, the compactness of the tissue has prevented penetration of the substrate and/or other reagents.

Magnification X 5300

Staining: ATP substrate.



PLATES 5:25 and 5:26

Electron micrographs taken from the pedicel region of infected millet ovaries 10 days post inoculation and prepared for the localization of phosphatase activity.

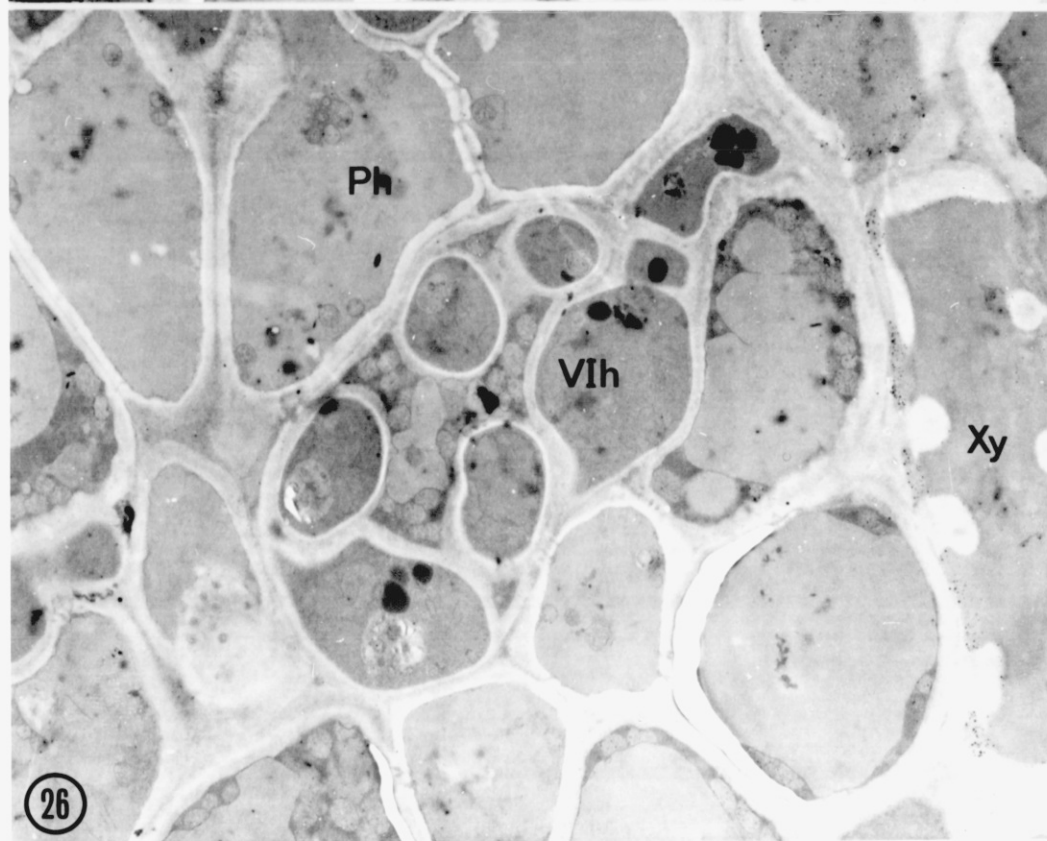
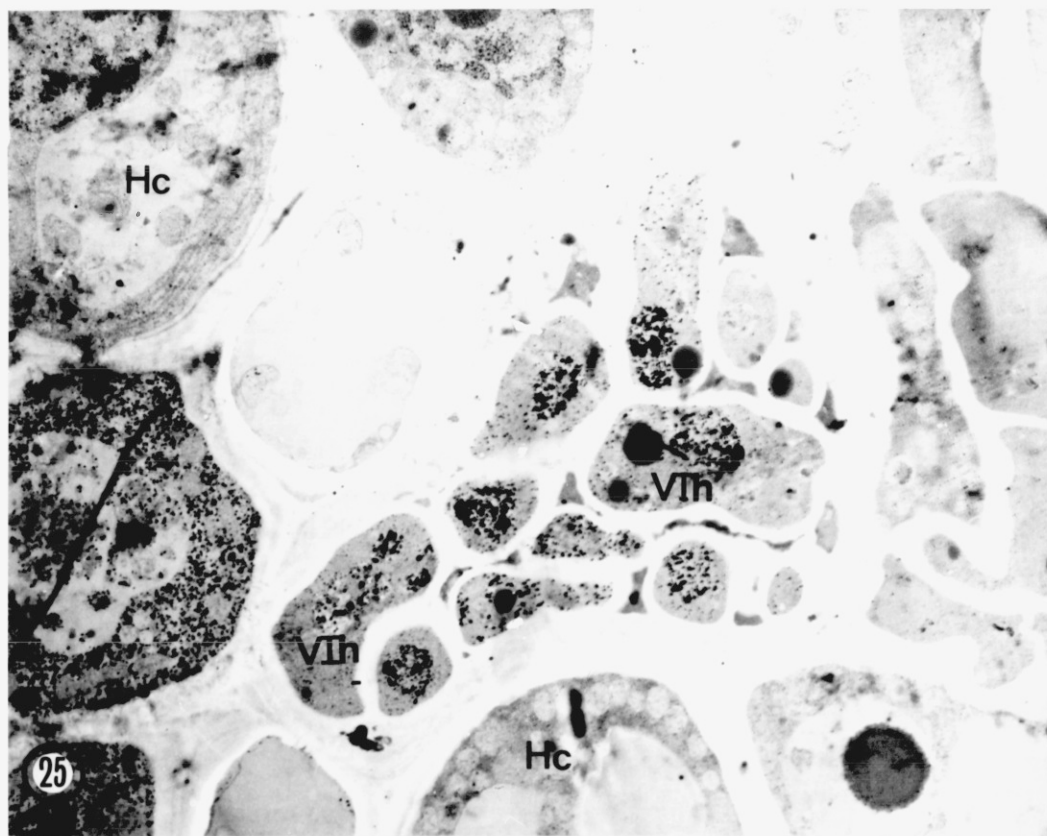
No deposits of lead are observed on the plasma membrane of host cells within the pedicel region. Phosphatase activity is located in some fungal hyphae (VIh) and within disrupting host cells (Plate 5:25). Heavy deposits of lead are located in the outer most tissues of the pedicel but no activity on fungal or host membranes is observed within the core of the pedicel. This could be due to poor penetration of the substrate or other media during preparation.

PLATE 5:25 Magnification X 5300

 Staining: β -glycerophosphate substrate

PLATE 5:26 Magnification X 5200

Staining: ATP substrate.



SECTION SIX

THE STRUCTURE AND DEVELOPMENT OF THE WHEAT OVARY BEFORE AND AFTER POLLINATION.

6.1. THE STRUCTURE OF THE UNPOLLINATED WHEAT OVARY.

The structure of the unpollinated ovary of wheat, or megagametophyte as it is often referred to in the literature, and its development during fertilization has been documented by You and Jensen, 1985. The ovary prior to fertilization is composed of a mature embryo-sac containing an egg cell with two synergids at the micropylar end, a central cell with two polar nuclei, which are usually in close proximity to the egg apparatus, and a mass of 20-30 antipodal cells at the chalazal end (Bennett et al, 1975; You and Jensen, 1985). The embryo-sac is surrounded in turn by the nucellus, the inner then outer integuments and the ovary wall, which is composed of an inner epidermis, parenchyma cells and an outer epidermis (Figure 6:1). Thin osmiophilic cuticle-like lamella line the inner surface of the inner epidermis, both the outer and inner surfaces of the outer and inner integuments and the outer surface of the nucellar epidermis (Figure 6:1). There are 6 cuticle-like lamella in total (Morrison, 1975).

6.2. Fertilization and the development of the tissues associated with the embryo.

A pollen tube, formed from a germinating pollen grain on the surface of the stigma, penetrates and grows down through the style, around the integuments (Morrison, 1975) and enters one of the synergids via the micropyle (You and Jensen, 1985). After passing through the filiform apparatus it discharges its contents causing further degeneration of that synergid. This enables the two sperm

cells to fuse with either the plasma membrane of the egg cell or the central cell. The nuclei then penetrate and enter the cytoplasm of the egg or the central cell where one fuses with the egg nucleus and the other with the fused polar nuclei (Jensen, 1974). The product of fertilization with the egg is the embryo and with the polar nuclei, the endosperm. Initially there is rapid division of the primary endosperm nucleus giving rise to coenocytic endosperm which gradually cellularizes so that further development of the endosperm is by cell division and cell expansion (Frazier and Appalanaidu, 1965; Mares et al, 1975; Simmonds and O'Brien, 1981). The endosperm cells immediately surrounding the initially slow developing embryo are distinct in that they have dense cytoplasm, few vacuoles and do not accumulate starch or protein bodies (Smart and O'Brien, 1983). Embryo growth becomes rapid about 15-20 days post anthesis (d.p.a.) and it is during this period that the axial organs (coleoptile, shoot and root primordia and coleorhiza) are initiated. A wide space forms around the developing embryo, the surrounding distinct endosperm cells having disrupted, apparently by hydrolysis (Smart and O'Brien, 1983). Endosperm cells nearest to the enlarging embryo never amass large quantities of starch or protein, remaining empty throughout the growth of the embryo (Smart and O'Brien, 1983). It is these cells that form the depleted layer in the mature caryopsis (Brown and Morris, 1890). The outer-most endosperm cells (developing germ aleurone cells) continually produce daughter cells which move into the endosperm tissue. These cells on the ventral side of the caryopsis, opposite the embryo, form the groove aleurone cells which are larger and develop thick walls (Morrison et al, 1978; Simmonds and O'Brien, 1981). The walls do not invaginate to form ingrowths

(Kiesselbach and Walker, 1952; Zee and O'Brien, 1971a; Felker and Shannon, 1980; Smart and O'Brien, 1983) though in one case the characteristic wall ingrowths of transfer cells have been reported to develop, 15 d.p.a. (Ayre and Angold, 1979).

6.3. THE DEVELOPMENT OF TISSUES ASSOCIATED WITH NUTRIENT TRANSPORT.

After pollination the outer integument degenerates as does the cuticle-like lamellae lining it on both sides. Two cuticles develop further thickening on the outer surface of the inner integument, the outer cuticle, and between the inner integument and the nucellar epidermis, the inner cuticle, (Morrison, 1975; Cochrane and Duffus, 1979). These two cuticles surround the developing embryo-sac except in the region of the placenta-chalaza (Cochrane and Duffus, 1979; Lingle and Chevalier, 1985).

The placenta-chalaza differentiates and develops in the groove region of the pericarp with the initiation of the endosperm (Frazier and Appalanaidu, 1965; Lingle and Chevalier, 1985). It forms 3 distinct tissues the nucellar projection, the chalaza and the vascular tissue within the pericarp. All 3 tissues extend along the length of the grain on the ventral side and are symplastically connected with one another (Frazier and Appalanaidu, 1965). They are not however, symplastically connected to the groove aleurone cells which lie adjacent to the placenta-chalazal tissue, being spacially separated by the endosperm cavity. The walls of the nucellar projection invaginate so that transfer cells are formed (Cochrane and Duffus, 1980; Smart and O'Brien, 1983). In barley it is found that the cells of the nucellar projection adjacent to the endosperm cavity become obscure as their walls thicken and eventually their protoplasts die, so that a large apoplastic area, which may

facilitate unloading of assimilates into the endosperm cavity, is formed (Cochrane and Duffus, 1980). The chalazal cells accumulate polyphenolic materials, which are probably tannins, and their walls become lignified during the grain filling phase of caryopsis development (Zee and O'Brien, 1970b; Cochrane, 1983; Lingle and Chevalier, 1985)

Phloem sieve tubes within the ventral vascular bundle of the pericarp are continuous with those in the rachilla. Xylem tracheids, however, are not continuous being separated by a core of unconnected tracheary elements (termed thick-walled cells) in the region of attachment to the rachilla. These thick-walled tracheary elements have been described by Zee and O'Brien (1970a) as modified tracheary elements whose differentiation is abnormal. The core of modified tracheary elements is surrounded by a ring of sieve tubes and their associated transfer cells (Zee and O'Brien, 1971a; O'Brien et al, 1985).

The structure of a mature wheat grain is shown in Figure 6:2. The cuticles become fused and harden forming the seed coat, or testa. The ovary is now composed entirely of endosperm cells and the embryo. The embryo has differentiated so that the plumule with protective coleoptile, radicle with protective coleorhiza and the scutellum are formed (Bradbury et al, 1956). The placenta-chalazal tissue is no longer visible as it has been crushed by the enlarged embryo and endosperm.

6.4. NUTRIENT TRANSPORT WITHIN THE DEVELOPING WHEAT CARYOPSIS.

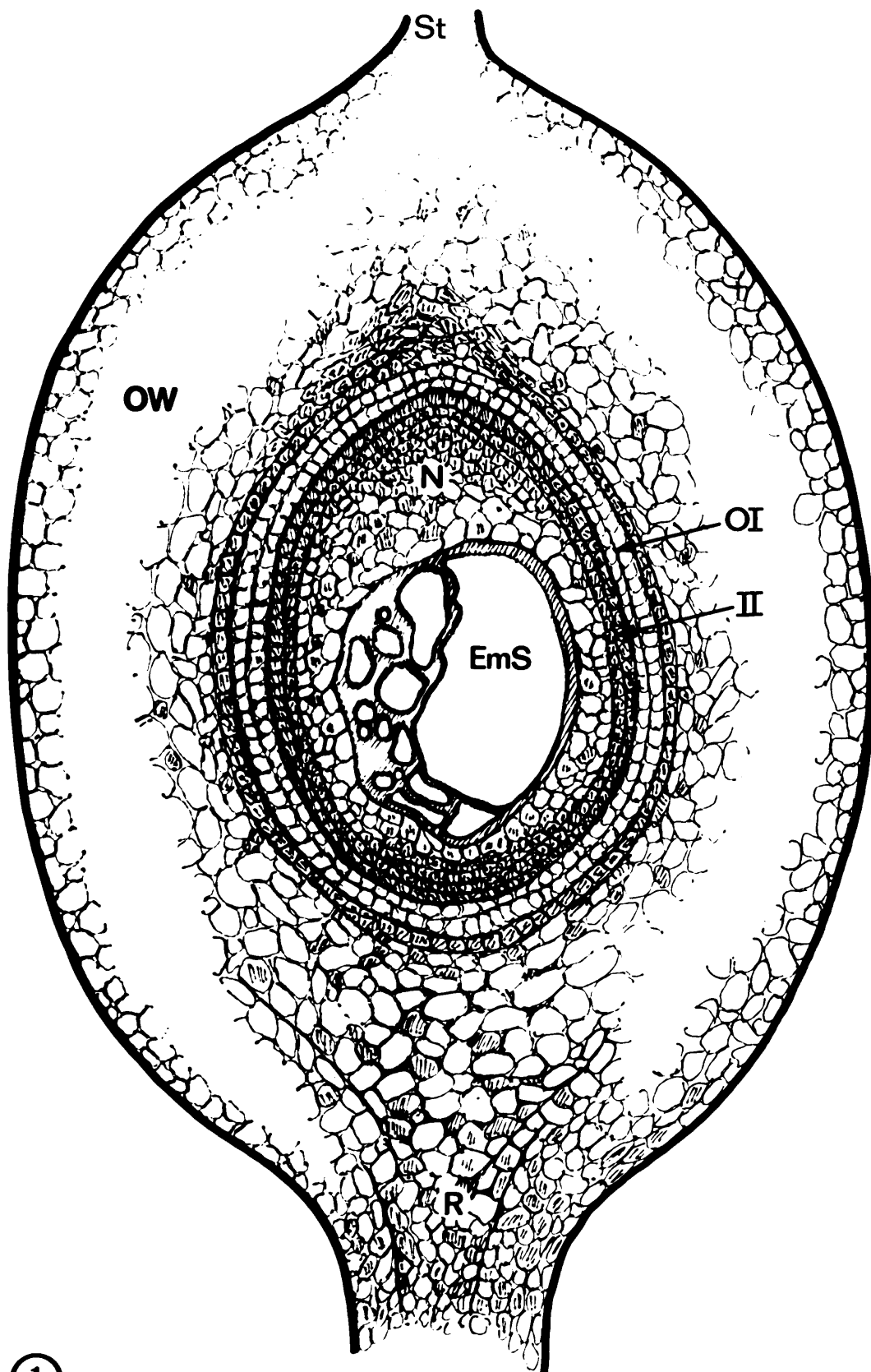
It has been proposed that nutrients are transported into the developing ovary from the vascular bundle at the base of the crease, moving in the symplast from the phloem of the pericarp vascular bundle, via the chalaza and out into the endosperm cavity (apoplast) through the nucellar projection (Frazier and Appalanaidu, 1965). Cuticles partially surrounding the nucellus and inner integument restrict nutrient movement in the symplast from the pericarp vascular bundle to the chalaza region (Morrison, 1975; Lingle and Chevalier, 1985). Subsequently nutrients are taken up from the apoplast of the endosperm cavity into the symplast of the developing endosperm by the groove aleurone cells.

The mechanisms proposed to be involved with the transport of assimilates into the developing caryopsis will be discussed, in the subsequent chapters, in relation to the transport of nutrients into the colonising pathogen.

FIGURE 6:1

A diagram of a longitudinal section through an
unpollinated wheat ovary.

The embryo-sac (EmS) is surrounded by the nucellus (N), the inner integument (II), the outer integument (OI) and the ovary wall (OW). A pollen tube would enter via the stigma (St) and penetrate down around the integuments to the micropylar region (the end nearest the rachilla (R)).

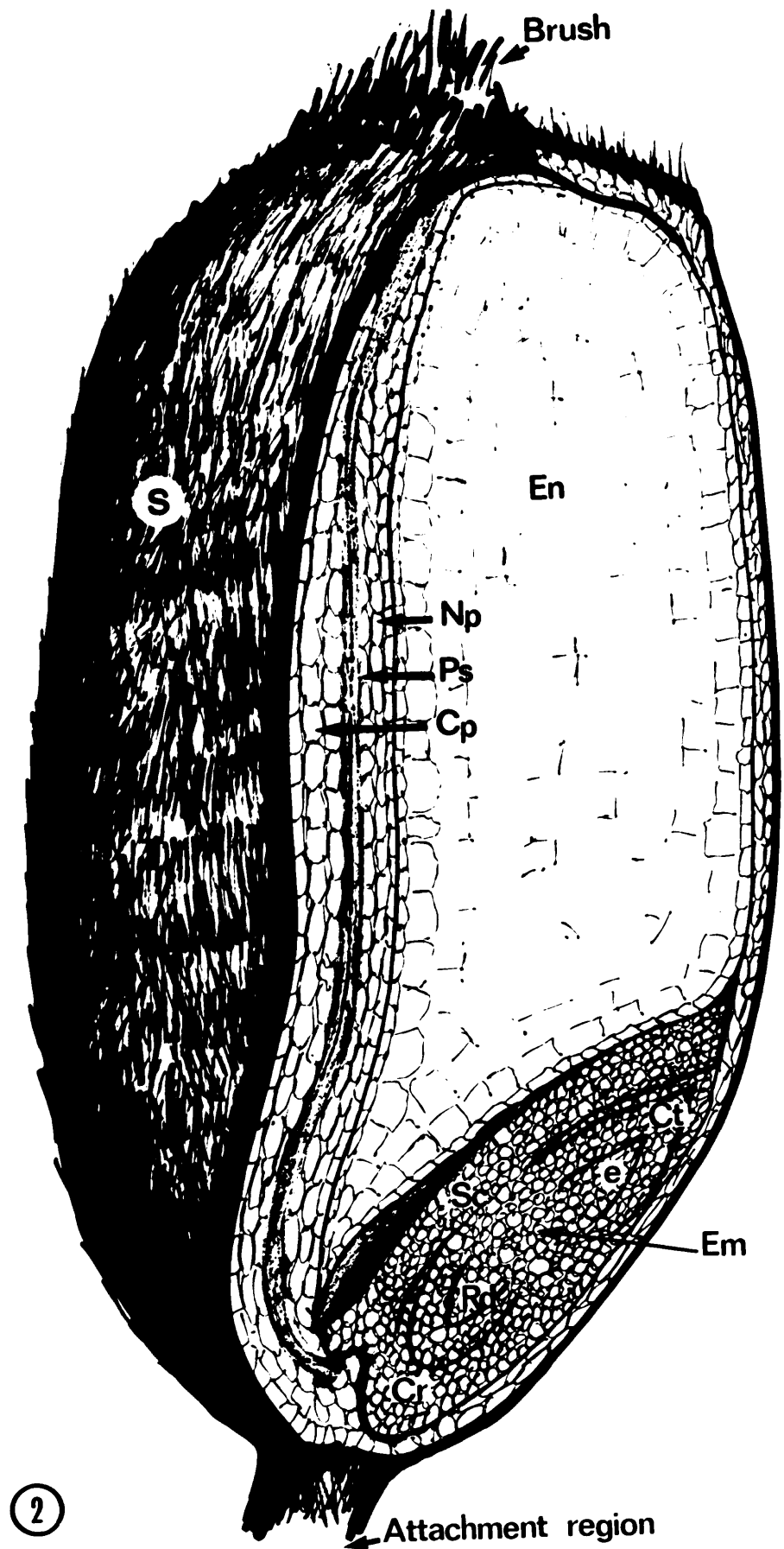


①

FIGURE 6:2

A diagram of a longitudinal section through
a mature wheat caryopsis.

The embryo (Em) has differentiated into the epicotyl (e) and radicle (Rd) surrounded by the coleoptile (Ct) and coleorhiza (Cr) respectively. The scutellum (Sc) lies adjacent to the endosperm (En)., On the ventral side there are remains of the nucellar projection (Np), pigment strand (Ps) and chalazal pad (Cp) tissues. The seed coat (S) comprises the remaining ovary wall, integuments and cuticles which fuse and harden.



SECTION SEVEN

THE COLONISATION OF WHEAT BY *TILLETIA CARIES*.

7.1. INTRODUCTION.

The infection of wheat by *T.caries*, a member of the Ustilaginales, has been described by Swinburne (1960,1963). Intercellular and intracellular dikaryotic hyphae, produced from *T.caries* teliospores contaminating the wheat seed coat or surrounding soil, penetrate the coleoptilar epidermis of the germinating wheat grain. Intercellular hyphae penetrate towards the growing point, reaching it at the 5th leaf stage, from where hyphal segments are subsequently passively carried up with the ear during the extension phase (Swinburne,1963). As the host megagametophytes develop the intercellular mycelia proliferate destroying the ovary tissues and virtually all the hyphal segments become spores. After fusion of the fungal nuclei to form a diploid, the spore wall thickens (Webster,1980). When the inflorescence emerges from the boot sheath the ovaries are found to have been replaced by maturing spores. Disease symptoms only become visible during flowering, the infected plants being slightly shorter, the flag leaf and inflorescence steel green in colour and within each floret the ovary is olive green, in comparison with the white of unpollinated ovaries. The anthers and stigmas are stunted (Fischer and Holton, 1957).

Studies on *T.caries* have included the infection process and growth within the host prior to flowering (Swinburne,1960,1963 and references therein), the formation of teliospore wall layers (Hess and Weber, 1976; Hess and Gardner,1983) and many comparative investigations of spore shape and ornamentations used in the identification of different species within the Ustilaginales

(Henry, 1984; Mordue and Ainsworth, 1984; Mordue, 1986). However, the development of the fungus within the host ovary prior to and during teliospore formation has not been investigated.

This study was undertaken to describe the relationship which the parasite forms with the wheat ovary and to compare it with the relationship which an uninfected pollinated embryo-sac establishes with its parent tissue. Particular attention is given to observations relating to a mechanism of nutrient transport from the host to the parasite.

Wheat ovaries infected with *T. caries* were taken at various stages during the development, colonisation and replacement of the unpollinated wheat ovary. Pilot examinations of fresh tissues were made prior to processing for electron microscopy to check that infection had occurred. The relationship of the host tissue to the developing fungus was therefore investigated by serial thick sectioning ($0.5\mu\text{m}$) and light microscopical examination of embedded material (section 2.3.2.). Where hyphae were observed in close association with the host cells the block-face was re-trimmed, thin-sectioned and stained for electron microscopy (section 2.3.2.). Unpollinated and pollinated ovaries from uninfected plants were prepared in the same way for comparative developmental studies. Infected or pollinated ovaries too large for fixative or resin penetration ($>5\times 3\text{mm}$), unless cut into eight or more pieces, were fractured and prepared for observation in the scanning electron microscope (section 2.6.2.).

7.2. RESULTS

7.2.1. COLONISATION BY THE FUNGUS

Infected ovaries dissected from the fifth node stage (Zadoks 1978) were very small (0.25mm in diameter), undifferentiated and indistinguishable from uninfected material (PLATE 7:1). No embryo-sac or integuments were visible. It was impossible to find any hyphae with the light microscope but with transmission electron microscopy (T.E.M.) a few small intercellular hyphae (0.7-1.7µm diameter) were observed (PLATE 7:2 and 7:3). They occupied junctions between three or more host cells approximately one third of the radius from the centre of the ovary.

At the 6th node stage, the ovary was larger and anthers were visible with the light microscope (PLATE 7:4). The ovary had differentiated into a concentrically layered structure (PLATE 7:5). Outside a central zone consisting of 10 layers of cells which extended to approximately one third of the radius from the centre, fungal cells (1.5-2.0µm diameter) were now common at the periclinal junctions of the cells (PLATES 7:5 and 7:6). Hyphae were fewer within the central 10-layer zone and were particularly sparse in the extreme centre (PLATE 7:6). Occasional hyphae were observed at the junction between the ovary and the developing vascular tissue in the pedicel and between cells of the anther filaments and outer protective tissues.

At the boot stage fungal development had progressed still further. The proliferating hyphae had formed spores which partly surrounded the central 10-layer zone in a cup-shaped layer approximately 0.12mm thick (PLATE 7:7). A mound of host cells extending from the surrounding ovary wall filled the open part of

the cup and within this zone the host tissue continued to divide forming cells in anticlinal columns with respect to the ovary wall (PLATE 7:7). Vegetative hyphae occurred sporadically among the spores but were more prolific at the inner and outer margins of the cup, growing between the host cells forming more spores so that the cup thickened.

When the inflorescence emerged from boot, sporulation continued in the cup-shaped region (PLATE 7:8). The mound tissue had enlarged and flattened, extending along the length of the ventral side. The cup-shaped region had become crescent-shaped as the ovary continued to enlarge. Vascular tissue, including xylem tracheids and phloem sieve tubes, was visible at the base of the ovary (PLATE 7:8), progressively differentiating along the ventral side adjacent to the mound (PLATE 7:9). At this stage hyphae were seen growing between the cells of the mound region (PLATE 7:10) and between those cells of the pericarp vascular tract, where some were found in xylem tracheids (PLATE 7:11). Hyphae were never observed in phloem sieve tubes or in any host cells in or below the pedicel. Within the mound region, although predominantly intercellular, some hyphae penetrated the host cell wall and developed intracellularly (PLATES 7:9 and 7:12). The ovary continued to enlarge and at maturity the volume of the infected structure was 1.5 - 2.0 times that of a mature wheat grain. Within the host the matured teliospores (PLATE 7:13) filled the tissue enclosed by a thin ovary wall. Although hyphae colonised the pericarp vascular tract and mound region they never sporulated in these regions (PLATE 7:14).

At maturity the infected ovaries were distinguishable from uninfected ovaries by the characteristic deep olive green colour in

the outer epidermis. When cut open at different stages with a razor blade, spherical particles (sporeballs) within the ovary were visible to the naked eye, and these changed from white through orange-brown to dark brown as the teliospores matured, before ultimately the whole mass blackened. The bunt ball becomes dehydrated at maturity and the remaining host cells collapse so that only a thin outer covering of host wall tissue restrains the loosely packed teliospores.

7.2.2. CYTOLOGY OF THE DEVELOPING FUNGUS

Fungal hyphae, 1.5-2.0 μ m in diameter, were seen to proliferate, within the ovary at the onset of host cell differentiation, into concentric layers. Hyphae were distinct from the host cells, when stained with toluidine blue and observed with a light microscope, as their cytoplasm was a much darker purple. Within the central 10-layer zone electron microscopy showed that the hyphae had a very dense granular cytoplasm, frequent small lipid droplets, a few vacuoles, some mitochondria and a nucleus (PLATE 7:15). Outside this zone hyphae proliferated adjacent to the concentric layers and occasionally between them (PLATE 7:16). Short lengths were visible enabling simple septa to be observed, the configuration of some of these suggesting that clamp connections had been formed (PLATE 7:16).

Developing spores were distinguishable from hyphae by their larger diameter, a less dense cytoplasm and a conspicuous nucleus (PLATE 7:17). Some hyphal compartments were seen to have two nuclei (PLATE 7:16) but developing spores contained only one (PLATES 7:16 and 7:17).

The thickness of the spore wall provided an indication of the degree of maturity. Spore initials arising on intercalary or terminal hyphae had a thin, almost invisible, wall (PLATE 7:17). PLATE 7:17 shows that each spore was separated from the parent hypha, which had degenerated, by a cross wall. The spores, at this stage, contained very conspicuous contents; mitochondria, some endoplasmic reticulum, a nucleus approximately 1.4 times larger in diameter than the nuclei of fungal hyphae, with a prominent nucleolus, vacuoles and lipid bodies. As the spore enlarged, from 3-5 μ m to 5-8 μ m in diameter, so did the vacuoles and the number and size of lipid bodies. As the spore wall thickened up to 5 layers became distinguishable (PLATE 7:24). The primary layer thickened initially (PLATE 7:18) and then additional lenticular thickenings were formed. In section these appeared like small crescent shapes at intervals along its innermost surface (PLATE 7:19). The spore contents remained easily distinguishable and layers of endoplasmic reticulum, interspersed with microbodies, were visible near the developing wall (PLATE 7:19). Between the crescent shaped thickenings, a more electron opaque material was then deposited (PLATES 7:20 and 7:21) so that at this stage it lined the inner surface of the wall. The third layer, a narrow and very electron dense deposit, was laid down and coated the whole of the inner surface (PLATES 7:20 and 7:21). At this stage spore contents were no longer clearly discernable and in some cases fixed poorly (PLATE 7:21). Only a few spores (PLATE 7:22, 7:23 and 7:24) were observed to have a fourth layer, an electron opaque layer lining the third layer. Most spores at this stage of maturity did not fix normally and resin penetration was poor. An additional, very thin superficial layer became discernable where it became detached

from the outer surface of the primary layer (PLATE 7:25 and 7:26) as the 2nd layer was deposited. All spore wall layers stained differentially, the first two being more electron dense when specifically stained for polysaccharides (PLATES 7:20 and 7:22) and the second two more electron dense when stained using the PACP method (PLATES 7:21 and 7:23). The characteristic ornamentation formed from the development of these wall layers was observed with the scanning electron microscope (PLATE 7:13).

7.2.3. CYTOLOGY OF THE HOST.

As the ovary enlarged at the 6th and 7th node stage, concentric layers became prominent in the centre of the ovary. The central cell was approximately twice the length of the surrounding cells with only a small vacuole visible (PLATE 7:5). Each of the cells immediately surrounding the central cell, in a zone approximately 2 layers in thickness, had a large vacuole and a small amount of groundplasm which contained a large nucleus at one end (PLATE 7:5). The remaining cells of the central 10-layer zone contained a large nucleus at their centre, a vacuole at one or both ends, cytoplasm with many ribosomes, small chloroplasts, mitochondria and occasional strands of endoplasmic reticulum (PLATE 7:15). Some cells within this region were dividing. All other cells within the undifferentiated ovary were highly vacuolated, had large nuclei and only occasionally showed signs of division. Two osmophilic cuticular-like lamellae were visible with the electron microscope surrounding the cells of the central 10-layer zone.

Most host cells did not show any response to fungal hyphae lying adjacent to them but some, which had been separated by the extending hyphae, showed signs of degeneration (PLATE 7:16). Their

contents were disrupted, many vesicles were seen in the cytoplasm, but a few chloroplasts remained visible (Figure 7:16). Eventually these degenerating cells collapsed and the developing fungal teliospores filled the space which they had occupied (PLATES 7:16 and 7:17).

During the latter stages of development intercellular colonisation by fungal hyphae and subsequent sporulation continued from the ovary wall tissue into the cavity. However colonisation within the mound tissue was more limited than elsewhere. There were fewer hyphae which though predominantly intercellular sometimes penetrated the living host cells. Between the hyphal wall and the invaginated host plasmalemma a thin wall-like matrix material was visible if sections were stained using the PATCH-SP method (section 2.3.2.) (PLATES 7:27 and 7:28). This layer was of uniform thickness and similar to the host cell wall in density and structure. The invaginated plasma-membrane was indistinguishable from the remaining host plasmalemma and that in uninfected cells when stained with PACP, PATCH-SP or uranyl and lead salts (section 2.3.2.) (PLATE 7:29 and 7:30). Most internal membranes stained weakly with the PACP method.

PLATE 7:1

A light micrograph of a wheat ovary (O) infected with *Tilletia caries* dissected from the rachis (R) at the fifth node stage. The ovary is small and indistinguishable from uninfected material of the same age.

Magnification X 210

Staining: Toluidine blue

PLATES 7:2 and 7:3

Electron micrographs of cells within an infected wheat ovary dissected from the fifth node stage. Small intercellular hyphae (h) are visible at the junctions between 3 or more host cells. The host cells have large nuclei (n), many of which are dividing (Plate 7:2). The size of the vacuoles (v) varies in different cells.

cp - cell plate

PLATE 7:2

Magnification X 4000

Staining: Uranyl acetate and lead citrate

PLATE 7:3

Magnification X 5250

Staining: Uranyl acetate and lead citrate

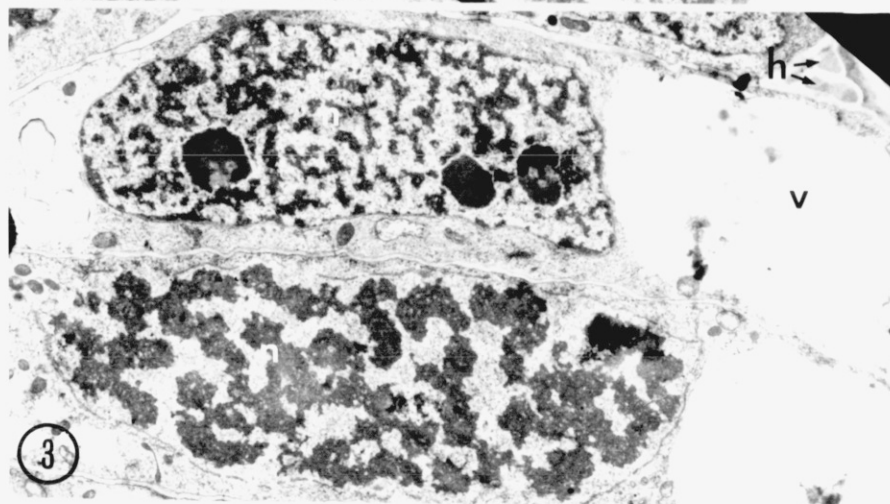
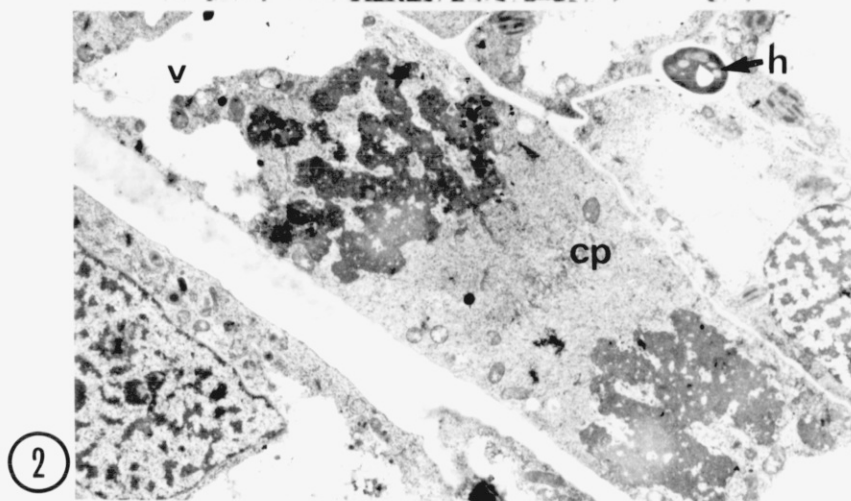


PLATE 7:4

A light micrograph of a longitudinal section through an infected wheat ovary (O) dissected from the 6th node stage. It is approximately 3 times larger than an unpollinated ovary. Anthers (A) are visible within the floral cavity and vascular tissues (V) can be seen in the pedicel (Pd).

Magnification X 240

Staining: Toluidine blue



PLATES 7:5 and 7:6

Light micrographs showing parts the infected ovary
in Plate 7:4 in more detail.

PLATE 7:5

The centre of the ovary (O) has differentiated to form a concentrically layered structure around a central cell (Cc). The central cell is approximately twice the length of the surrounding cells. Intercellular hyphae are present between the concentric layers and they are especially prolific outside the central 10 layer zone.

Magnification X 500

Staining : Toluidine blue

PLATE 7:6

Intercellular hyphae (h) are particularly sparse
in the extreme centre of the 10-layer zone.

Magnification X 1250

Staining: Toluidine blue.

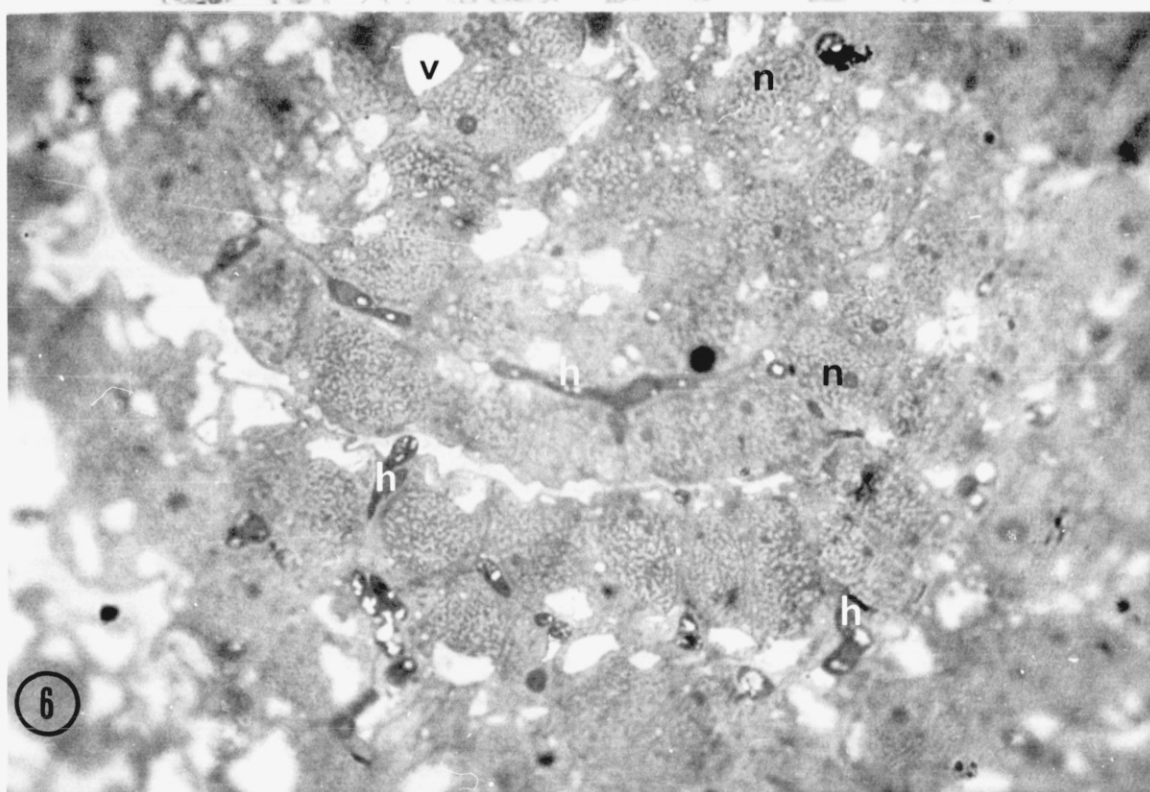


PLATE 7:7

A light micrograph of an infected wheat ovary taken at the boot stage. Fungal development has progressed so that the proliferating hyphae outside the 10-layer zone have formed spores (s) which envelope the 10-layer zone on three sides, producing a cup-shaped layer approximately 0.1mm thick. Host cells of the 10-layer zone are joined to the ovary wall by cells which are in anticlinal columns with respect to the ovary wall (OW). Vascular tissue (V) is visible in the pedicel (Pd).

Magnification X 300

Staining: Toluidine blue



PLATE 7:8

A longitudinal section of a wheat ovary infected with *T. caries* as the ear emerges from boot. Sporulation continues in the cup-shaped region and the host cells which previously filled the cup have now flattened out forming a crescent-shaped mound (M) on the ventral side (Vs). Vascular tissues (V) are visible as they progressively differentiate along the ventral side of the ovary.

Magnification X 310

Staining: Toluidine blue



PLATE 7:9

A longitudinal section through an infected wheat ovary after emergence from boot. Vascular tissues (V) are seen in the ventral wall near the mound tissue (M), xylem tracheids (Xy) being most conspicuous. Hyphae occur between the host cells of the mound tissue and are visible within the xylem tracheids.

Magnification X 310

Staining: Toluidine blue



PLATES 7:10 and 7:11

Light micrographs showing parts of Plate 7:9
(infected ovaries) in more detail.

Plate 7:10

Hyphae extend into the mound tissue (M) growing intercellularly (h). Spores (s) are first formed from hyphae growing between the host cells at the inner most edge of the ovary wall. The spores (s) are distinct from hyphae (h) as they are larger and have greater lipid (Lb) content.

Magnification X 1340

Staining: Toluidine blue

Plate 7:11

Intercellular hyphae (h) growing between the cells of the mound region and within the xylem tracheids (Xy) associated with the mound tissues. Some hyphae are seen to penetrate cells, associated with the mound tissue, forming intracellular hyphae (Ih).

Magnification X 1560

Staining: Toluidine blue

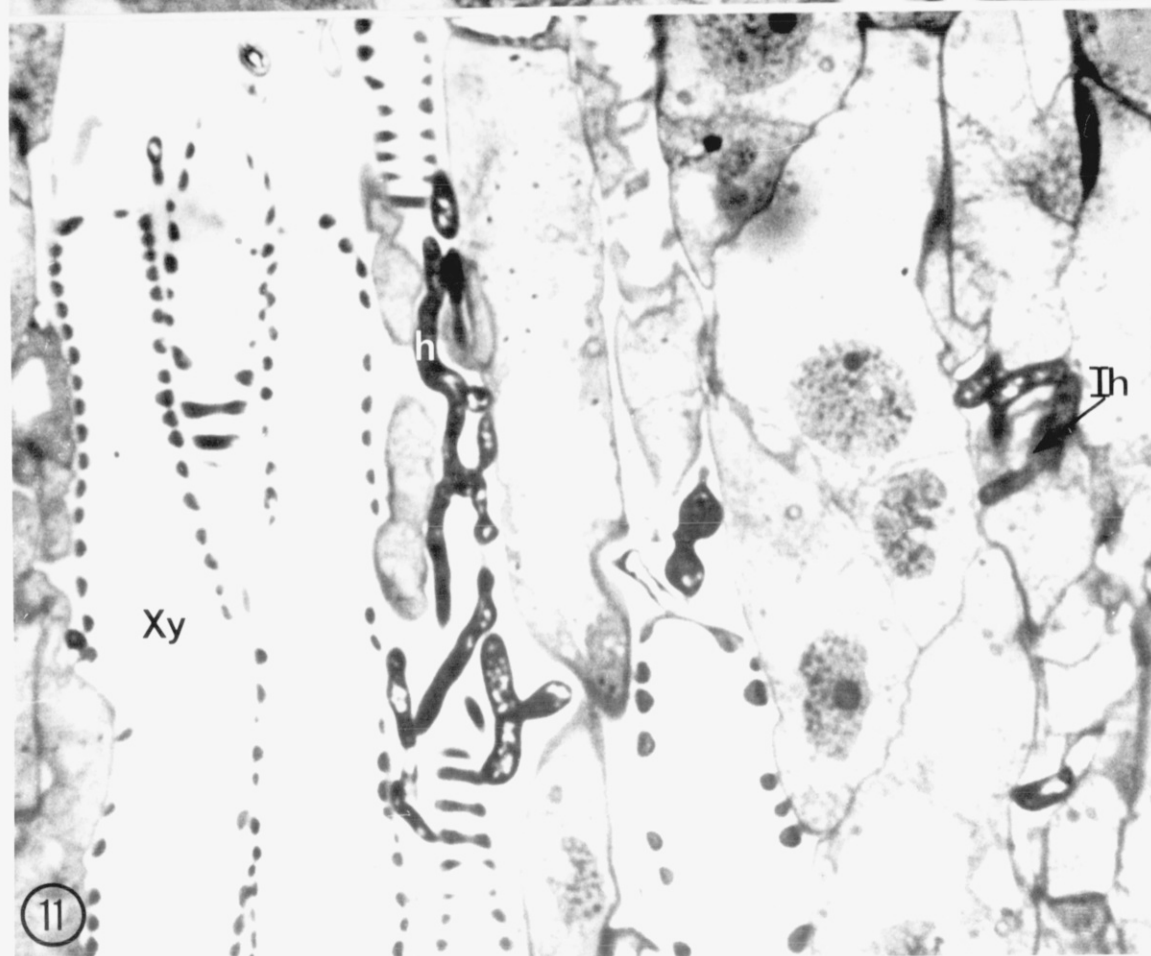
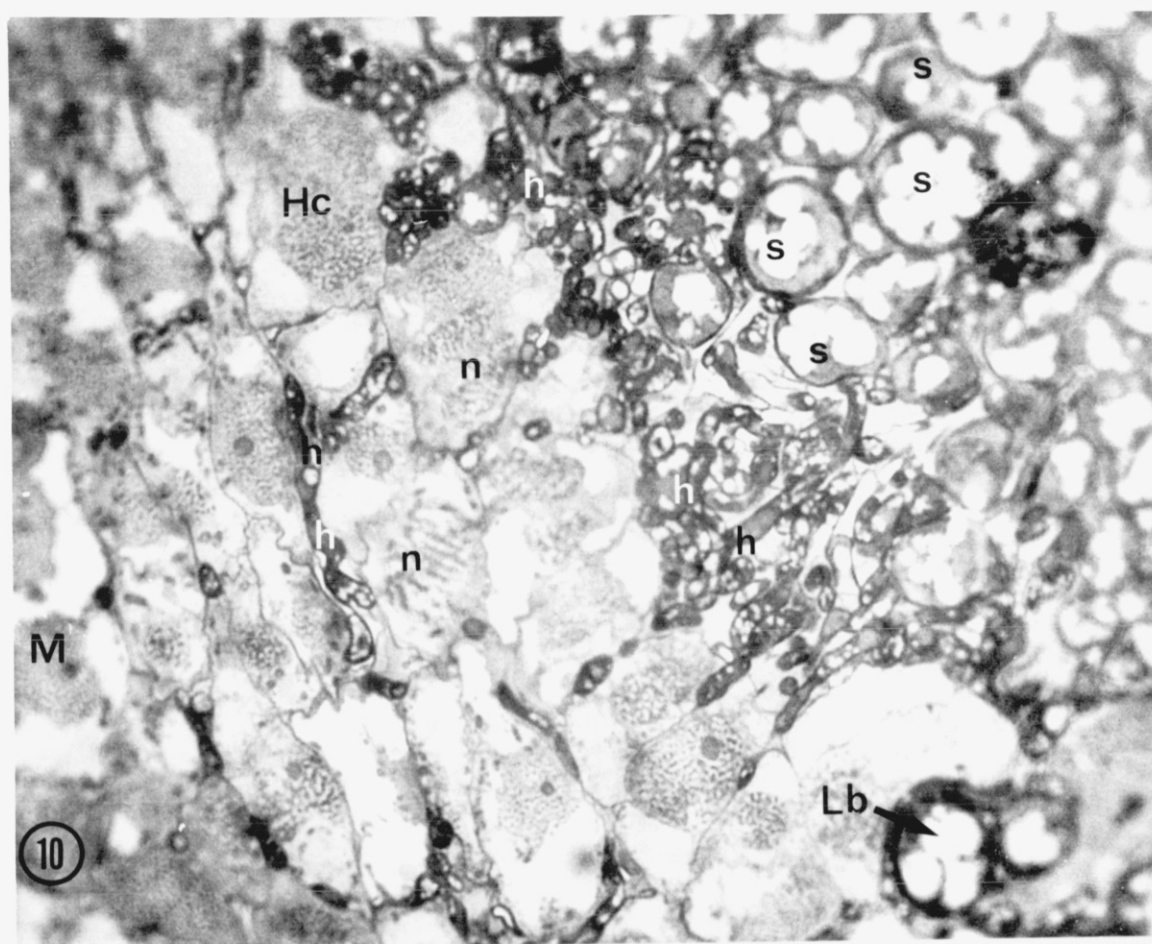


PLATE 7:12

An electron micrograph of cells within the mound region in an infected wheat ovary confirming the presence of intracellular hyphae (lh). The intracellular hypha is surrounded by host wall-like material and an invaginated host plasma-membrane. The fungal nucleus (Fn) and nucleolus are prominent. The host cells have large nuclei (n) and are connected to each other by many plasmodesmata (pd).

Magnification X 11750

Staining:Uranyl acetate and lead citrate



PLATE 7:13

A scanning electron micrograph showing the sporogenous hyphae (si) at the periphery of the spore region (s) adjoining host cells (Hc). The mature teliospores (T) show the reticulation pattern characteristic of *Tilletia caries*.

Magnification X 2650

PLATE 7:14

A scanning electron micrograph showing a median longitudinal fracture through an infected wheat ovary. The ovary wall (OW) is wide and on the ventral side (Vs) the mound tissue (M) is easily visible. The cavity in the middle of the specimen was occupied by mature and maturing teliospores but as these were not bound to each other or the host tissue they are lost during preparation. The stunted stigma (St) of the infected ovary is visible. Higher power observations showed that vascular tissues (V) are present in the ventral wall of the ovary.

Magnification X 530

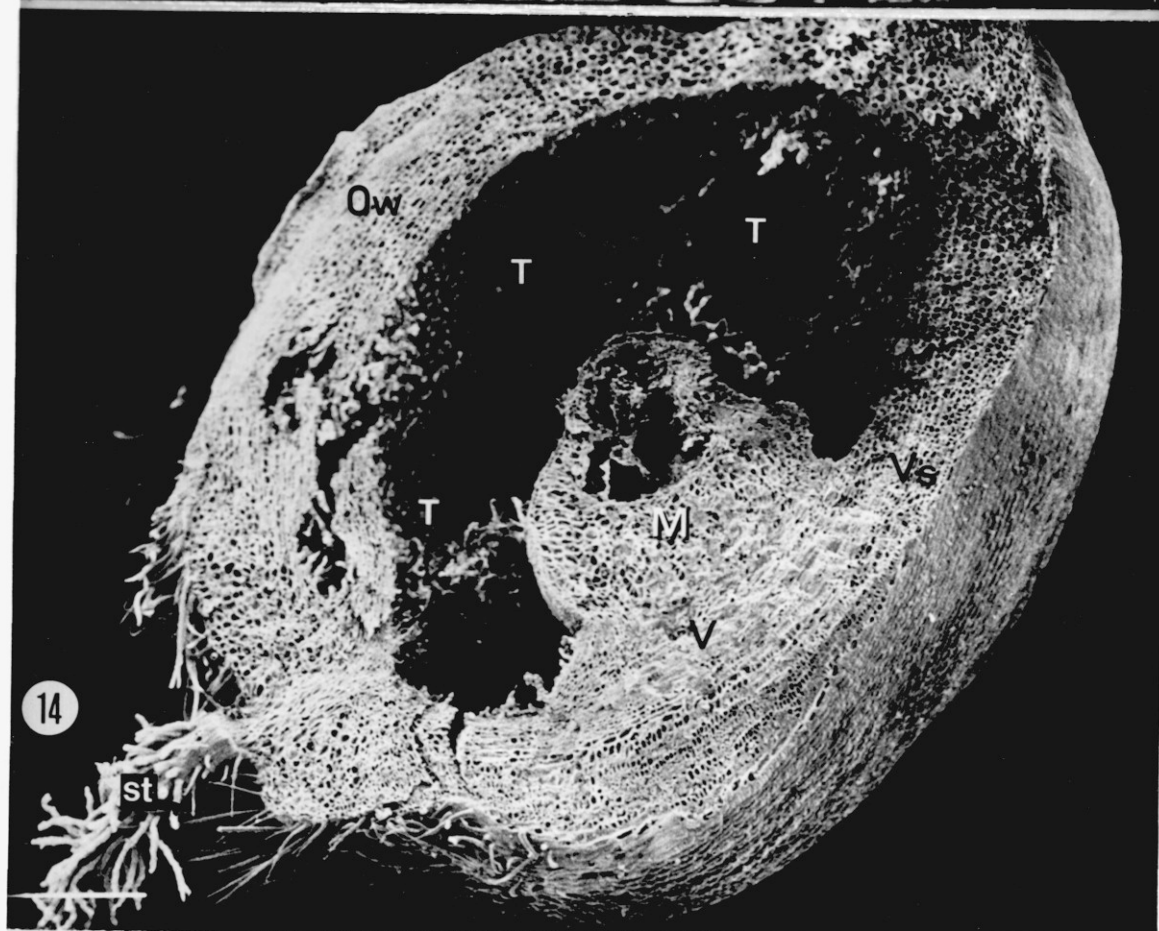
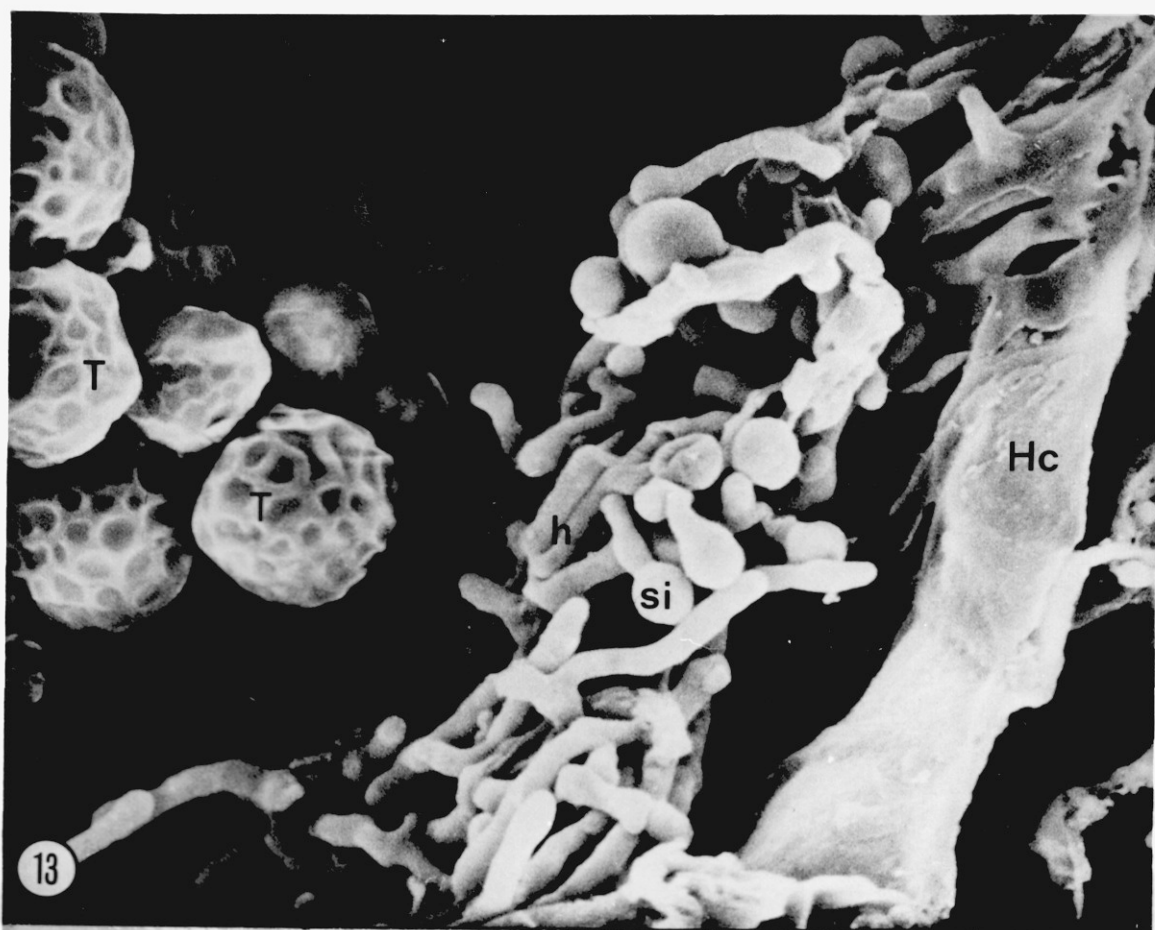


PLATE 7:15

An electron micrograph of the central region in an infected wheat ovary dissected at the 6th node stage. Intercellular hyphae (h) occupy junctions between three or more host cells. Each host cell contains a large nucleus (n) and vacuole (v). Ribosomes are numerous as are chloroplasts (c), mitochondria (m) and plasmodesmata (pd-arrowed). Occasional strands of endoplasmic reticulum (ER) are visible within the cytoplasm. The fungal cytoplasm is more dense than the host's so that they are distinct with both light and electron microscopy.

Magnification X 6400

Staining: Uranyl acetate and lead citrate

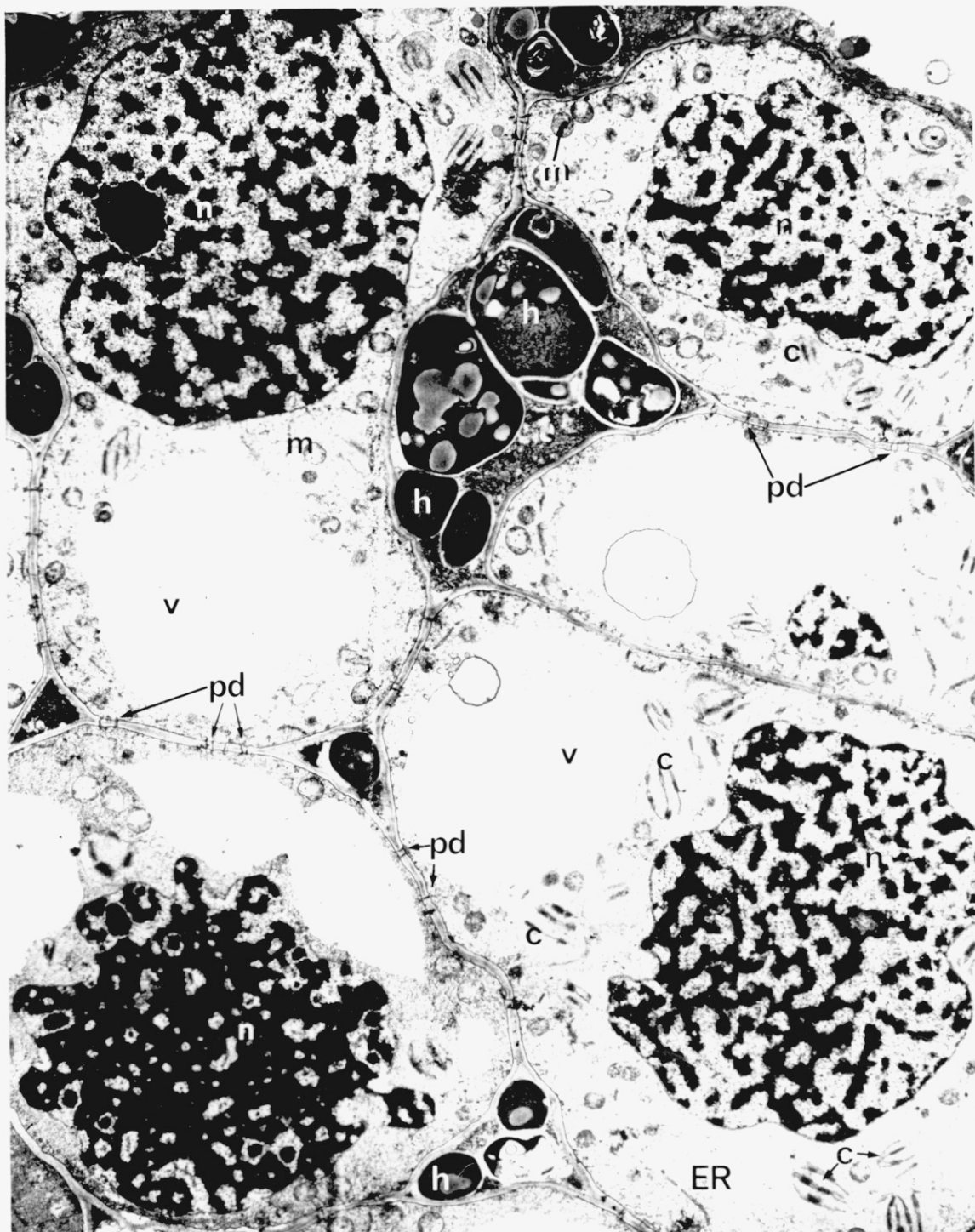


PLATE 7:16

An electron micrograph of the central region of an infected wheat ovary dissected at the 6th node stage showing hyphae (h) penetrating between the cells of the concentric layers. Short lengths of hyphae are visible enabling simple septa (sp) to be observed, the configuration of some suggesting that clamp connections (cc) have been formed. Developing spores (s) are distinguishable from hyphae (h) by their larger diameter and less dense cytoplasm. They have a single conspicuous nucleus (Fn). Two nuclei (Fn) are observed in some hyphal compartments. Host cells, when separated by the extending hyphae, showed signs of degeneration. Mainly vesicles (ve) are present within the lightly staining cytoplasm which contains a few chloroplasts (c). Plasmodesmata are seldom visible between the degenerating cells which are eventually replaced by the developing teliospores.

Magnification X 5300

Staining: Uranyl acetate and lead citrate



PLATE 7:17

An electron micrograph taken from an infected wheat ovary prior to emergence from boot. Spore initials (si) at the edge of the cup-shape have thin, almost invisible, walls, a large conspicuous nucleus (Fn), a vacuole (v), endoplasmic reticulum (ER), many mitochondria (m) and lipid bodies (Lb). Each spore is separated from the degenerating parent hypha by a cross wall (cw).

Magnification X 8400

Staining:Uranyl acetate and lead citrate



PLATE 7:18

An electron micrograph showing two maturing

T. caries spores.

The spores enlarge as they mature, as do their vacuoles (v) and lipid bodies (Lb). The primary wall layer (W1) initially thickens and then lenticular thickenings (Lt) are formed.

Magnification X 7500

Staining: PATCH-SP

PLATE 7:19

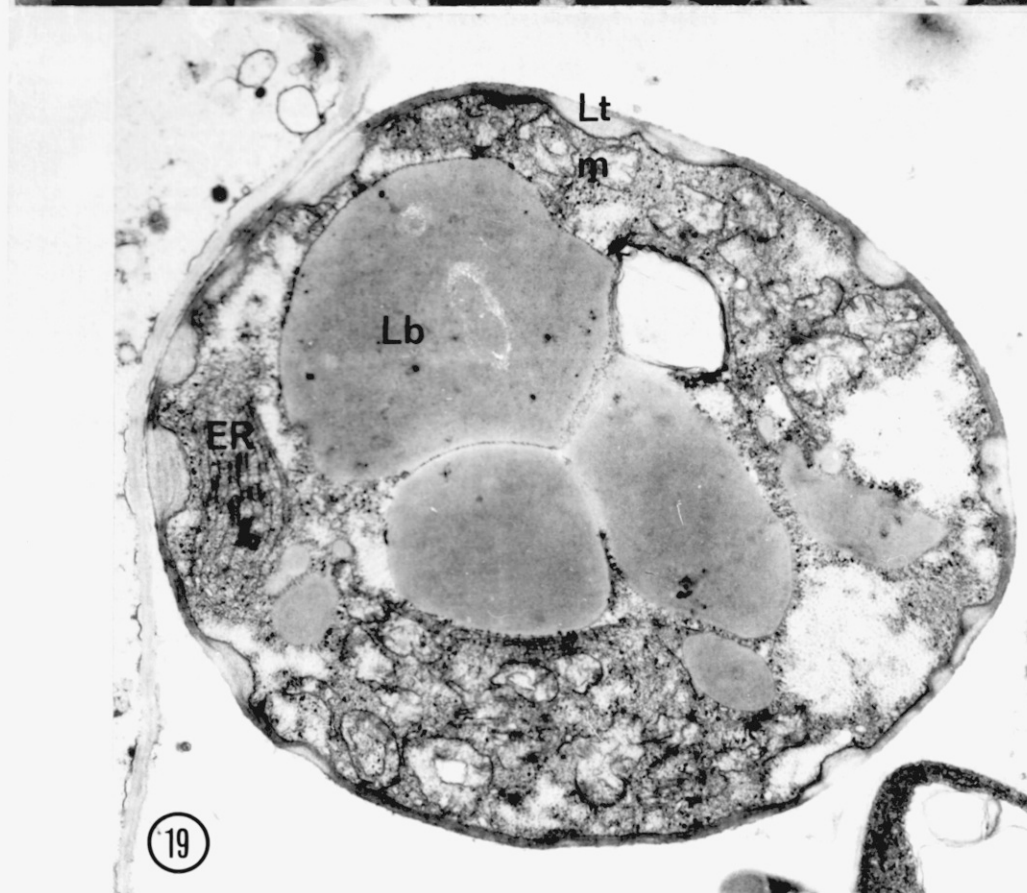
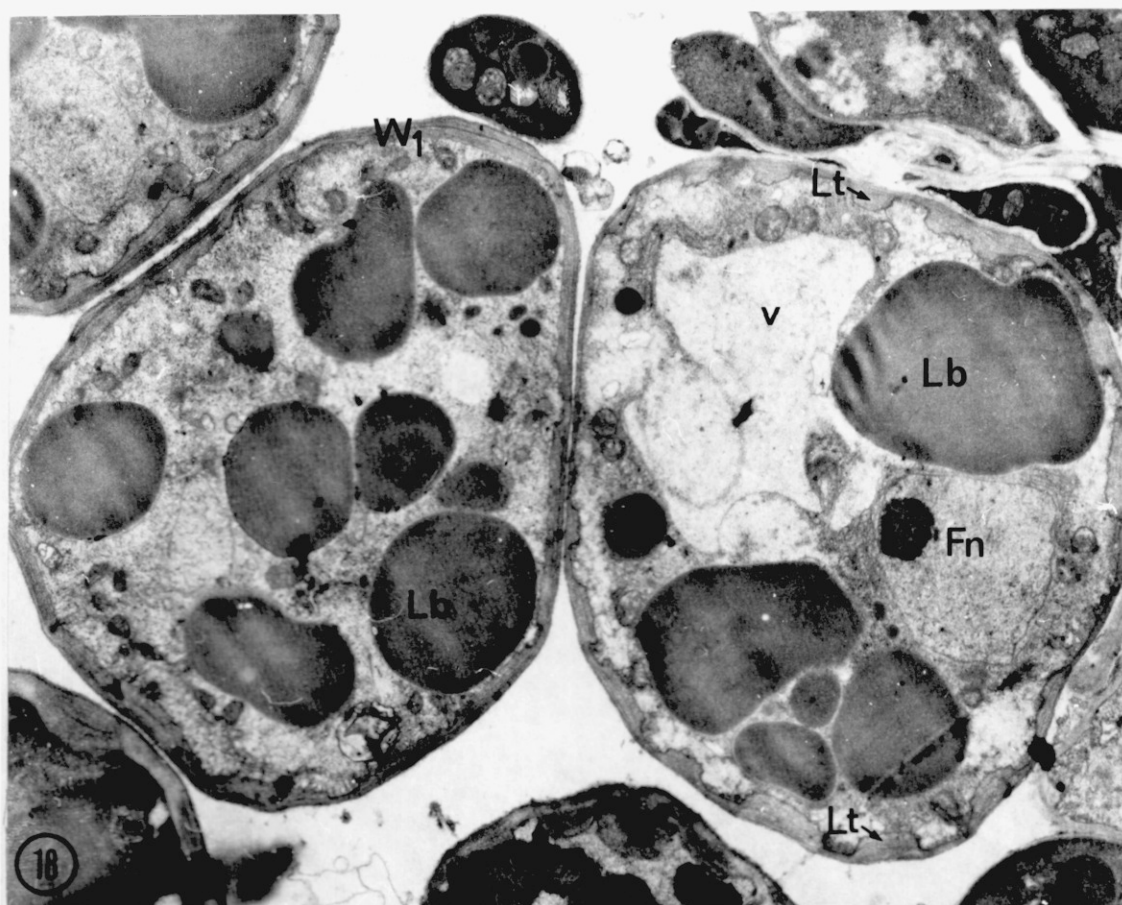
An electron micrograph showing a maturing

T. caries spore.

The lenticular thickenings (Lt) appear as crescent-shapes shown at intervals along the innermost surface of the primary wall (W1). Many mitochondria (m) remain visible and layers of endoplasmic reticulum (ER), interspersed with microbodies, occur near the developing wall.

Magnification X 11300

Staining: Uranyl acetate and lead citrate



PLATES 7:20 ; 7:21 ; 7:22 and 7:23

Electron micrographs of maturing *T. caries* teliospores.

PLATE 7:20

A second more electron dense material (W2) has been deposited between the lenticular thickenings (Lt) so that it lines all or nearly all of the inner surface of the spore wall. The third layer (W3) is just visible lining the whole of the inner surface of the second layer.

Magnification X 4700 Staining: PATCH-SP

PLATE 7:21

All three wall layers (W1, W2 and W3) are now distinguishable, the third (W3) being distinct from the first two by its greater electron density.

Magnification X 5600 Staining: PACP

PLATE 7:22

A fourth (W4) thicker and continuous wall layer has been deposited on the inner surface of the third layer (W3). Using this staining technique the first two layers (W1 and W2) are electron dense whilst the latter two (W3 and W4) are electron transparent. Spore contents are poorly fixed.

Magnification X 4750 Staining: PATCH-SP

PLATE 7:23

The third and fourth wall layers (W3 and W4) are more electron dense than the first two (W1 and W2) using this staining technique.

Magnification X 6250 Staining: PACP

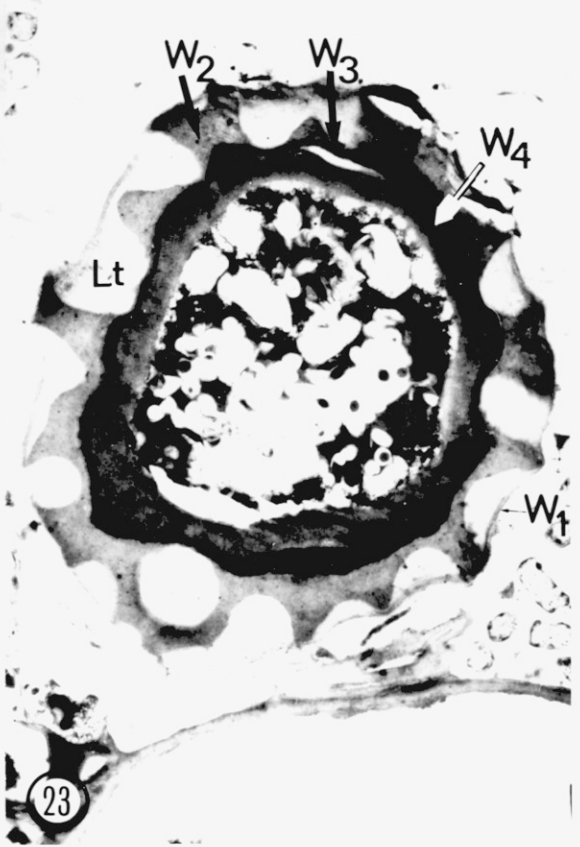
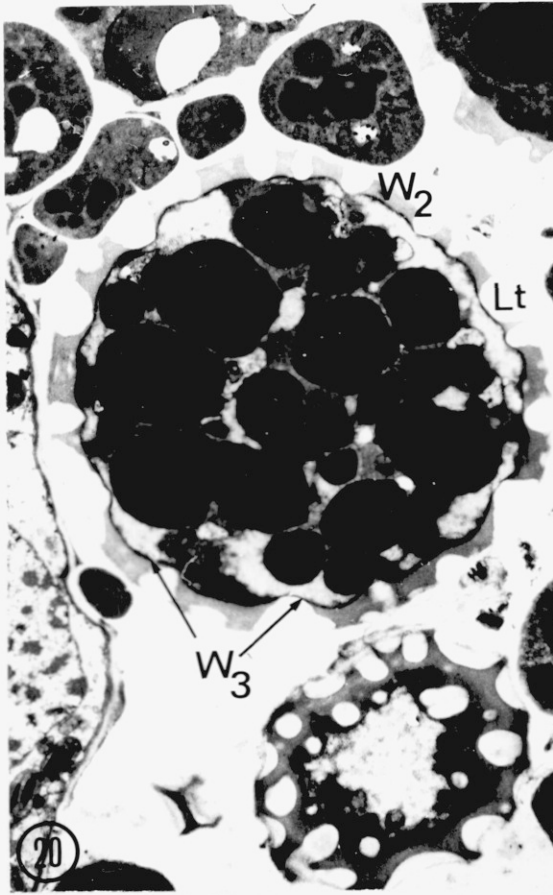


PLATE 7:24

An electron micrograph of a mature teliospore of

T. caries.

All four wall layers (W1, W2, W3 and W4) are distinct, the third (W3) being thinnest.

Magnification X 11750

Unstained

PLATES 7:25 and 7:26

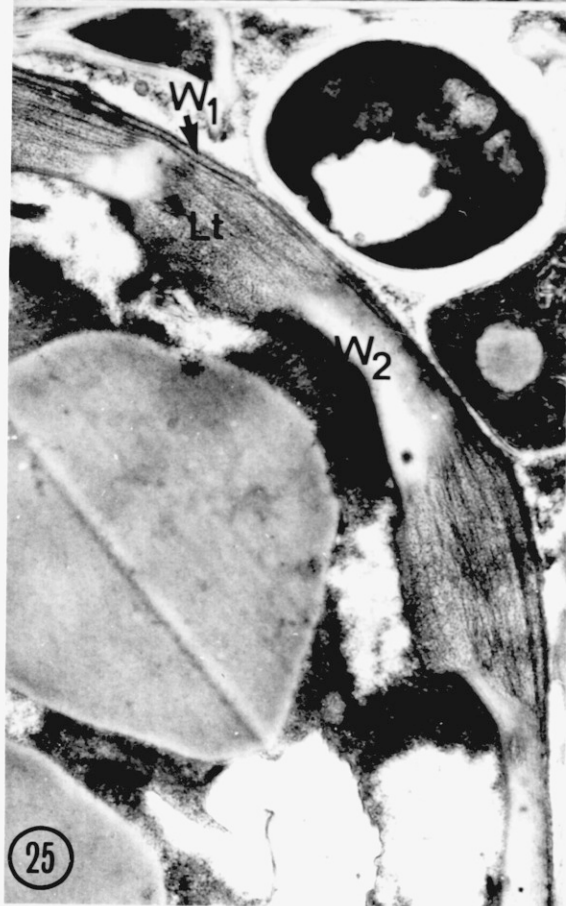
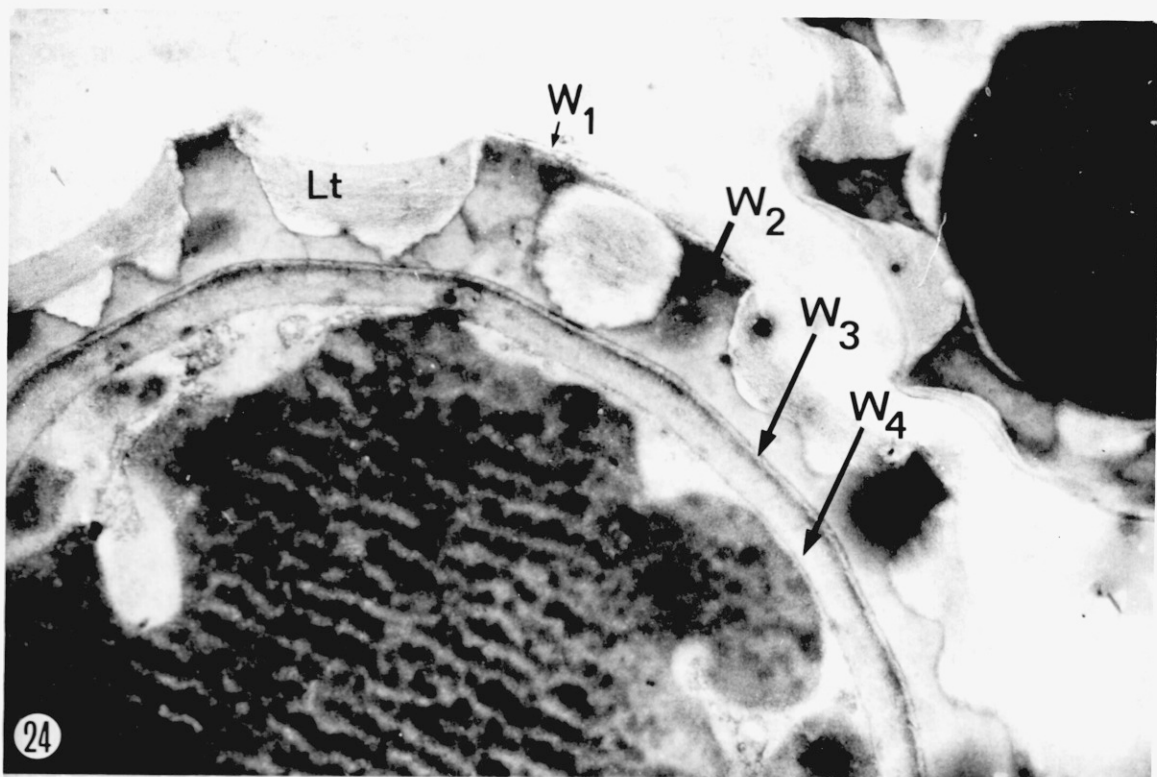
Electron micrographs of maturing teliospores of *T. caries* showing that a thin layer becomes distinct as it detaches from the outer surface of the spore during the deposition of the second wall layer (W2).

Plate 7:25 Magnification X 18150

Staining: PATCH-SP

Plate 7:26 Magnification X 13300

Staining: PATCH-SP

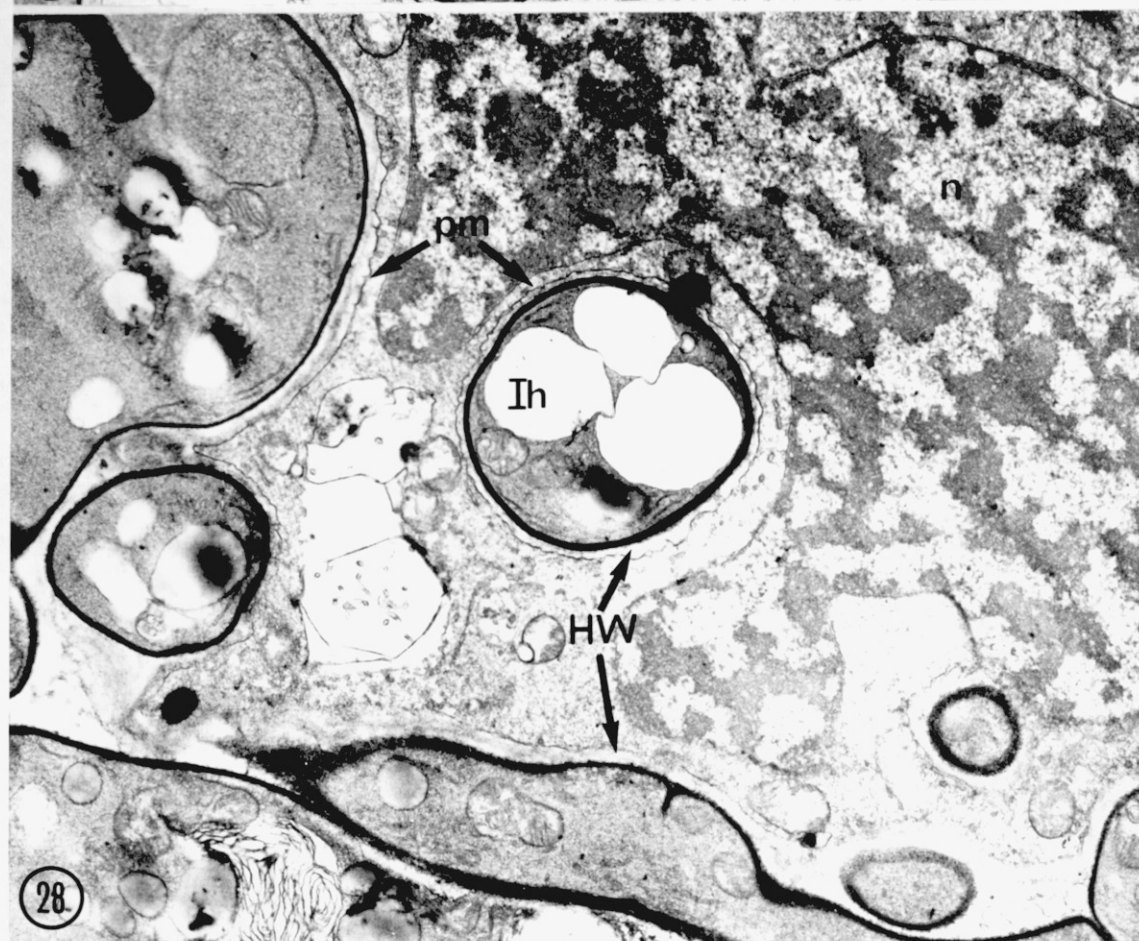
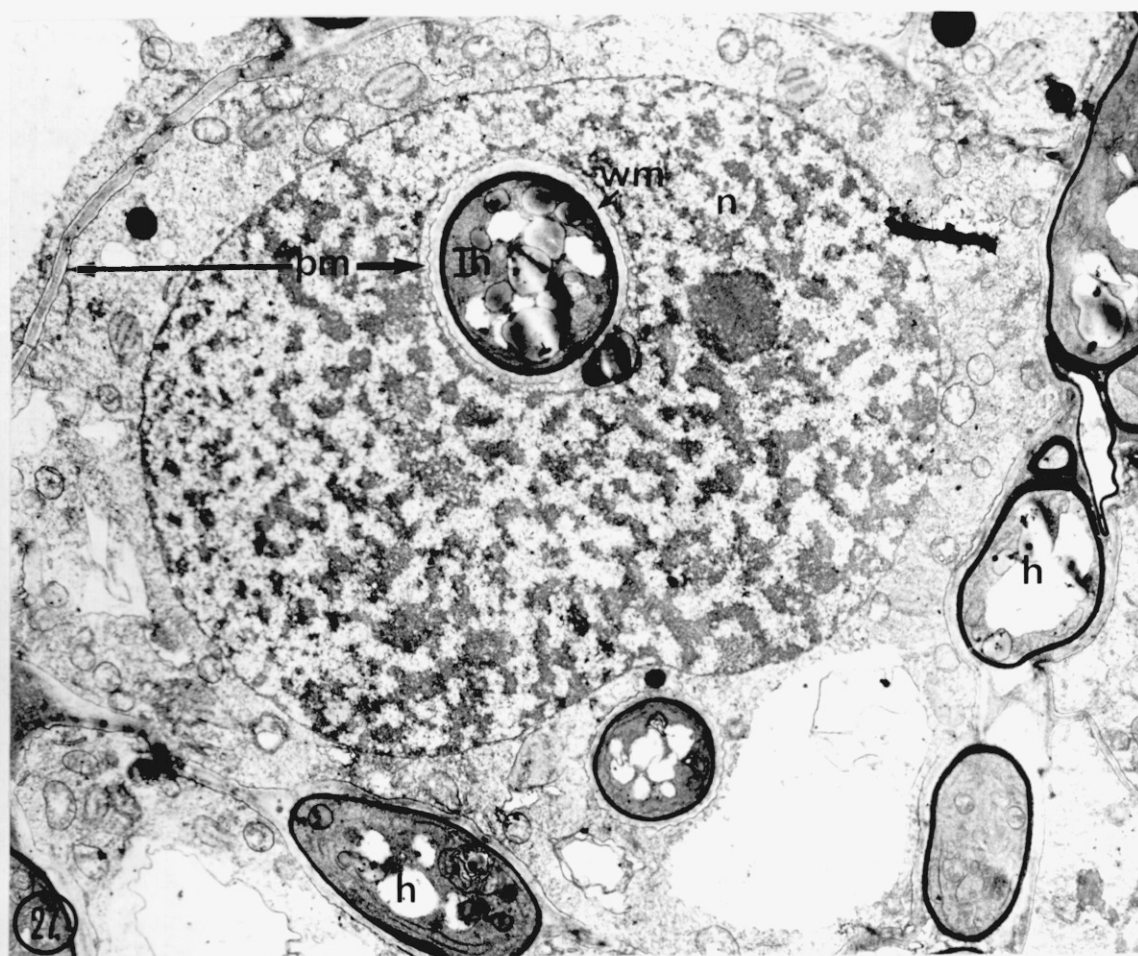


PLATES 7:27 and 7:28

Electron micrographs showing that intracellular hyphae (lh) are formed within cells of the mound tissue. Each intracellular hypha is always surrounded by a wall-like matrix (wm) and a host plasma membrane (pm) indistinguishable from the host plasma membrane lining the invaded cell. The fungal cell wall stains intensely compared to the host cell wall (HW). In each micrograph, one intracellular hypha is closely associated with the host nucleus.

Plate 7:27 Magnification X 7800
 Staining: PATCH-SP

Plate 5:28 Magnification X 12900
 Staining: PATCH-SP



PLATES 7:29 and 7:30

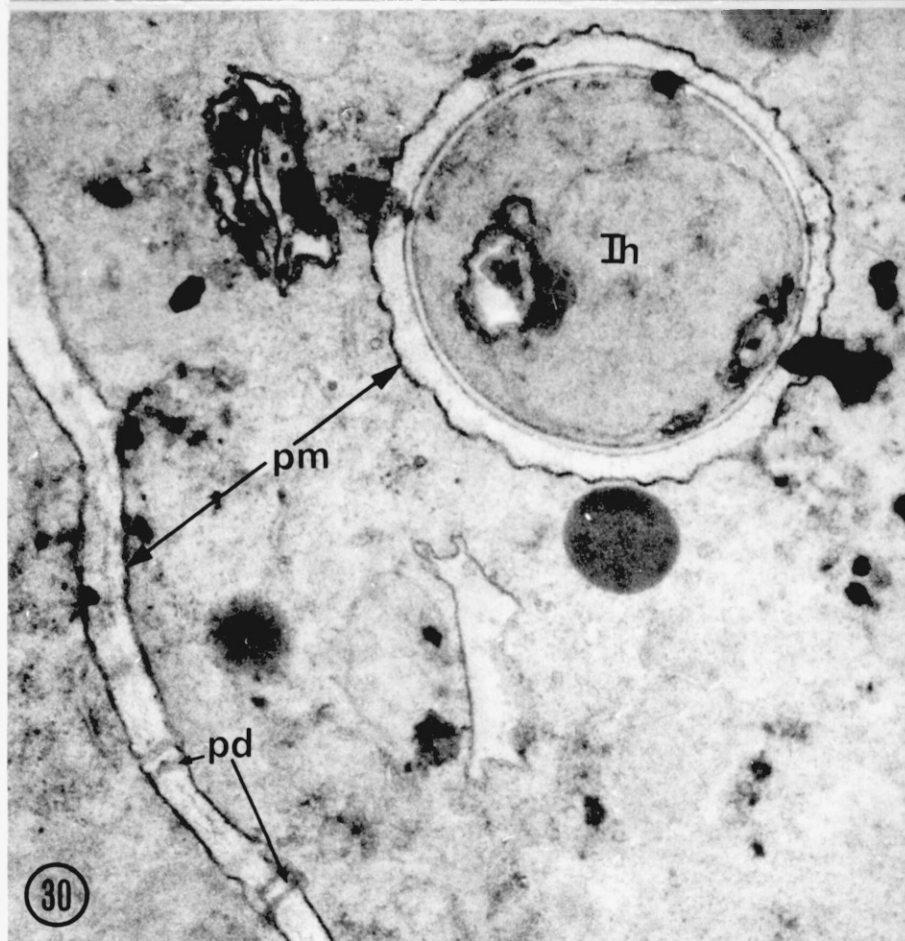
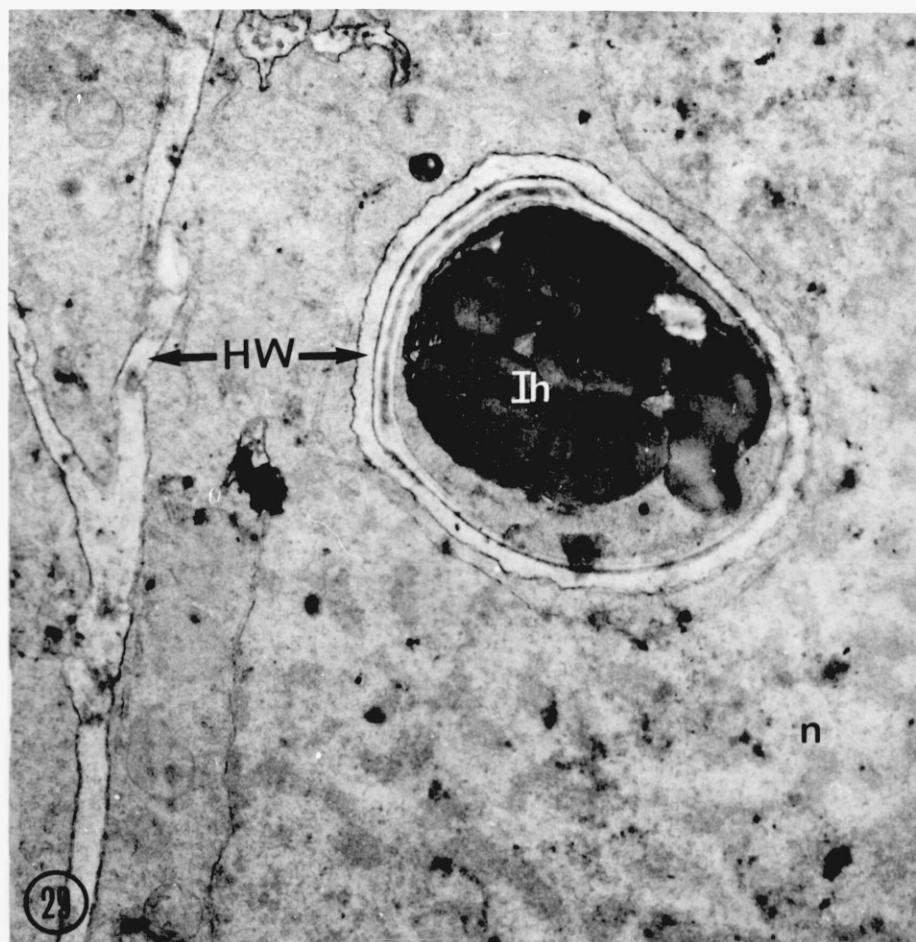
Electron micrographs showing intracellular hyphae (lh), within the mound region, surrounded by a host plasma-membrane (pm) indistinguishable from the membrane lining the host cell wall. Most internal membranes stain weakly with this technique. The wall-like material (wm) surrounding the intracellular hypha stains similarly to the host cell wall (HW).

Plate 7:29 Magnification X 16500

Staining : PACP

Plate 7:30 Magnification X 27100

Staining : PACP



SECTION EIGHT

THE DEVELOPMENT OF *CLAVICEPS PURPUREA* IN WHEAT.

8.1. INTRODUCTION.

Claviceps purpurea infection of wheat and rye and the subsequent colonisation of the host has previously been investigated by Luttrell (1980), and Shaw and Mantle (1980). It was shown to infect these hosts during flowering. The fungus either penetrates the stigmatic or ovary wall surface, growing via the transmitting tissue in the style around the outer integument surrounding the unpollinated ovule, to the base of the ovary (Shaw, 1979). Colonisation continues both into the ovule and outward between the cells of the ovary wall. Intercellular hyphae were shown to degrade the middle lamella of cell walls pushing the host cells apart (Shaw and Mantle, 1980). These cells invaginate and their contents degenerate. The parasite establishes at the base of the ovary forming an interface between the developing stroma above and the living host cells of the rachilla below. Fungal hyphae were not observed in the rachilla of wheat, confined by a plate of host cells at the base of the ovary (Shaw, 1979), though in rye they were found in and around the central vascular bundle within the rachilla (Luttrell, 1980). Above this interface fungal stroma, termed sphacelial tissue, destroys and replaces all the ovary tissue. Conidia, formed from conidial pockets within the top of the sphacelium, exude from the floret in a honeydew secretion 7-10 days after artificial inoculation. Sphacelial tissue develops acropetally from the base, a generative zone, and it is here that sclerotial hyphae are first visible. The sclerotial hyphae proliferate producing the characteristic sclerotium, the overwintering stage of the parasite.

The association of parasitic hyphae within the pedicel tissue of both millet (This thesis) and rye (Luttrell, 1980) led to a further investigation of *C. purpurea* on wheat. Photographs presented by Shaw and Mantle (1980) of the interface region contain no vascular tissue within the pedicel and it is possible that an association of hyphae within this region was overlooked.

The relationship between *C. purpurea* and the wheat host was examined at the stage when honey-dew formation began. The parasite having established within the host would still be developing as sclerotial growth is only beginning. Samples were taken between 7 and 10 d.p.i. (section 2.2.2.) and processed for light microscopy (section 2.4),, scanning and transmission electron microscopy (sections 2.3. and 2.6.2.).

8.2.

RESULTS.

8.2.1. DEVELOPMENT OF THE FUNGUS WITHIN THE HOST.

Observations by scanning electron microscopy showed that sphacelial tissue of *C. purpurea* was well established within the unpollinated wheat ovary at the onset of honey-dew formation, approximately 7-10 days post inoculation. The fungal mass had proliferated extensively within the host and also outside the ovary wall filling the gap between it and the protective tissues (PLATE 8:1). Fungal tissue was seen to end at the base of the ovary where the glumes attached to the pedicel (PLATE 8:2). Above the stromal interface, formed between the host and the parasite at the top of the pedicel, the darker staining sclerotial tissue had begun to differentiate (PLATE 8:3). The sclerotial hyphae were bulbous and compacted containing many lipid bodies compared to the narrower, longer and highly vacuolated hyphae of the sphacelial tissue.

Conidial pockets were visible within the developing sphacelial tissue (PLATE 8:3). When median sections were obtained fungal hyphae were observed to have penetrated further into the pedicel region beyond the stromal interface. They grew down towards a core of thick-walled cells (PLATE 8:3). Colonisation within the pedicel was restricted to the vascular tract, hyphae lying adjacent to xylem tracheids and phloem sieve tubes or at the periphery of their corresponding parenchyma cells (PLATES 8:6, 8:7, 8:8 and 8:9). Electron microscopy showed that at the top of the pedicel hyphae completely filled the host cells (PLATES 8:4 and 8:5). Hyphae seen at the core of thick-walled cells either occupied regions between parenchyma cells surrounding the core of thick-walled cells, some which were transfer cells (PLATE 8:9), or within or between the cells of this region (PLATES 8:10 and 8:11).

8.2.2. CYTOLOGY OF THE HOST.

No host cells were visible within the mass of hyphae above the pedicel. At the top of the pedicel only host cell walls remained, contents having been replaced by fungal hyphae (PLATE 8:4). Host cells partially invaded had no distinct contents and fungal hyphae were surrounded by an osmiophilic material (PLATE 8:5). Neighbouring uninvaded cells were at various stages of disorganisation. Their cytoplasm and contents became unrecognisable, vacuoles enlarged and vesicles were numerous (PLATES 8:5 and 8:6). Further towards the core of thick-walled cells the cytoplasm of invaded cells and of those cells surrounding them became less disrupted (PLATES 8:7 and 8:8). Fungal hyphae were mainly restricted to the periphery of the cells enclosed by a cell wall-like material (PLATE 8:8). This material was less electron opaque than the host cell wall and stained

similarly to the parts of the host wall invaginated in transfer cells when stained with uranyl and lead salts , PACP or PATCH-SP (PLATES 8:7 and 8:9). The PACP staining property of the plasma membrane adjacent to the wall-like material surrounding the hyphae was difficult to distinguish due to the proximity and similar staining property of the wall-like material (PLATE 8:12). Where an invaginated plasma membrane was distinct it stained similarly to the host plasma membrane (PLATE 8:8).

In one case an intracellular hypha, lodged between the wall ingrowths of a transfer cell, caused the formation of an electron transparent material similar in texture to encasements formed around haustoria (PLATE 8:13).

8.2.3. CYTOLOGY OF THE FUNGUS.

Intracellular fungal hyphae and those within the core of thick-walled cells were distinguishable from those at the top of the pedicel because their cytoplasm was much denser and only lipid bodies were visible (PLATES 8:9, 8:10 and 8:11). As the hyphae neared the top of the pedicel their cytoplasm became lighter, lipid bodies were still numerous, vacuoles larger and nuclei were prominent (PLATE 8:5). Each hyphal segment contained two nuclei which lay adjacent to each other, some contained more than two (PLATE 8:5). Septa were frequently seen in hyphae occupying positions between the host cells and at their periphery (PLATES 8:7 and 8:10).

PLATES 8:1 and 8:2

Scanning electron micrographs of wheat ovaries
longitudinally fractured 7-10 days post inoculation.

PLATE 8:1

The ovary is completely replaced by the fungal
stroma (St). Only a few cells of the ovary wall
(OW) and those in the pedicel (Pd) remain.

Magnification X 83

PLATE 8:2

The fungal stroma (St) has replaced all of the
ovary tissue and a stromal interface (I) between
the fungus and the host is established at the top
of the pedicel (Pd).

Magnification X 100

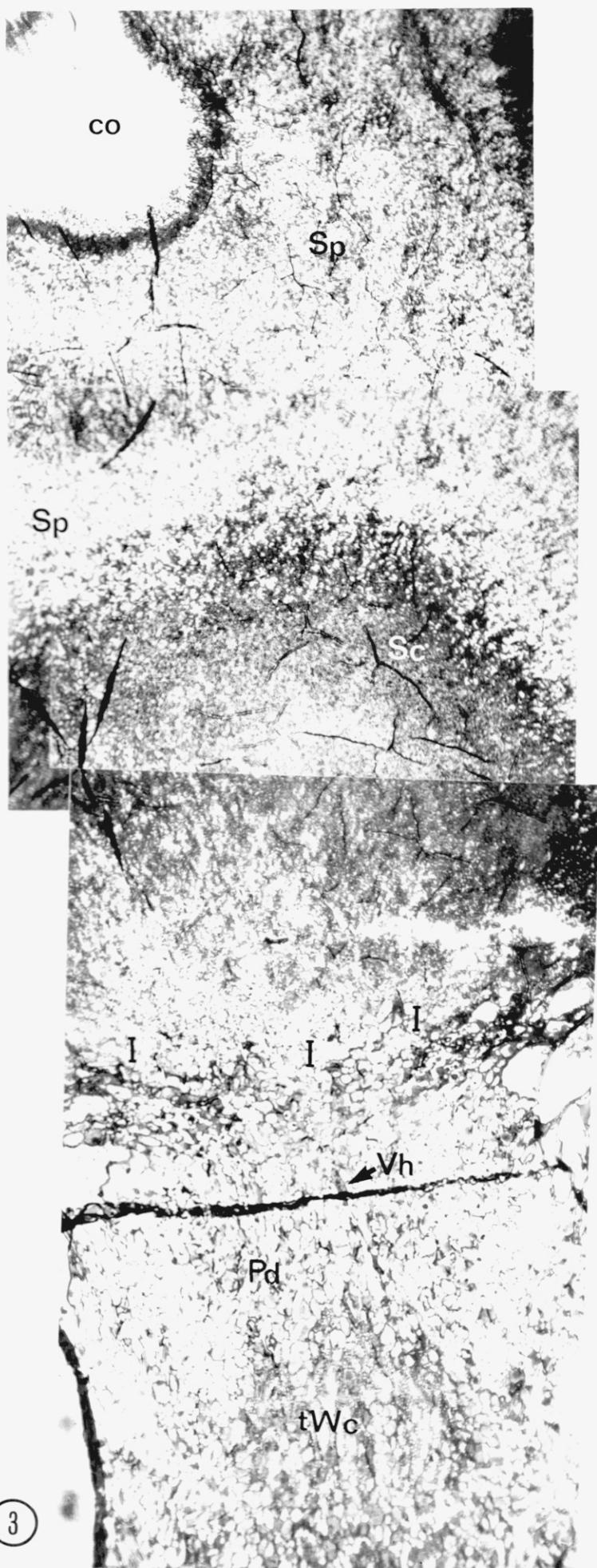


PLATE 8:3

Light micrograph showing a longitudinal section through a wheat ovary 7-10 days post inoculation. Conidial pockets (Co) have developed within the sphacelial tissue (Sp) and darker staining hyphae of the sclerotial tissue (Sc) occur just above the stromal interface (I). This median section shows that some hyphae (Vh) penetrate into the pedicel tissue growing down towards the core of thick-walled cells (tWc).

Magnification X 140

Staining: Toluidine blue



PLATES 8:4 and 8:5

Electron micrographs of an ovary 7-10 days after infection.

PLATE 8:4

Host cells in the upper part of the pedicel are filled with hyphae (h). No organised contents are visible within the cells and only the host cell wall remains (HW).

Magnification X

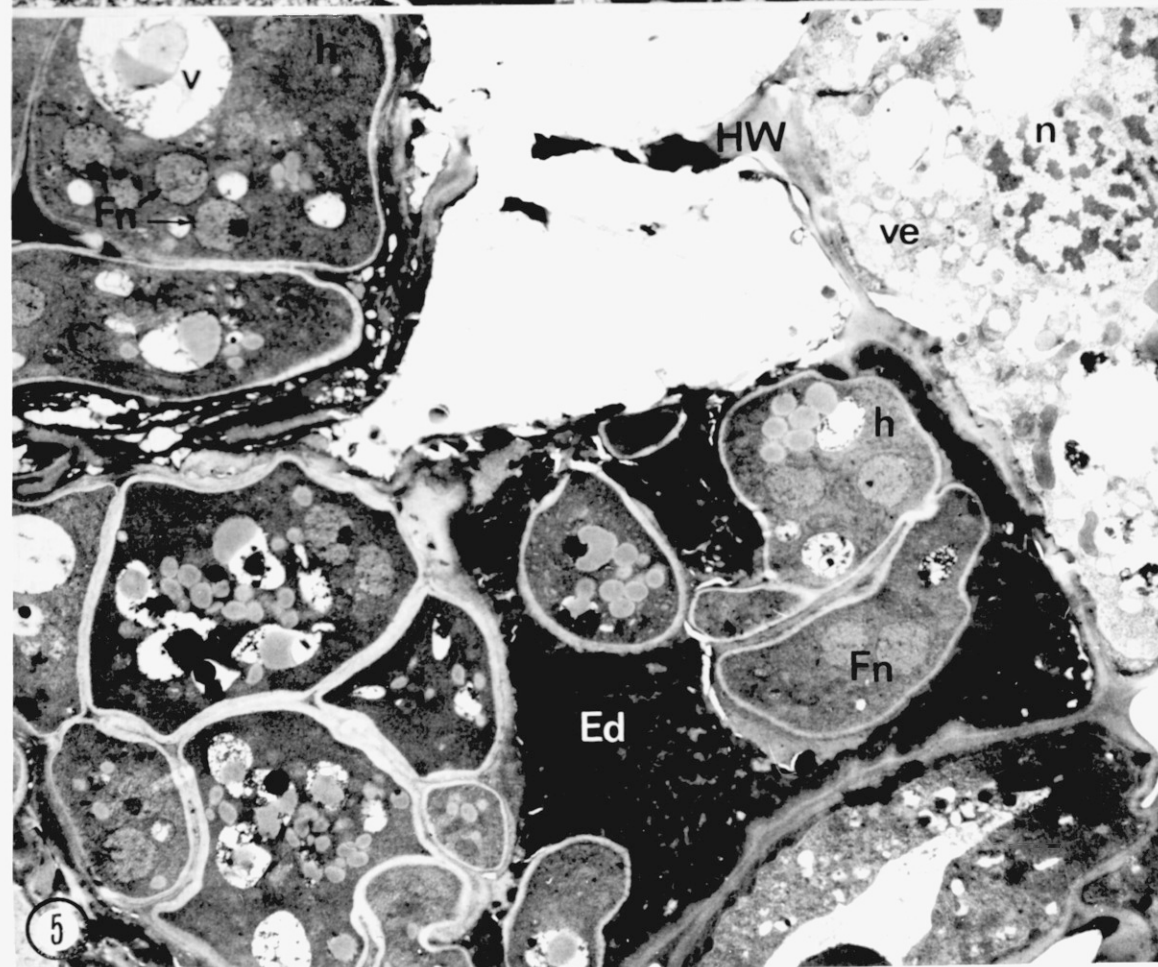
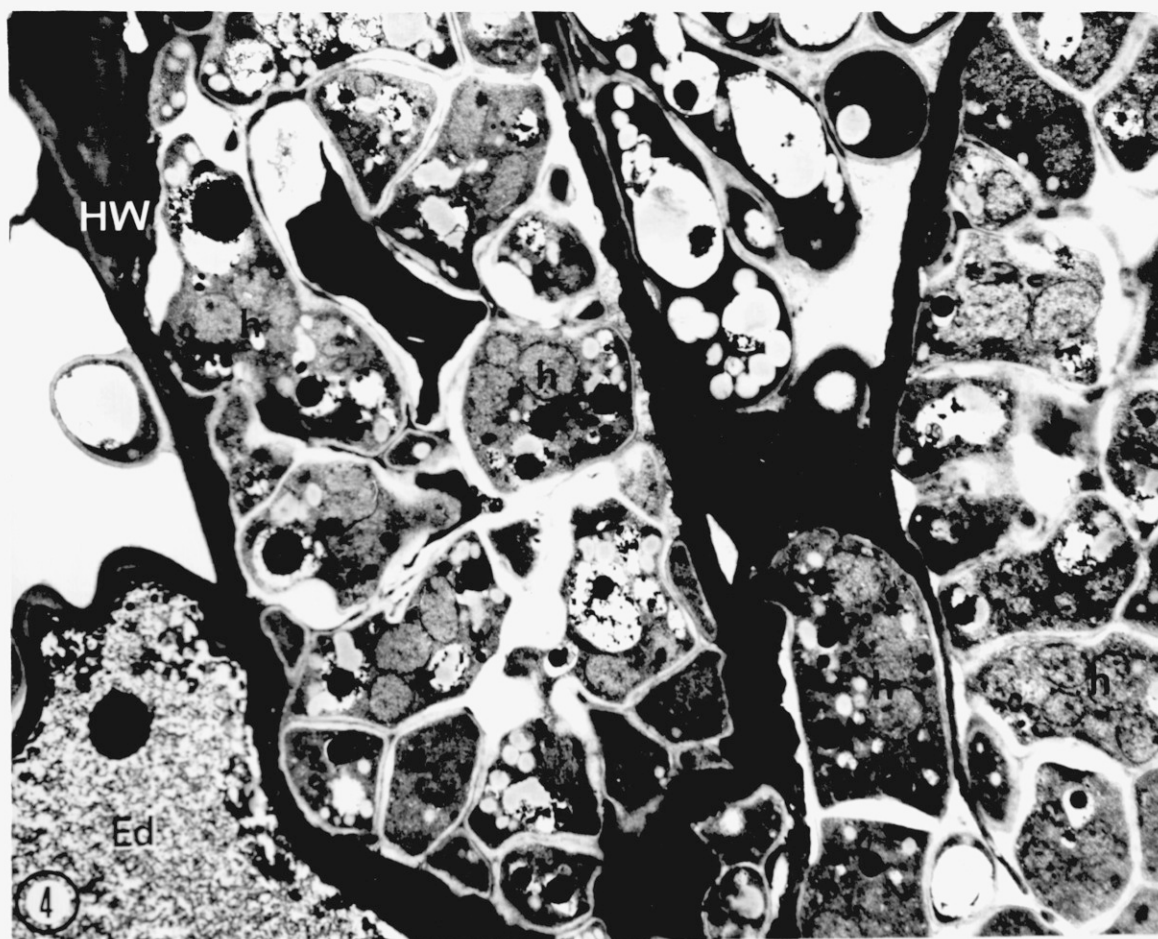
Staining PATCH-SP

PLATE 8:5

Not all host cells in the upper part of the pedicel are packed with fungal hyphae, some still have recognisable organelles. Some cells adjacent to host cells occupied by fungal hyphae are disrupting, their cytoplasm containing many vesicles (ve). An osmophilic material (Ed) fills the spaces within the host cell occupied by hyphae. Most hyphal segments have two nuclei (Fn) lying adjacent to each other but some hyphal segments had more than two.

Magnification X 5130

Staining: PATCH-SP



PLATES 8:6 and 8:7

Electron micrographs of infected pedicel tissue

7-10 days after inoculation.

PLATE 8:6

In the basal region of the pedicel fungal hyphae (h) occupy host cells adjacent to host vascular tissues, especially those adjacent to phloem sieve tubes (Ph). Host cells not invaded by fungal hyphae contain visible nuclei (n) and a densely staining cytoplasm.

Magnification X 4250

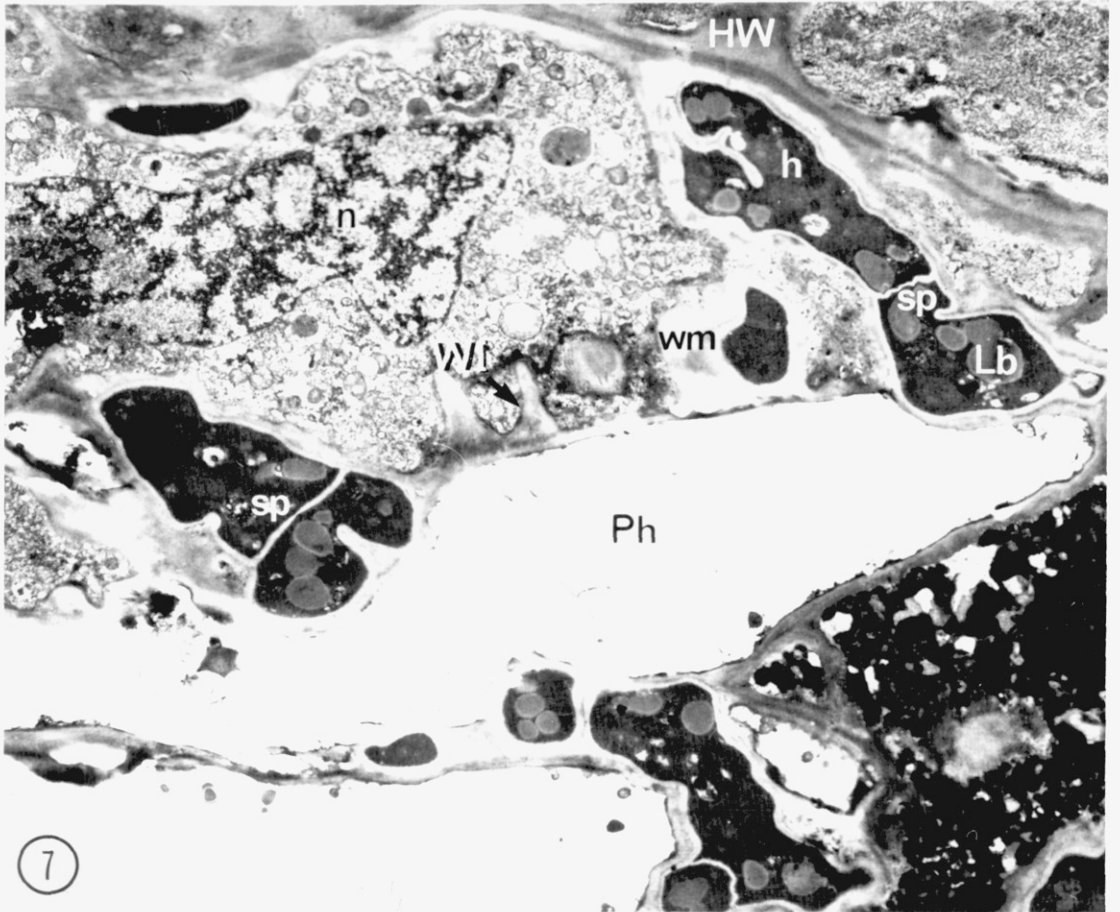
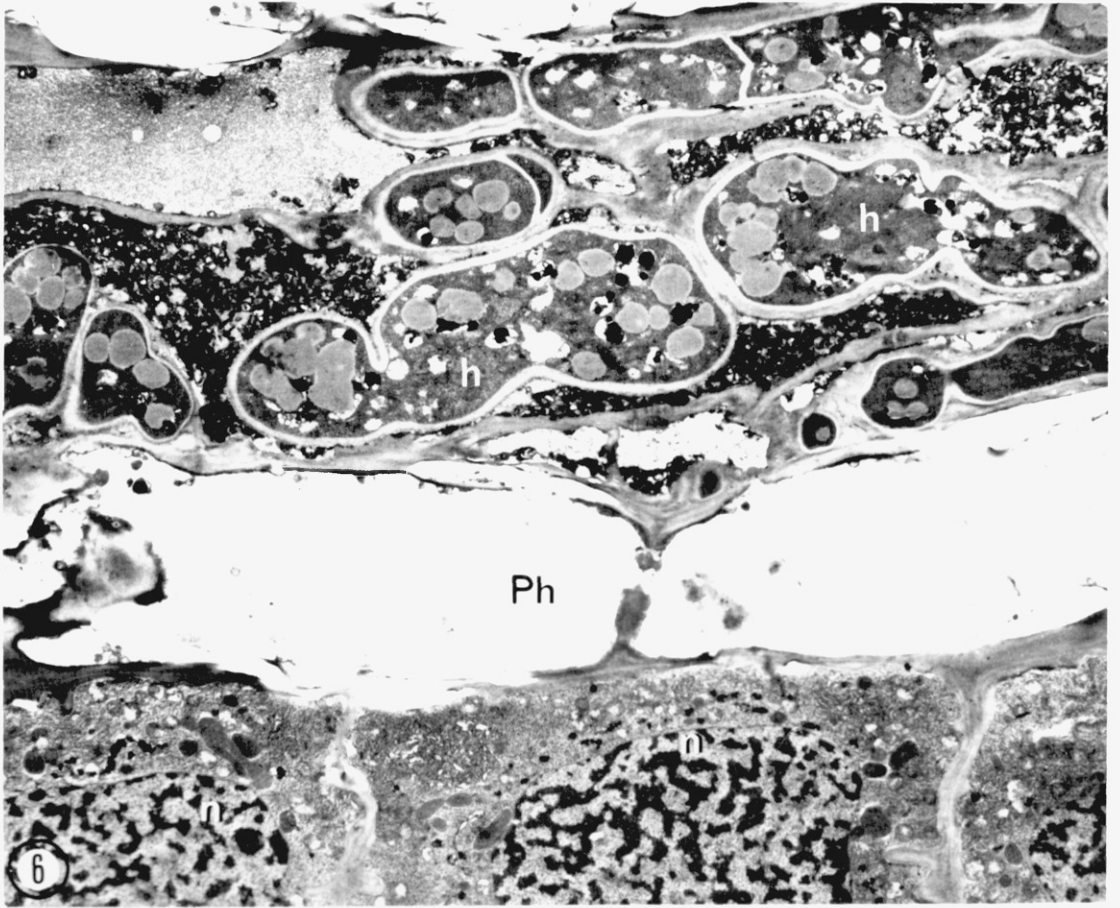
Staining: PATCH-SP

PLATE 8:7

Fungal hyphae within a transfer cell, adjacent to a phloem sieve tube (Ph), are surrounded by a material (wm) which stains lighter than the host cell wall (HW), but similarly to the wall ingrowths (WI). Invading fungal hyphae initially colonise the periphery of the host cell. Septa are sometimes seen but most hyphal contents are obscured by their densely staining cytoplasm. Some lipid bodies (Lb) are visible in fungal hyphae.

Magnification X 5130

Staining: PATCH-SP



PLATES 8:8 and 8:9

Electron micrographs of infected pedicel tissue
7-10 days after inoculation.

PLATE 8:8

Intracellular hyphae invading the host cells near
the core of thick walled cells (tWc). All hyphae
are surrounded by light wall-like material (wm).
Some hyphae occupy cells whose contents have
disrupted.

Magnification X 4250

Staining: PATCH-SP

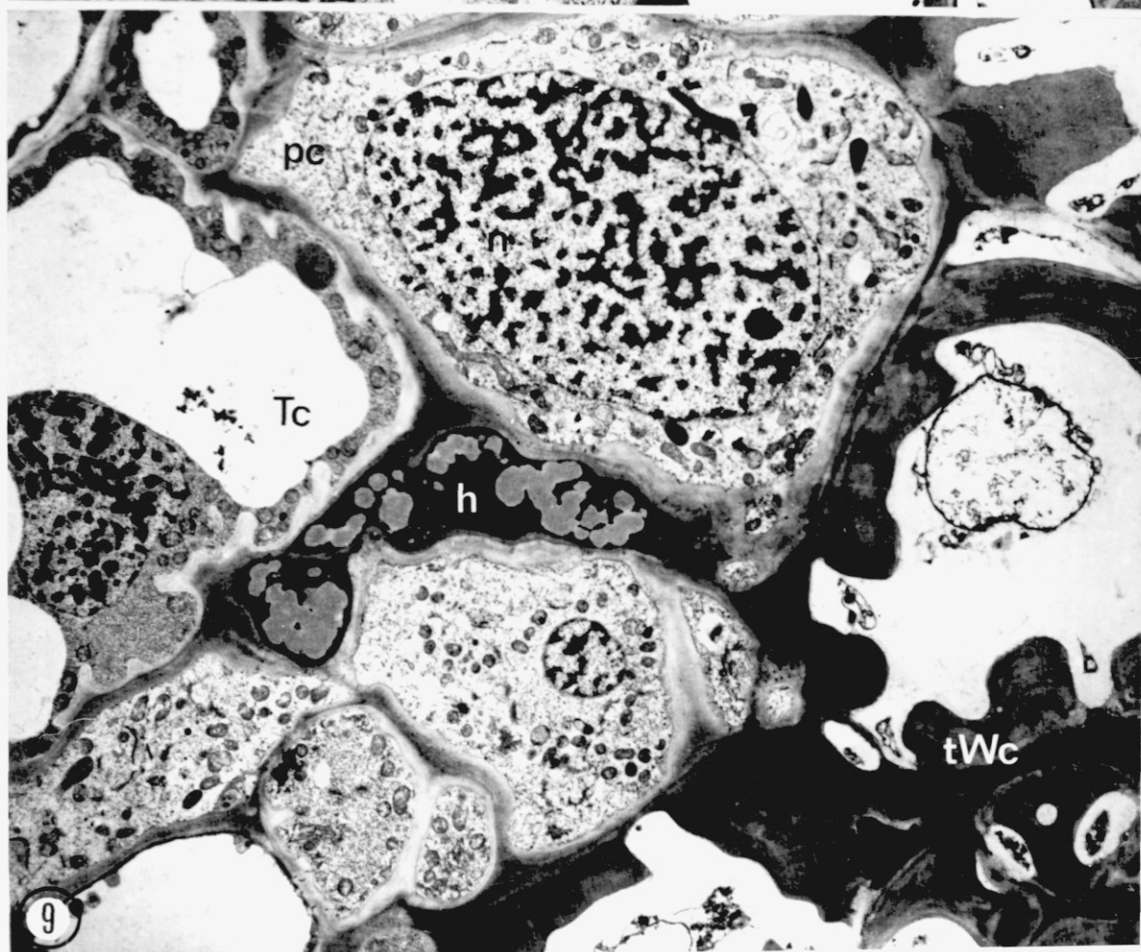
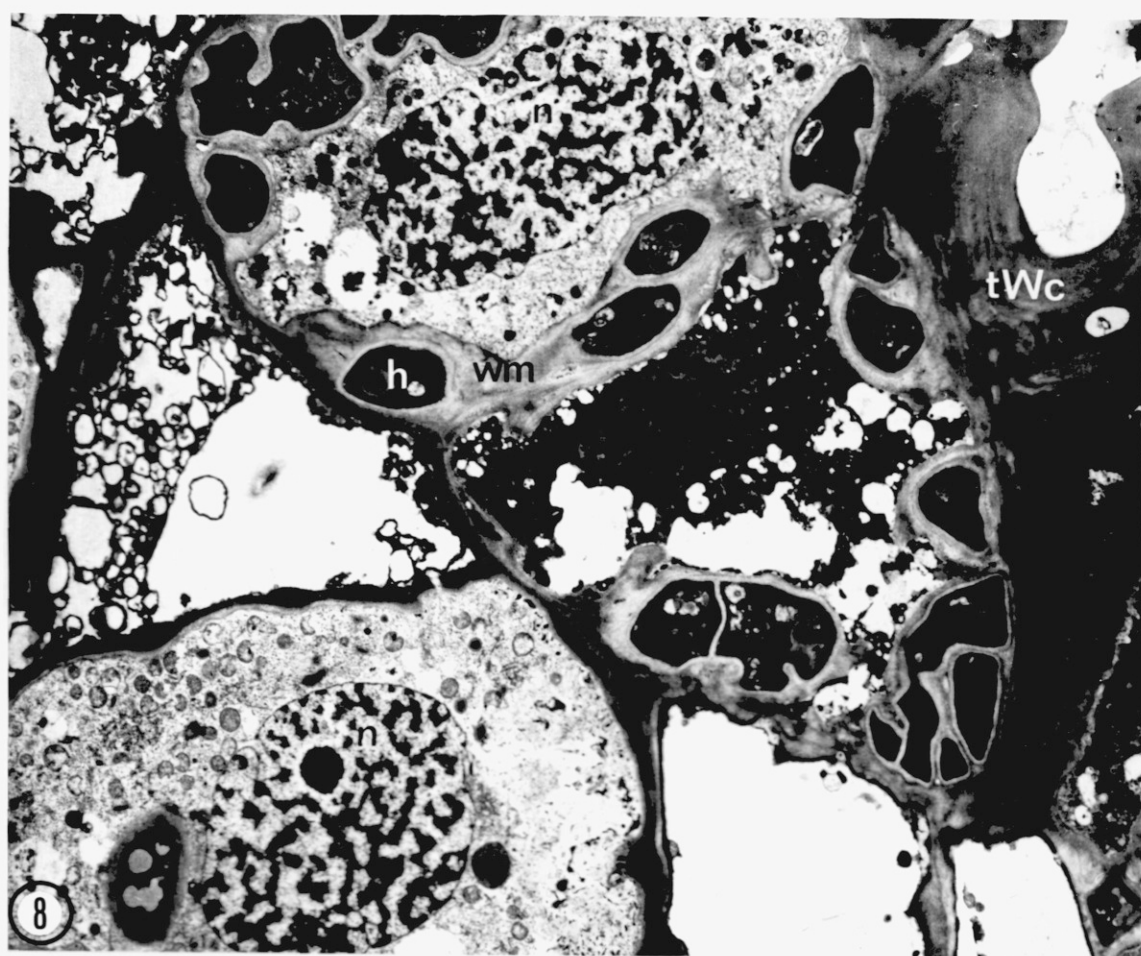
PLATE 8:9

Intercellular hypha (h) lying adjacent to a
transfer cell (Tc) in the pedicel.

Thick walled cells (tWc), parenchyma cell (pc)

Magnification X 4250

Staining: PATCH-SP



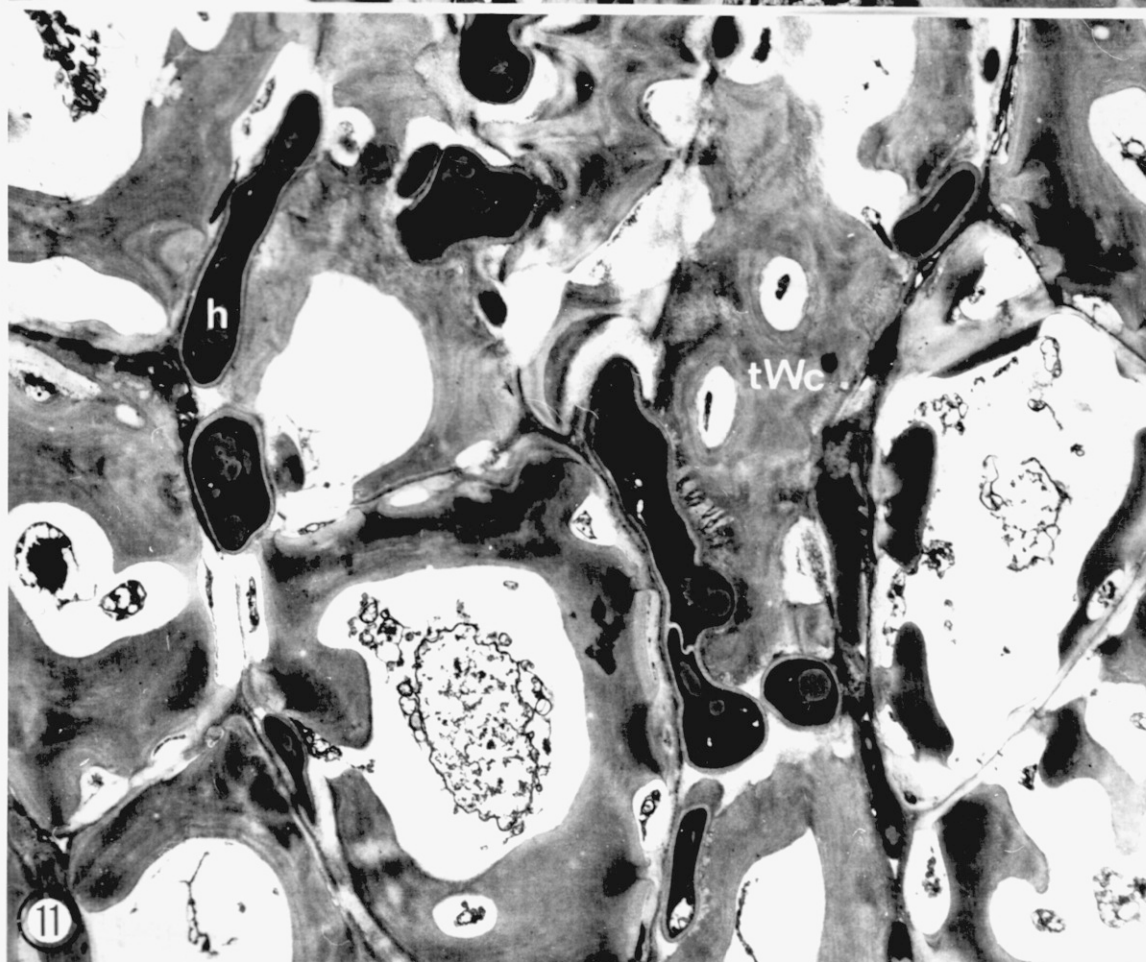
PLATES 8:10 and 8:11

Electron micrographs of infected pedicel tissue
7-10 days after inoculation.

Many fungal hyphae (h) are observed within the core of thick walled cells (tWc). The contents of the thick walled cells range from normal, to disorganised to none. The fungal hyphae are distinct due to their intensely staining cytoplasm and their lipid bodies (Lb).

PLATE 8:10 Magnification X 4250
 Staining: PATCH-SP

PLATE 8:11 Magnification X 4250
 Staining: PATCH-SP



PLATES 8:12 and 8:13

Electron micrographs of infected pedicel tissue

7-10 days after inoculation.

PLATE 8:12

Hyphae (h) growing at the periphery of the host cell sometimes have an electron dense material (Ed) surrounding them. This material stains similarly to parts of the host wall but the staining is not consistent. The plasma membrane (pm) surrounding this material is, in some regions, difficult to distinguish.

Magnification X 5350

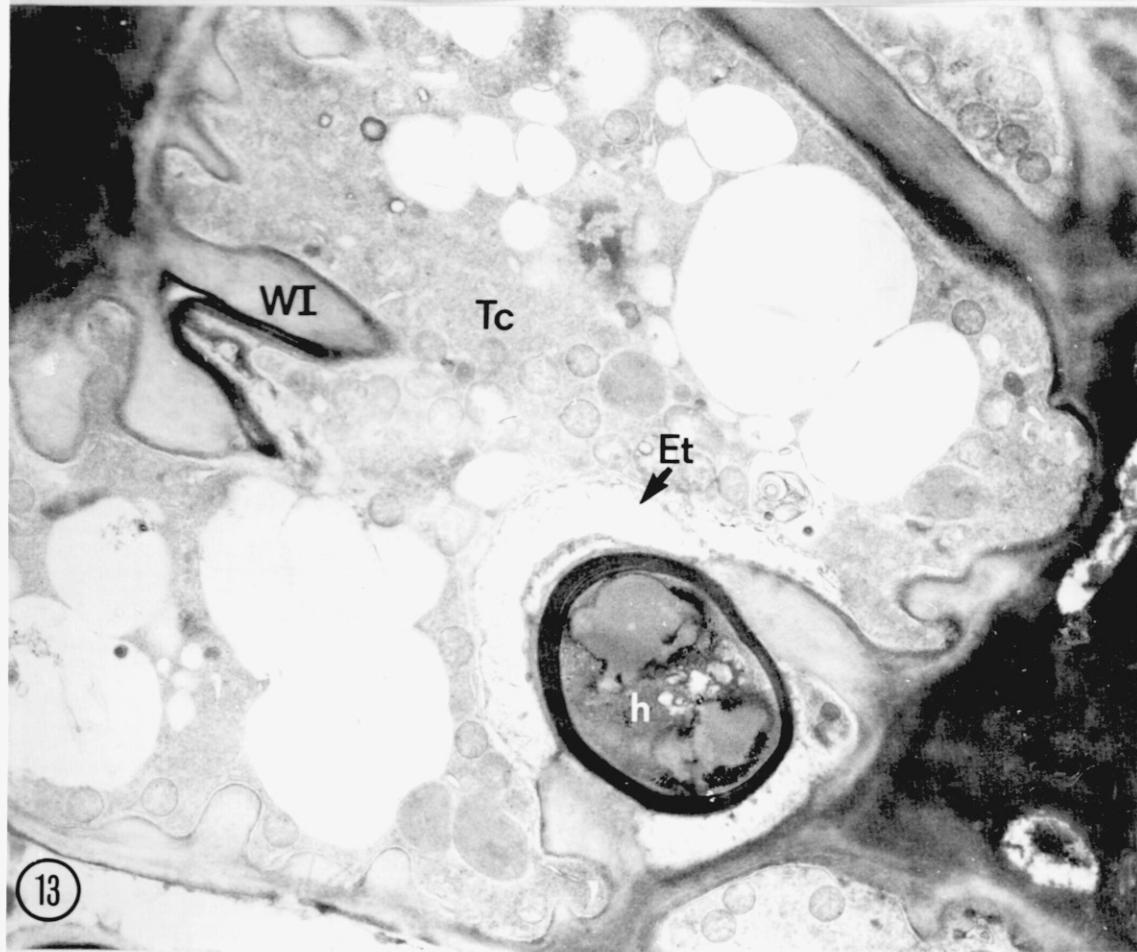
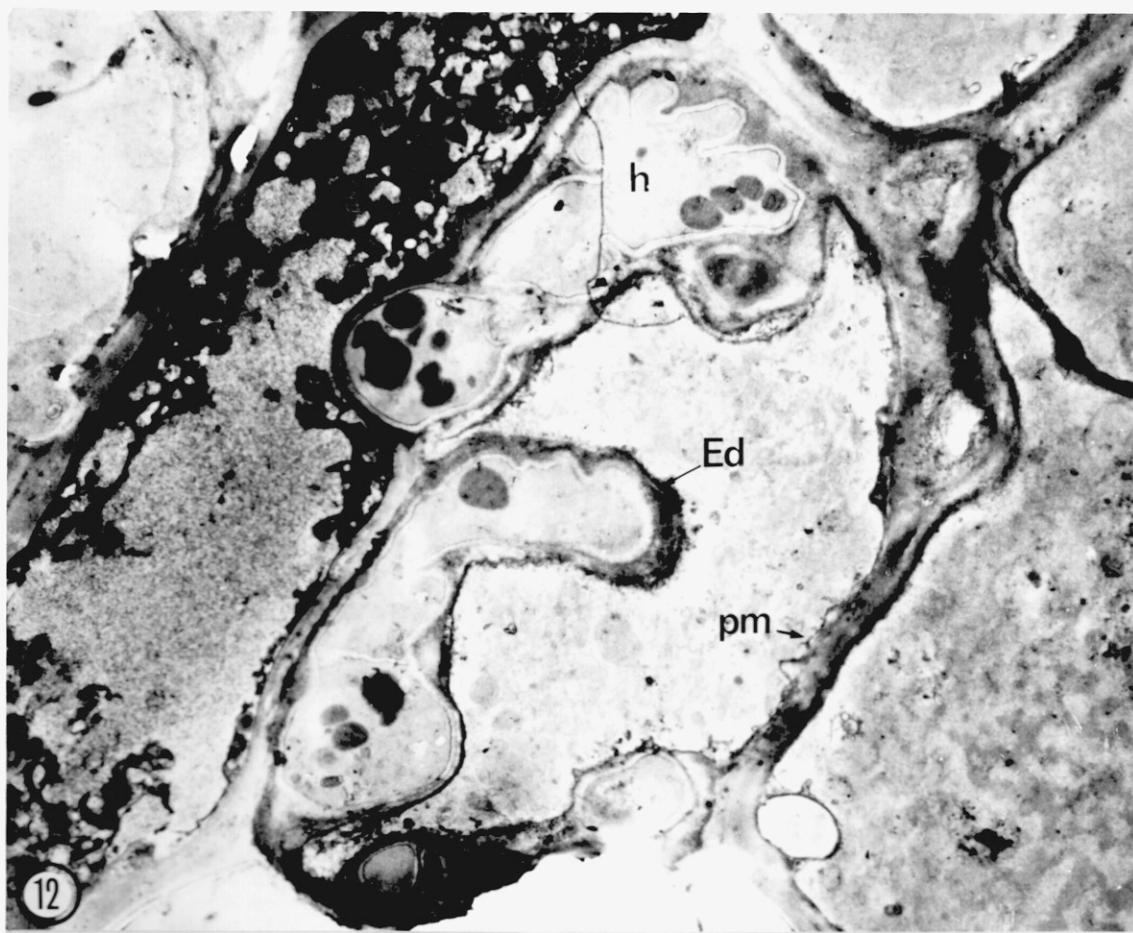
Staining: PACP

PLATE 8:13

An electron transparent (Et) material is visible where a fungal hypha (h) has grown between two wall ingrowths (WI) of a transfer cell. This material is much lighter than the wall ingrowths and has a texture similar to callose found in collars and encasements around haustoria.

Magnification X 9400

Staining: PATCH-SP



SECTION NINE

INFECTION OF WHEAT BY

TILLETIA CARIES AND *CLAVICEPS PURPUREA*.

9.1. INTRODUCTION

Observations from the present investigation suggested that if *T.caries* infection replaced the seed and that infection by *C.purpurea* displaced the seed then it was possible for both to occur in the same ovary. Infection of the wheat inflorescence by more than one parasite has previously been observed (Hanna, 1932;) where both smut and bunt symptoms were expressed on same ear (the former at the top and the latter at the base of inflorescence). Infection of the ovary with two pathogens is a less common phenomenon though infections of wheat with *T.caries* and *C.purpurea* have been observed by Hanna (1938) where the bunt ball remained fused to the tip of the developing sclerotium. Because the anthers become trapped by the expanding ovary during the formation of the bunt ball and the stigmas wither the path of *C.purpurea* infection through the stigma (Shaw and Mantle, 1980) is excluded and is instead directed towards the base of the ovary (Willingale and Mantle, 1987b).

A brief survey of wheat ovaries infected with both *T.caries* and *C.purpurea* was undertaken to clarify the ideas, already set out in this thesis, that bunt (and smuts generally) replace the host tissues whilst *Claviceps* species displace them.

9.2. RESULTS

C.purpurea sphacelial tissue was well established 10 days post inoculation in all samples which had been previously infected with *T.caries*. *C.purpurea* hyphae colonised all the host cells enveloping the cup-shaped layer of *T.caries* teliospores (Plates 9:1 and 9:2).

C.purpurea hyphae were abundant in the ventral wall of the ovary having entered near the base of the ovary. Sub-cuticular growth on the epidermal cells of the ovary wall in or near the crease region was evident (Plate 9:3). Once established at the base of the ovary *C.purpurea* growth continues acropetally displacing the *T.caries* teliospores into the upper portion of the ovary (Plates 9:1 and 9:2). Transverse sections of the infected structure showed that host cells were still visible in the placenta-chalazal like region (PLATE 9:3) [the mound region described in section 7.2.1.1. *T.caries* teliospores filled the cavity whilst *C.purpurea* hyphae colonised all remaining host tissues, including the vascular tissues in the pericarp on the ventral side (Plates 9:3 and 9:5). *C.purpurea* hyphae were observed, in median sections, to enter the pedicel, as described in section 8.2.1., penetrating down to the core of thick-walled cells.

Teliospores of *T.caries* filled the cavity into which the now *C.purpurea* colonised placenta mound-like tissue projected and conidia from *C.purpurea* freely mixed with the teliospores adjacent to the remaining mound tissue (Plate 9:6). However the majority, if not all, of the conidial pockets formed by *C.purpurea* hyphae were produced on the outer surface of the sphacelium (Plates 9:7, 9:8 and 9:9). *C.purpurea* hyphae were not observed to colonise any part of the cavity filled with *T.caries* teliospores, they remained separated as two domains by a compacted layer composed of fungal hyphae and/or host cells (Plates 9:10 and 9:11).

PLATE 9:1

Scanning electron micrograph showing a longitudinal fracture through a wheat ovary infected with both *T.caries* and *C.purpurea*. The *C.purpurea* stroma has displaced the *T.caries* teliospores into the upper portion of the ovary. A few host cells are visible in the pedicel (Pd).

Magnification X 33

PLATE 9:2

Scanning electron micrograph showing longitudinal and transverse fractures of a wheat ovary infected with both *T.caries* and *C.purpurea*. The teliospores (T) of *T.caries* are totally surrounded by the fungal stroma (St) of *C.purpurea*.

Magnification X 33

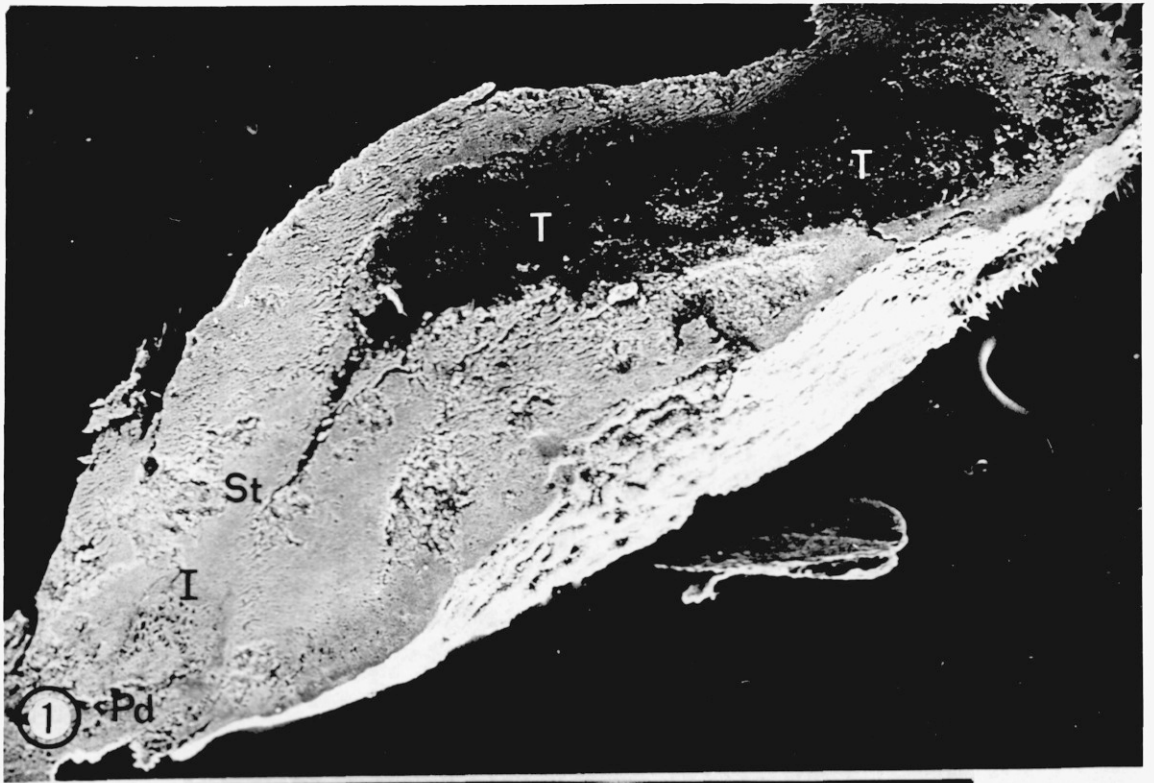


PLATE 9:3

Light micrograph showing a median transverse section through a wheat ovary. *T. caries* teliospores (T) form a cup-shape around *C. purpurea* hyphae and a mound of host cells, similar to the placenta-chalazal (PC) in pollinated tissue. The ovary wall (OW) is also distinguishable and the region of the placenta-chalaza which normally contains vascular tissue is filled with *C. purpurea* hyphae (h).

Magnification X 175

Staining : Toluidine blue

PLATE 9:4

An electron micrograph showing *C. purpurea* hyphae (h) growing beneath the cuticle (cu) of the epidermal cells of the ovary wall (OW).

Magnification X 3900

Staining: Uranyl acetate and lead citrate

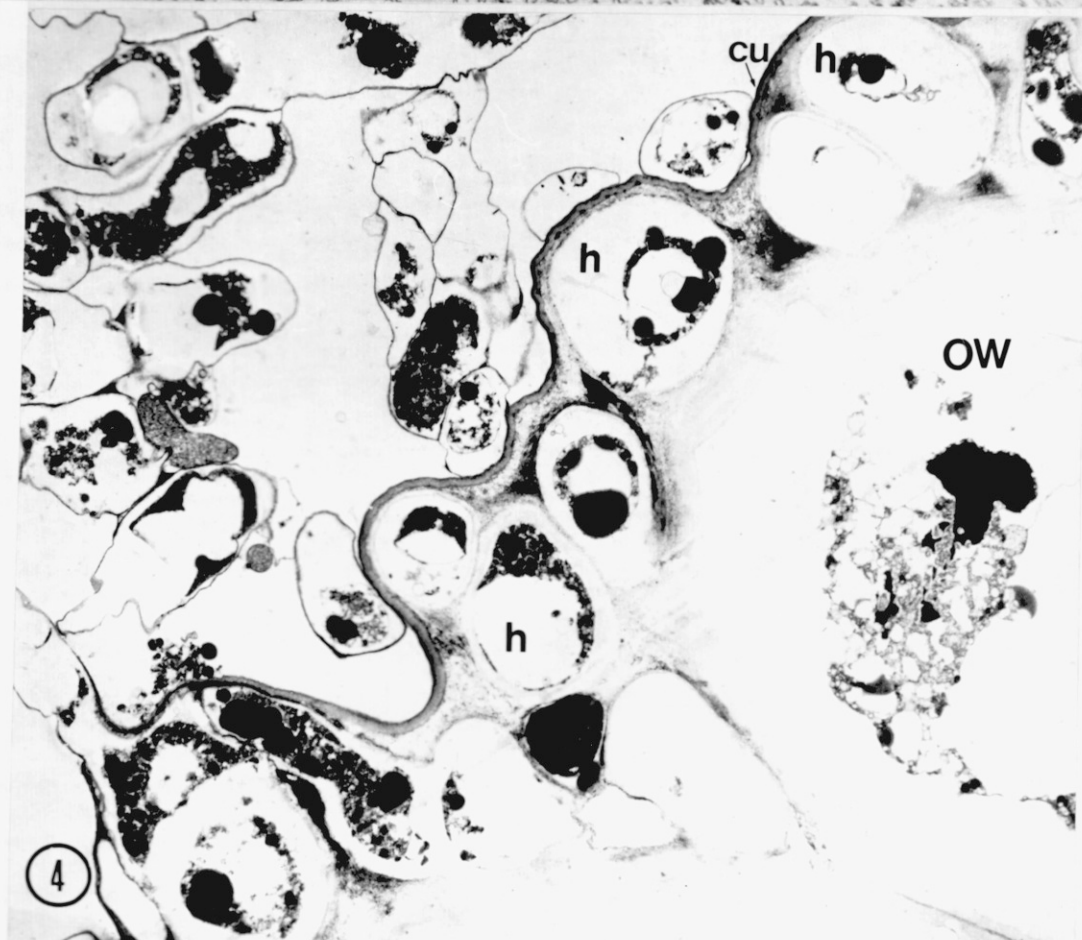
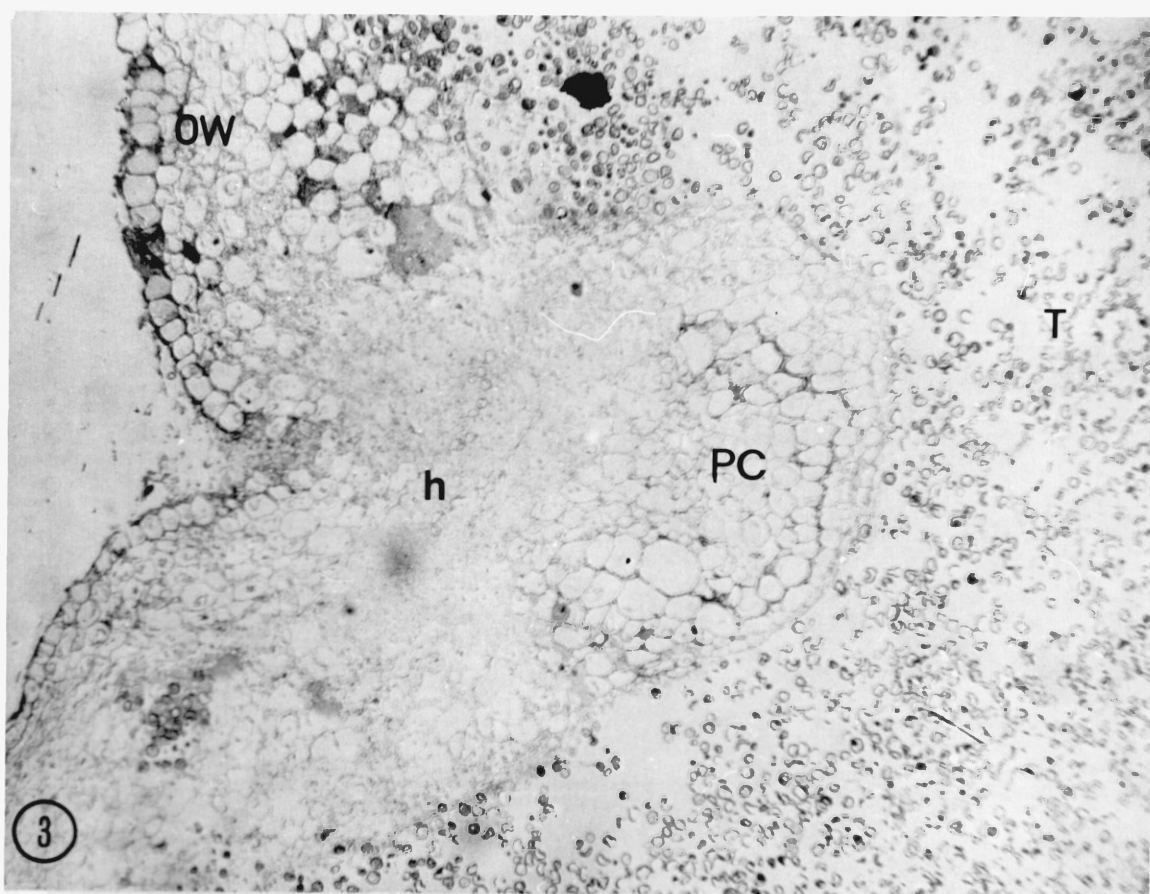


PLATE 9:5

Light micrograph showing part of Plate 9:3.

C. purpurea hyphae (h) have replaced the vascular tissues of the placenta-chalaza leaving only the xylem tracheids (Xy) remain visible. *T. caries* teliospores (T) fill the cavity into which the placenta-chalazal tissue projects.

Magnification X 425

Staining : Toluidine blue

PLATE 9:6

Electron micrograph of the placenta chalazal tissue where the two pathogens meet. Adjacent to the placenta-chalazal tissues *C. purpurea* conidia (Co) occur among the *T. caries* teliospores (T) within the intercellular spaces.

Magnification X 1700

Staining:Uranyl acetate and lead citrate

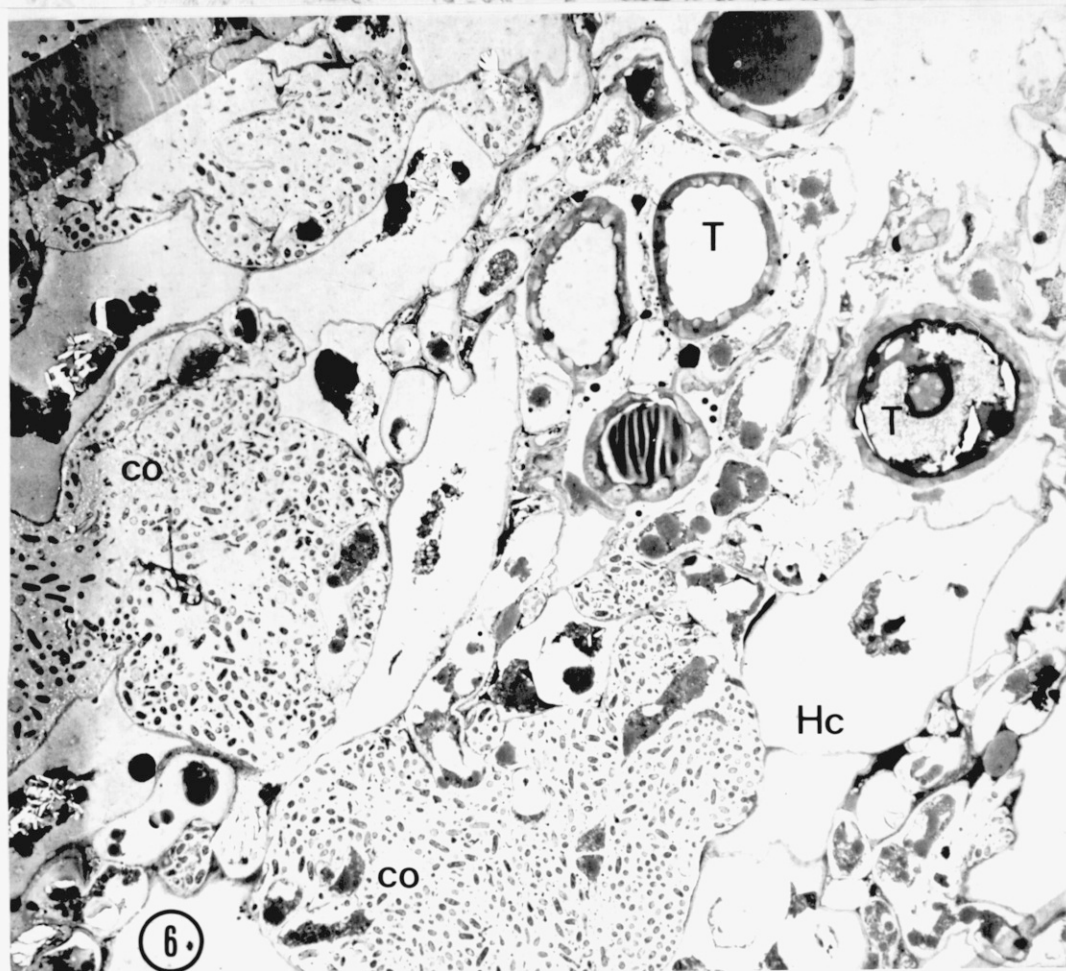
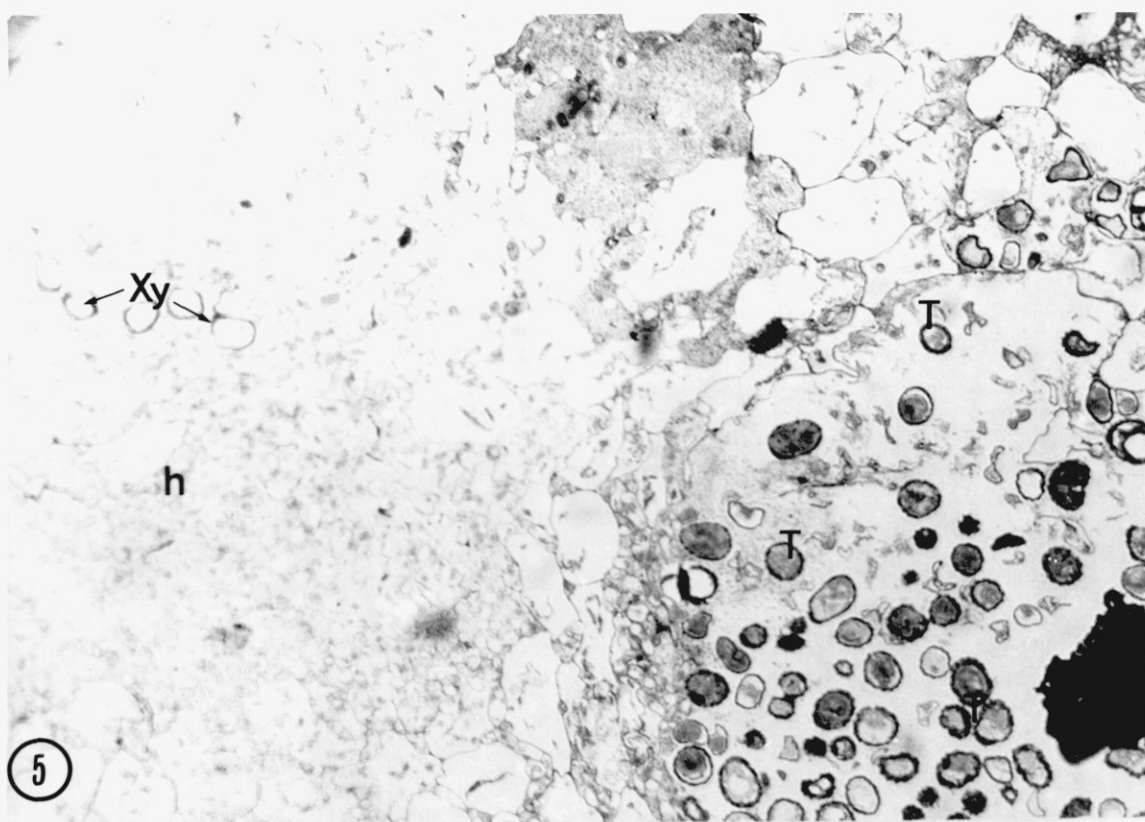


PLATE 9:7

Light micrograph of a conidial pocket (Co) produced from the sphacelial hyphae (Sp).

Magnification X 936

Staining : Toluidine blue

PLATE 9:8

Scanning electron micrograph showing the labyrinth of conidial pockets (Co) produced on the outer surface of the sphacelium (Sp).

Magnification X 30

PLATE 9.9

Scanning electron micrograph showing the conidial pockets (Co) located in the outer surface of the sphacelium (Sp) and the central region occupied by *T. caries* teliospores.

Magnification X 33

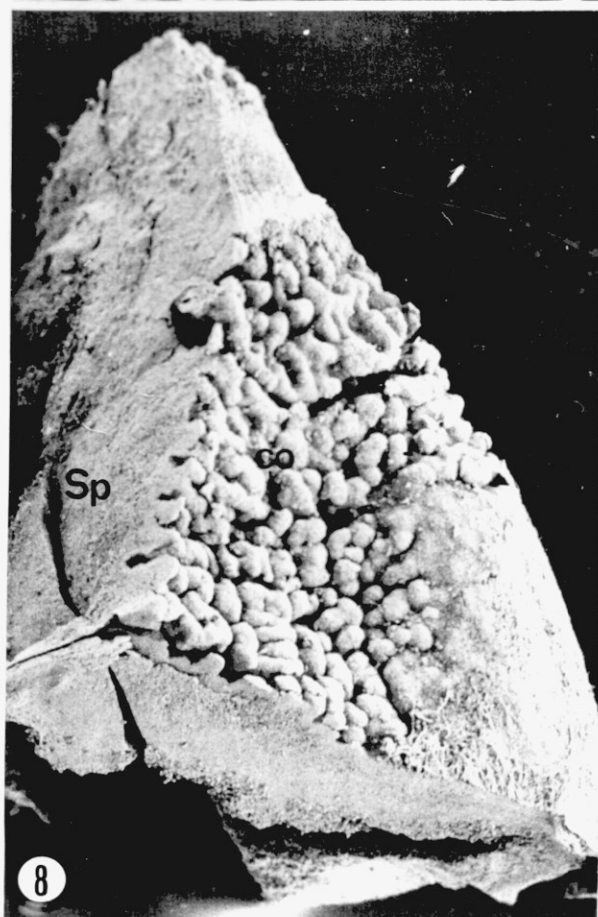
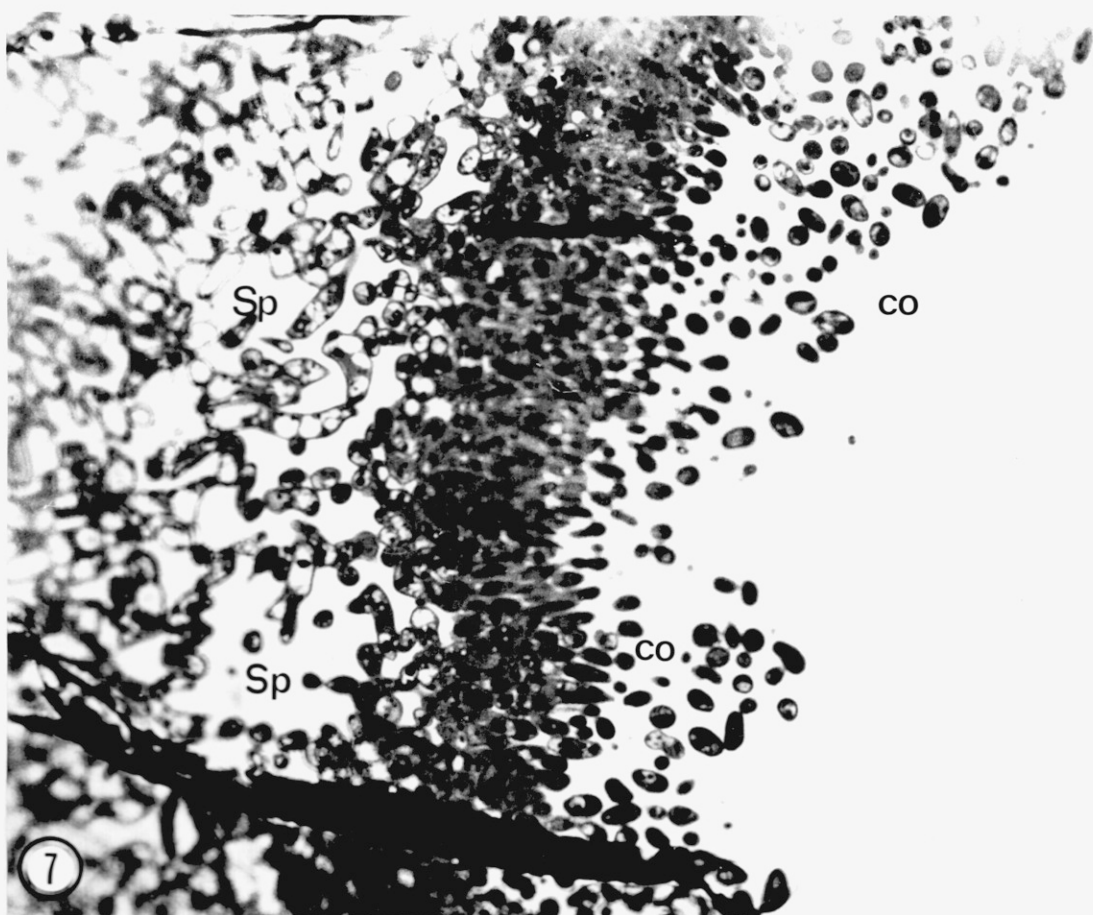


PLATE 9:10

Light micrograph showing the interface (I) of compacted tissue formed between the *C. purpurea* stroma (St) and the *T. caries* teliospores (T).

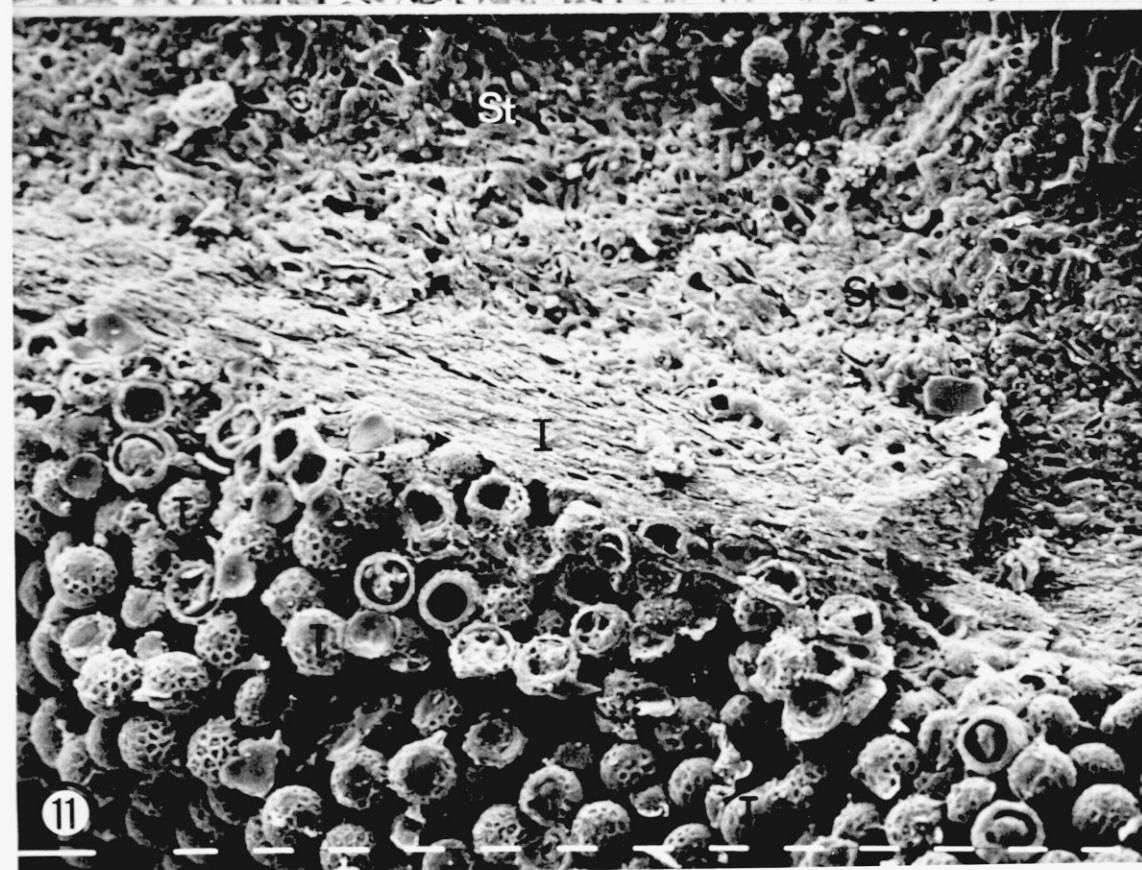
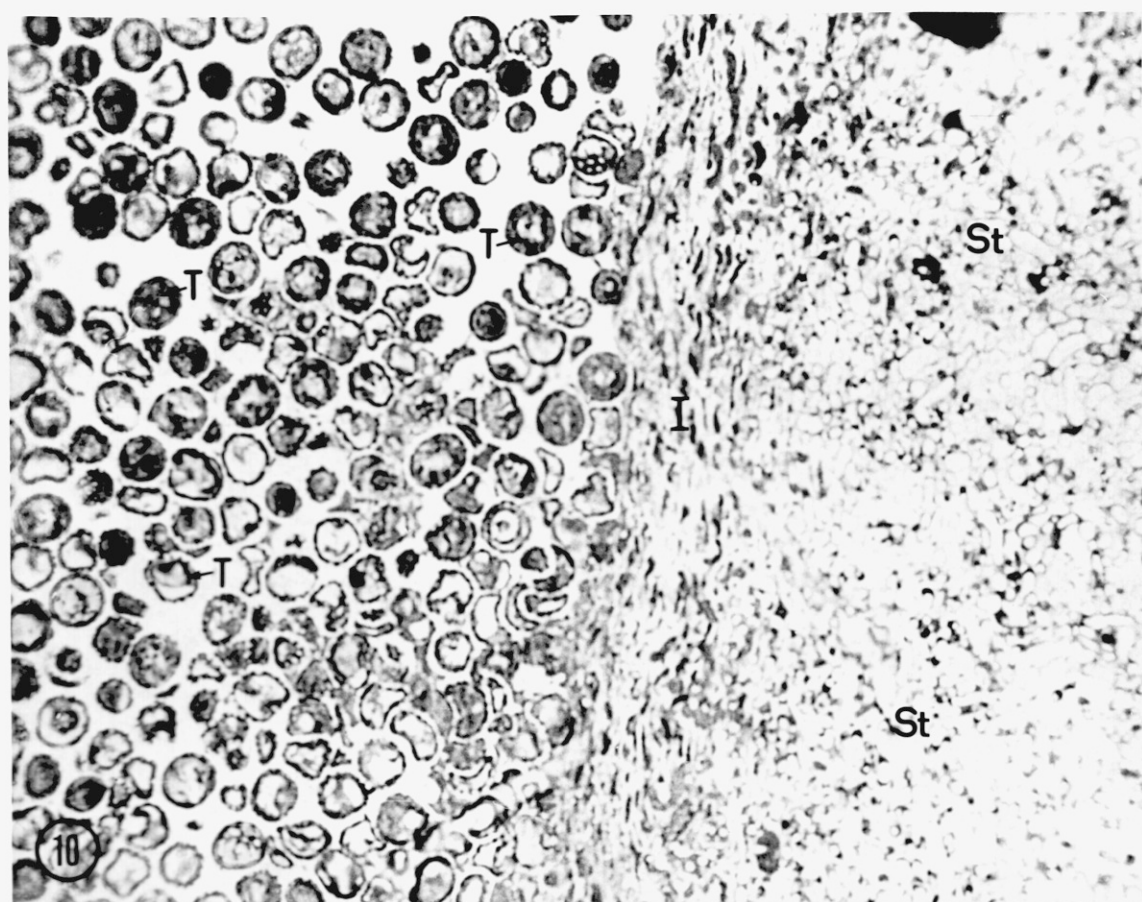
Magnification X 430

Staining: Toluidine blue

PLATE 9:11

Scanning electron micrograph showing no mixing between the two pathogens, they remain isolated by the interface (I) of compacted tissue.

Magnification X 550



SECTION TEN

DISCUSSION.

10.1 INITIAL INFECTION AND DEVELOPMENT OF *T. CARIES* AND *T. PENICILLARIAE* IN WHEAT AND MILLET OVARIES.

Tilletia caries and *Tolyposporium penicillariae* both infect and establish themselves within their respective host ovary. However they initially enter their hosts in different ways. *T. caries* enters the host tissue at the seedling stage but only sporulates in the ovary, whereas *T. penicillariae* infects the ovary via the stigma and style during flowering and sporulates immediately.

Temperature, soil moisture content and depth of sowing all influence the infection of wheat seedlings by *T. caries*. A damp, cold period and deep seed sowing retards the seedling growth enabling hyphae of *T. caries* to infect the leaf node primordia and the apical growing point so that transmission of the hyphae occurs to the nodes and the inflorescence initials (Swinburne, 1960; 1963). This period of low temperature is most important. Swinburne (1963) showed that if hyphae had not reached the growing point prior to the elongation of the internodes above the 4th or 5th leaf base, no teliospores developed in the ovary because the apical meristematic tissue had removed itself from the source of infection.

Only very few hyphae, all of which were small, were observed by electron microscopy during early stages of ovary development. Hyphae were not found in the rachilla or in the rachis of the inflorescence. Swinburne (1963) observed that hyphae occupied regions within the apical region and particularly in the nodes

prior to the elongation phase. Hyphae subsequently developed further only in the nodes or in the ovaries. In the present work the absence of hyphae from the rachilla indicates that the fungus maintains itself in the meristematic tissue of the main and lateral apices and the flower primordia. Thus the fungus remains isolated from all of the differentiating tissues except those of the ovary at flowering. As suggested by Willingale and Mantle (1987b) the use of fluorescent techniques would help to locate exactly the early stages of fungal development in the inflorescence.

In contrast *T. penicillariae* infects millet during flowering (Bhatt, 1946). The mechanism of entry is not precisely known although experiments carried out by Bhatt (1946) and observations from section 4.2.2. strongly suggest entry into the ovary through the stigma and style. Environmental conditions greatly influence the initial infection stage and subsequent establishment of the pathogen. It was shown in section 4.2 that high humidity is essential for the sporidia of *T. penicillariae* to infect the millet ovary. The exact temperature is less important. These results are similar to those described by Patel and Desai (1969) who first recognised the importance of high humidity for infection to occur and used polythene bags to maintain the condition. Polythene bags are also used for a short period after inoculation with the fungus when screening millet for resistance (Wells et al, 1987).

10.2 COLONISATION AND MODIFICATION OF THE HOST TISSUES

BY *T. PENICILLARIAE* OR *T. CARIES*.

Following infection *T. penicillariae* colonises an already differentiated ovary whereas *T. caries* develops in the wheat ovary as the ovary differentiates. Once established within the ovary both pathogens grow and replace the host tissues with teliospores. This is achieved in different ways in the two hosts. *T. caries* hyphae penetrate between the cells of the differentiating ovary, causing them to disrupt, leaving a space to be filled with spores. Continuation of this process eventually leaves only the placenta-chalazal mound and a few layers of the ovary wall surrounding a spore filled cavity. *T. penicillariae* hyphae also colonise a cavity but this is formed by the expansion, rather than the disruption, of the ovary wall. The cells and intercellular spaces of the ovary wall enlarge forming the gall-like development and as the nucellus and ovary wall tissues part a large cavity is produced. Similar reactions during colonisation have been described in other plant-smut fungus interactions (*Ustilago esculenta* on *Zizania caduciflora* (Chan and Thrower, 1980); *U. esculenta* on *Z. latifolia* (Yang and Leu, 1978)). It has been proposed that development of the *gau sun* (the swollen culm of infected *Zizania* species) is due to the production of plant growth substances by the fungal mycelium causing an imbalance within the host apex and subsequently abnormal development of the inflorescence (Chan and Thrower, 1980). Examination of the liquid found within the cavity of the millet ovary, during the early stages of infection by *T. penicillariae*, may show the presence of growth substances.

Similarly the culture medium of the fungus may also accumulate growth factors as found with *U.esculenta* (Chan and Thrower, 1980).

In both pathogen interactions the development of spores occurs between the ovary wall and the nucellus. The position is easily recognised in the millet ovary colonised by *T.penicillariae* as all the pre-pollination tissues are present. (This is not in agreement with Bhatt (1946) who described hyphae between the aleurone layer and the ovary wall, tissues which only occur in a pollinated ovary). Although the position of development of *T.caries* seems at first to be different it is probably the same. The 10-layer zone, around which fungal development begins, probably corresponds to the embryo-sac and nucellus tissues. It is surrounded by a cuticle-like lamella similar to those located on the outer surfaces of developing integuments (Tilton and Lersten, 1981). The cells within the 10-layer zone have an internal organisation similar to the tissues within the differentiating ovary of *Ornithogatum candatum* (lilaceae) (Tilton and Lersten, 1981) which later develop to form the embryo-sac, nucellus and integuments. Hyphae of *T.caries* thus initially colonise the region equivalent to that between the ovary wall and the nucellus.

10.3 FORMATION AND DEVELOPMENT OF SPORES.

Details of the maturation of the teliospores produced from the sporogenous hyphae of *T.caries* are in agreement with the results of Hess and Weber (1976). Spore initials are separated from the parent hypha by a septum. The primary wall, at this stage, is very thin due to the enlargement of the spore. The

diameter of the spore nucleus is 1.4 times greater than the hyphal nuclei indicating that fusion of nuclei has occurred prior to, or during, the initiation of the spore. The primary wall then thickens and the spore matures. The observations of spore maturation are identical to those of Hess and Weber (1976) except that they termed the third wall layer (W3 in this thesis) the partition layer and subsequently the fourth layer (W4 in this thesis) wall three (W3). The thin layer (W1) which detaches from the teliospore during the deposition of W2 may either be the primary wall which breaks due to the expansion of the spore or it corresponds to a structure termed a pellicle, found on the outer surface of other maturing spores e.g. urediospores of *Melampsora lini* (Littlefield and Bracker, 1971). Observations of the mature spores showed no sign of this outer layer implying that it had detached therefore corresponding to W1 rather than a pellicle, which remains around the spore. Both the pellicle and the partition layer (W3 in this thesis) have been described as comprising hydrophobic materials (Littlefield and Bracker, 1971; Hess and Gardner, 1983). The partition layer, composed of lamellar material, renders the spores impermeable so that spore contents remain unfixed, unless they are broken open prior to fixation (Gardner et al, 1975). The lamellar structure of this layer was not observed in the present study but its effect on the spore fixation was apparent. The layer gave an intense reaction with PACP, a stain thought to be specific for glycoproteins (Van der Woude, 1973), but it stained weakly with PATCH-SP, a reaction also noted in the striated zone in teliospores of the smut *Entorrhiza casparyana* (Fineran and Fineran, 1984). These authors

observed that this zone had similar staining and fracturing characteristics to those of lipid bodies and membranes in frozen etched plant tissues and to the paraffinic hydrocarbons that are associated with the walls of fungal spores including *Tilletia* (Weete, 1980).

The development of spore wall layers in *T. penicillariae* teliospores was not investigated as mature infected ovaries were only examined by scanning electron microscopy. A few spores had begun to differentiate in earlier samples processed for transmission electron microscopy and at least two wall layers were observed, the outer one composed of a polysaccharide. A material, also polysaccharide in composition, surrounded the aggregates of hyphae and the differentiating spores within the cavity. It seems likely that this binds the developing teliospores together so that they form sporeballs and/or that it comprises nutrients.

10.4 THE FORMATION OF INTRACELLULAR HYPHAE AND THE HOST RESPONSE.

Both *T. caries* and *T. penicillariae* infections form intercellular^{and} intracellular hyphae as previously shown in several smut-host interactions (Fullerton, 1970). In the previous investigation intracellular hyphae resembled haustoria in that the host plasma membrane was invaginated and encapsulations were formed (Fullerton, 1970). The intracellular hyphae formed by *T. penicillariae* and *T. caries* also resemble haustoria as they are surrounded by a matrix lying between the fungal wall and the host plasma membrane. However the matrix surrounding the few intracellular hyphae of *T. caries*, formed in cells of the placenta-chalazal mound in wheat, have staining properties

indistinguishable from those of the host cell wall. Conversely the intracellular hyphae of *T. penicillariae* in the hilum region of the millet ovary are surrounded by a matrix whose staining properties differed from those of the host cell wall. It stained more intensely than the host wall after uranyl acetate and lead salts or PACP treatment. With PATCH-SP treatment the density was similar to that of the host wall, but the texture was more open. Similar distinctive staining reactions have also been observed in penetration matrices of *Peronospora pisi* infecting pea (Hickey and Coffey, 1978), *Tussilago farfara* infected by the monokaryon of *Puccinia poarum* and *Albugo candida* infecting *Brassica oleracea* (Coffey, 1983). Thus in *T. caries* there is no evidence other than position to distinguish the intracellular hyphae from intercellular structures. However in *T. penicillariae* the interfacial materials have similarities with those associated with fungal haustoria.

10.5 THE INFECTION AND COLONISATION OF HOSTS BY CLAVICEPS SPECIES.

Both *Claviceps fusiformis* and *C. purpurea* infect their hosts prior to pollination entering primarily through the stigma (Luttrell, 1980; Shaw and Mantle, 1980; Willingale and Mantle, 1985). Unlike smut and bunt infections the pathogens do not modify the ovary tissue but they displace the nucellus and its associated tissues, which would have formed the embryo and endosperm, and establish an interfacial region at the base of the ovary which extends into the pedicel.

The fungal stroma of *Claviceps fusiformis* develops above the interface as is observed in infections of wheat and rye by *Claviceps purpurea* (Luttrell, 1980; Shaw and Mantle, 1980).

Conidial pockets form in the upper part of the sphacelium and the sclerotial tissue differentiates acropetally from the generative zone at the interface. Observations suggest that the colonisation of the millet tissues is accompanied by host wall degradation as occurs in wheat infected by *C.purpurea* (Shaw and Mantle, 1980). As hyphae penetrate from cell to cell they constrict, form a cross wall and continue growing within the cell by multiple branching. A few hyphal segments observed contained two or more nuclei indicating the possible multinucleate nature of the developing pathogen and woronin bodies are associated with septa, also as described for *C.purpurea* (Shaw and Mantle, 1980).

Hyphae in both wheat and millet infections occur in association with the vascular tissues in the pedicel, but are observed only if median sections are taken through it. Hyphae occupy inter- and intracellular positions adjacent to phloem sieve tubes and their associated parenchyma cells. Colonisation does not continue beyond the core of thick walled cells which occur in wheat or the major group of xylem tracheids in the pedicel of millet. The colonisation of the pedicel has previously been observed in rye infected with *C.purpurea* (Luttrell, 1980) but not in wheat (Shaw and Mantle, 1980) or millet.

10.6

DEVELOPMENT OF INTRACELLULAR STRUCTURES AND THE HOST RESPONSE.

The initial penetration of a host cell by an intercellular hypha in millet is associated with the deposition of a callose-like material. This material is not associated with subsequent penetrations of neighbouring host cells by the intracellular

hypha. This suggests that the fungus, once it has entered a host cell, modifies the response of adjacent cells. *C. purpurea* grown *in vitro* has been shown to possess β -glucanase, an enzyme which hydrolyses callose (Dickerson et al, 1978). Thus it seems unlikely that the enzyme is produced in significant amounts at the initial penetration but that it may be formed soon afterwards. The lack of aniline blue fluorescence associated with hyphae within the infected pedicel of millet ovaries, 7-10 days after infection, indicates that callose is not a constituent of the matrix associated with the majority of intracellular hyphae in this region.

The intracellular hyphae of *C. fusiformis* are also coated with various materials. Around the hyphal wall there is a matrix which stains similarly to the penetration matrices around the necks of some fungal haustoria (Hickey and Coffey, 1978). It is fibrillar in structure and its specific staining indicates a composition distinct from that of the host cell wall. The intracellular hyphae of *C. purpurea* observed were invariably associated with a wall-like material which had staining properties similar to those of the wall ingrowths of transfer cells. Although intracellular hyphae do occur in the rachilla of wheat the majority of hyphae are intercellular and associated with the core of thick-walled cells. However in millet the majority of hyphae are intracellular and occupy cells associated with phloem sieve-tubes.

NUTRIENT TRANSPORT

In order to understand the transfer of nutrients from host to the fungi it is first necessary to consider the arrangement of tissues in the uninfected ovary where nutrients feed the developing embryo and endosperm tissues.

10.7 TRANSPORT OF NUTRIENTS INTO THE POLLINATED MILLET OVARY.

Observations of the placental tissues in the pollinated millet ovary are strikingly similar to those observed in other tropical grains (e.g. sorghum and maize). Examination of the literature with regard to the proposed movement of nutrients to and within maize and sorghum ovaries is presented so that a possible mechanism for nutrient flow into the millet ovule can be proposed.

Nutrients travelling towards the developing grain in the pedicel leave the phloem endings in the placental region and continue into a small zone at the base of the ovary. This zone, termed the chalazal pad in this thesis, is separated from the developing seed but not by the cuticles which surround the remaining inner ovary wall tissue. The zone has been identified previously in millet and in other cereals (*Pennisetum americanum* (L.) Leeke [Fussell and Dwarthe, 1980]; maize [Kiesselbach and Walker, 1956]; *Echinochloa crusgalli* var *frumentaceae* (Link) W.F.Wight [Zee and O'Brien 1971b]; Yellow foxtail millet (*Setaria italica* (L.) Beauv) [Rost and Lersten, 1973]; Sorghum [Sanders, 1955; Maness and McBee, 1986]). All the tissues of the placenta-chalaza are in symplastic continuity with the phloem sieve-tubes in the pericarp tissue of the ovary and in the species mentioned

above the chalaza is the terminal part of the maternal symplast. From the chalazal pad nutrients pass into the endosperm, but as it and the endosperm cells are not symplastically connected, nutrients must be off-loaded into the apoplast prior to entry into the symplast of the endosperm via the endosperm transfer cells. This is a major barrier to nutrient transport to the developing ovule (See Figure 10:1).

Unloading of assimilates from the pedicel of maize was shown to be unaffected by metabolic inhibitors (HgCl_2 ; CCCP; NaF; DNP) or variation in apoplastic pH suggesting a passive process (Porter et al, 1985). Calcium chloride, which affects the semi-permeability of membranes, inhibited unloading, a phenomenon also described in *Vicia faba* (Anderson, 1983), cotton leaf cells (Rehfeld and Jensen, 1973) and soya bean seeds (Thorne et al, 1983). In the soya bean ethylene dinitro tetra-acetic acid (EDTA) was shown to stimulate unloading, an effect attributed to the chelation of divalent ions, especially Ca^{2+} , from the plasma membrane causing a rapid leakage of ^{14}C -assimilates from them (Thorne et al, 1983). A similar effect was also demonstrated in the maize pedicel (Porter et al, 1985).

Solutes passively effluxing from the caryopsis vascular tissues are then freely available in the apoplast. Many authors have demonstrated the presence of invertase within the placenta-chalazal tissue which causes the hydrolysis of the main solute, sucrose, to hexose sugars. This transformation of carbohydrates has been extensively studied as a mechanism for maintaining a concentration gradient and phloem unloading during the uptake of sugars into various plant storage tissues (Thorne, 1985;

FIGURE 10:1

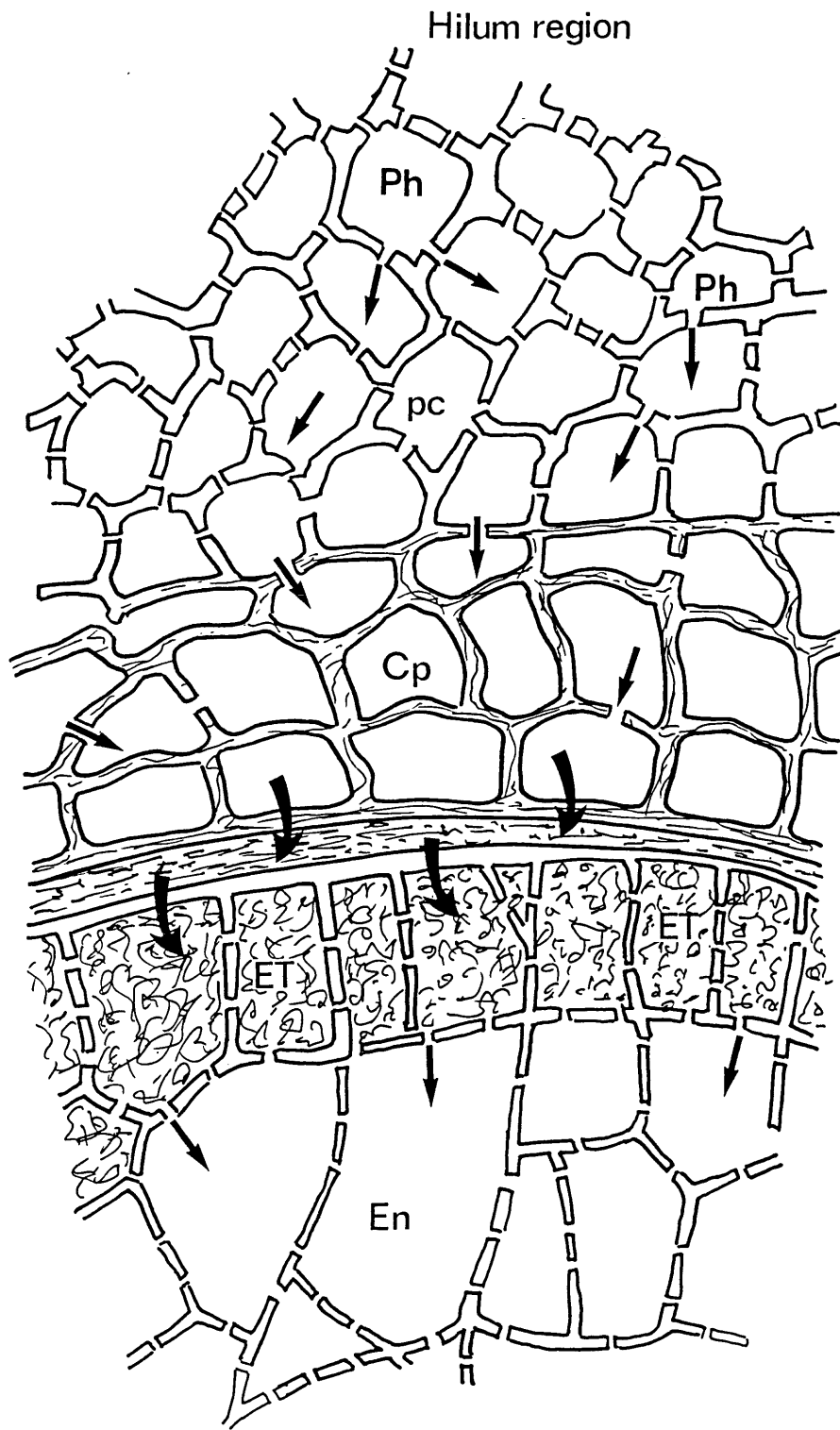
Diagram of a transverse section through a developing millet caryopsis showing the proposed transport pathway for nutrients into the developing seed.

Solutes efflux from the phloem sieve tubes (Ph) in the hilum region and are transported via the symplast to the chalazal pad (Cp). Here solutes pass out of the symplast into the apoplast from where they can be taken up by the endosperm transfer cells (ET) into the symplast of the endosperm (En).

Solute movement →

Transfer of solute ↪

(from symplast to apoplast or apoplast to symplast)



Wolswinkel, 1985). Hydrolysis of sucrose occurs during unloading from the placenta-chalazal tissue of maize (Shannon, 1968; Felker and Shannon, 1980) and sorghum (Maness and McBee, 1986). This process has been thought to be mediated by an acid invertase located in the walls of the placenta-chalazal tissue (apoplast) (Sorghum, Bhatia et al, 1980; Maize, Shannon and Dougherty, 1972). Hexose sugars were shown to accumulate in the placenta-chalazal region of maize (Felker and Shannon, 1980) and in the placenta sac (a large cavity between the placenta-chalaza and the endosperm) of the sorghum caryopsis (Maness and McBee, 1986). A similar transformation of sucrose into hexose sugars also occurs in the accumulation of storage products in sugar cane (Glasziou and Gayler, 1972). However, complete degradation and subsequent re-synthesis of sucrose was shown not to be required for normal caryopsis growth when using *in vitro* maize kernels (Cobb and Hannah, 1986). It is therefore possible that hydrolysis is not essential for transfer of solutes into the developing ovule, although its occurrence would maintain a concentration gradient for unloading of solutes from the maternal tissue.

Uptake of the monosaccharides into the endosperm is facilitated by the specialised endosperm transfer cells adjacent to the placenta-chalaza. These cells have wall ingrowths similar to those which have been described at sites where adverse surface area-volume relationships exist between donor and receptor compartments of the transport pathway (Gunning and Pate, 1969).

It is therefore concluded that from the work on maize and sorghum and the structural similarity of millet to these crops that nutrients travel in the symplast of millet to the placenta-

chalazal tissue where they passively efflux into the surrounding apoplast. The off-loading gradient is probably steepened by enzymic hydrolysis of sucrose in the apoplast of the chalazal pad enabling hexoses to accumulate prior to uptake by the endosperm transfer cells. This mechanism is consistent with others proposed for flux of assimilates into storage cells where regulation of nutrient transfer is facilitated by the permeability of the plasma membrane and the apoplastic sucrose concentration [general (Ho and Baker, 1982); *Vicia faba* (Wolswinkel, 1983; Soya bean (Thorne, 1985);]

Having established the probable processes by which nutrients enter the uninfected millet ovary it is now possible to consider the transfer of nutrients into the infected ovary.

10.8 TRANSPORT OF NUTRIENTS INTO THE MILLET OVARY INFECTED WITH *TOLYPOSPORIUM PENICILLARIAE*.

When the millet ovary is colonised by *Tolyposporium penicillariae* its development is modified. A cavity is formed between the ovary wall and the nucellus except in the hilum region where the two tissues remain symplastically connected as they are not separated by the cuticles. The cavity continues to enlarge as it is colonised and filled by the invading hyphae. Some hyphae penetrate cells of the nucellus and also enter the hilum region.

Hyphae within the hilum region colonise the host tissues as far as the upper part of the pedicel, particularly associating with the phloem sieve tubes. The presence of intracellular hyphae within the hilum cells of millet during the development of the smut ball is probably significant in the maintenance of a

nutrient supply to the developing spores. However these intracellular hyphae do not have the same relationship with the host cytoplasm which has been proposed (Gay, 1984) for haustoria of powdery mildews, downy mildews or rusts. Haustoria of these pathogens, although lying outside the host symplast, are separated from it by an interfacial membrane which is modified so that the separation is abnormal. The modified membrane around haustoria has been shown to be distinct from the normal host plasma membrane in its negative properties when treated with a reagent specific for plasma membranes (PACP procedure) or examination for ATPase activity (Spencer-Phillips and Gay, 1981; Woods and Gay, 1983). It has been proposed that this modified membrane aids nutrient transport from the host to the haustorium because normal components which control flow of solutes through the membrane are absent thereby allowing an uncontrolled leakage from that cell into the matrix surrounding the haustorium (Spencer-Phillips and Gay, 1981; Woods and Gay, 1983). However the invaginated membrane surrounding intracellular hyphae of *T. penicillariae* gave a positive reaction to the PACP stain. The attempt to localize ATPase activity was unsuccessful but since in all previous work the results of the two have been exactly correlated (Woods and Gay, 1987) a distinction seems unlikely. The significance of intracellular structures of *T. penicillariae* hyphae is therefore unclear with regard to a direct nutritional advantage over intercellular hyphae. The only possible advantage is contact with the surfaces of the host cells.

The majority of the fungus which grows and differentiates in the cavity can be considered as equivalent to the seed in

pollinated millet tissue. Nutrients passing from the maternal symplast to the pathogen must enter an apoplast, as observed in the transport of nutrients in the pollinated millet ovary. It seems likely that nutrients moving towards the ovary passively efflux from the pedicel (maternal symplast), as in pollinated tissue. However, because the placenta-chalazal tissues do not develop in the *T. penicillariae* infected millet ovary, the host invertase, proposed to be present in the chalazal pad region of the pollinated ovary, does not occur. The absence of a host invertase probably does not affect the mechanism of transport to the pathogen. Other biotrophic infections have been shown to result in an increase in the activity of acid invertase in the infected tissue (*Albugo tragopognis* on *Senecio squalidus* and *Puccinia poarum* on *Tussilago farfara* and *Poa pratensis* [Long et al, 1975]). In each of these infections carbohydrates, especially starch and fructans, accumulated in the infected area (Whipps and Lewis, 1981). Work on maize smut infections has shown that there is a progressive increase in the total acid invertase activity within infected tissue (Callow et al, 1980). This is correlated with a low sucrose and high hexose content within the infected maize tissue. Comparison of the invertase found in the infected tissue and in extracts of cultured haploid sporidia led these authors to suggest that the invertase activity was due to an enzyme of fungal origin and to a lesser extent a stimulation of host invertase (Callow et al, 1980). Their conclusions, however, differ from those proposed by Billett et al (1977) who attributed the increase in invertase activity to a single invertase component with an identical mobility on

polyacrylamide gels to one of two invertase bands detected in uninfected host plants. This would indicate that the fungus is even more dependent on the host mechanisms. Thus the presence of *T. penicillariae* hyphae associated with the undifferentiated maternal tissues of millet (i.e. in the hilum and upper pedicel) may be significant in the maintenance or production of invertase activity. The mechanisms of transport in the uninfected and infected flowers would be essentially identical if the host invertase is extracellular as suggested above. However, it is unlikely that the fungus could replace the role of an intracellular host invertase which produces hexoses before release by the maternal symplast.

In addition to the above other solute transformations may influence nutrient transport. In the *T. penicillariae* infected ovary some nucellar cells and a band of cells in the hilum, adjacent to them, accumulate starch within their chloroplasts. It has been suggested that this accumulation may occur as a result of abnormally high concentrations of hexoses within the tissues resulting from fungal infection (Long et al, 1975; Whipps and Lewis, 1981). The formation of starch grains within chloroplasts has also been simulated by supplying hexoses to healthy leaves (Bennett et al, 1971).

Flow towards the fungus may be assisted by fungal transformation of the host derived compounds. In Basidiomycotina and Ascomycotina the products of the conversion of sugars taken up result usually in arabitol and mannitol together with trehalose and glycogen. This maintains a flow to the fungus as it reduces the concentration of translocate and furthermore,

these products cannot be metabolized by the host (Smith, Muscatine and Lewis, 1969; Cooke, 1977).

The formation of the gall-like structure is probably due to an imbalance in the host growth hormones. The presence of high levels of auxins may effect the host's metabolism, as it has been shown, causing an increase in nutrient uptake of that tissue (Cooke, 1977). It has also been proposed that cytokinin affects the long distant transport of nutrients in plants, though as yet these ideas have not been substantiated (Cooke, 1977).

The majority of the invading fungus therefore occupies a position within the host similar to the absorptive tissues (endosperm transfer cells) found in pollinated tissue. The accumulation of starch within the hilum region, where intracellular hyphae are numerous, indicates the presence of high concentrations of hexoses within these tissues. The conversion of the main constituent sucrose, in both uninfected and infected tissues, into hexose sugars catalysed by an enzyme of fungal and/or host origin, would maintain a steep gradient for further off-loading of solutes to the pathogen. The fungus therefore replaces the position normally occupied by the seed and develops a relationship with the maternal tissues thereby utilizing the host's nutrient pathway, which normally develops in response to the developing seed.

10.9 TRANSPORT OF NUTRIENTS INTO THE WHEAT OVARY.

The structure of the developing wheat ovary differs from that observed in millet. It is therefore necessary first to consider this in relation to the flow of nutrients into the

pollinated wheat ovary, the variations from that proposed for the millet ovary and the consequences upon the localization of the pathogens within the host and their uptake of nutrients from the host.

The majority of nutrients travel to the rachilla in the phloem sieve tubes (symplast) and in the xylem tracheids (apoplast). A discontinuity, the significance of which will be discussed below, occurs in the xylem tracheids just below the attachment of the ovary to the rachilla. The xylem tracheids are interrupted by a core of thick-walled cells which have undergone abnormal tracheary development (Zee and O'Brien, 1970a; 1971a). Beyond this zone normal xylem tracheids occur along the crease region of the ovary wall. Phloem sieve tubes continue uninterrupted from the rachilla to the ovary and phloem transfer cells surround the xylem discontinuity (Zee and O'Brien, 1970a; O'Brien et al, 1985). Nutrients continue their path into the developing ovule via the cells of the pigment strand, chalazal pad and nucellar projection which are all symplastically connected. However, there is no symplastic continuity between the cells of the nucellar projection (of the chalazal region) and those of the developing seed as they are separated by the endosperm cavity (apoplast). Flow of nutrients to the developing seed is restricted to the placenta-chalazal region by the cuticles which line the maternal tissues except in this region (Morrison, 1975; Lingle and Chevalier, 1985). Nutrients therefore must pass into the endosperm cavity before they can enter the developing seed and be utilized in the endosperm. Therefore as

with millet, nutrients have to cross an apoplast as they pass from the maternal tissue to the developing seed.

(See Figure 10:2).

Within the rachilla restriction of flow of solutes has been demonstrated experimentally. Using 1% solution of acid fuchsin and ferric chloride Zee and O'Brien (1970a) showed that a barrier to free space transport exists at this discontinuity as these dyes accumulate at the core of thick-walled cells and do not move into the ovary. Similar results were obtained with both 8-anilino-1-naphthalene sulphonic acid (ANS) and calcofluor white M2R neither of which enter the wheat ovary, accumulating at the xylem discontinuity (Cook and Oparka, 1983). Therefore the lumens of the core of thick-walled cells do not offer a continuous pathway and movement of solution is therefore restricted to their cell walls. In experiments using lanthanum nitrate (pH 7.2) deposits were not detected in the inner wall layers of the thick walled cells nor in the upper parts of the core, indicating that movement of solutes within the free space is also limited to the outer wall regions (Zee and O'Brien, 1970b). However movement of fluorescein supplied to the cut stem of wheat entered the caryopsis via the phloem moving outward through cells of the vascular parenchyma, pigment strand and nucellar projection and later accumulated in the endosperm (Cook and Oparka, 1983). Fluorescein therefore moves from the xylem in the rachilla into the ovary by transport in the phloem. It probably enters the phloem at the xylem discontinuity via the transfer cells abundant in that region.

FIGURE 10:2

Diagram of a transverse section through a developing wheat caryopsis showing the transport pathway for nutrients into the developing seed.

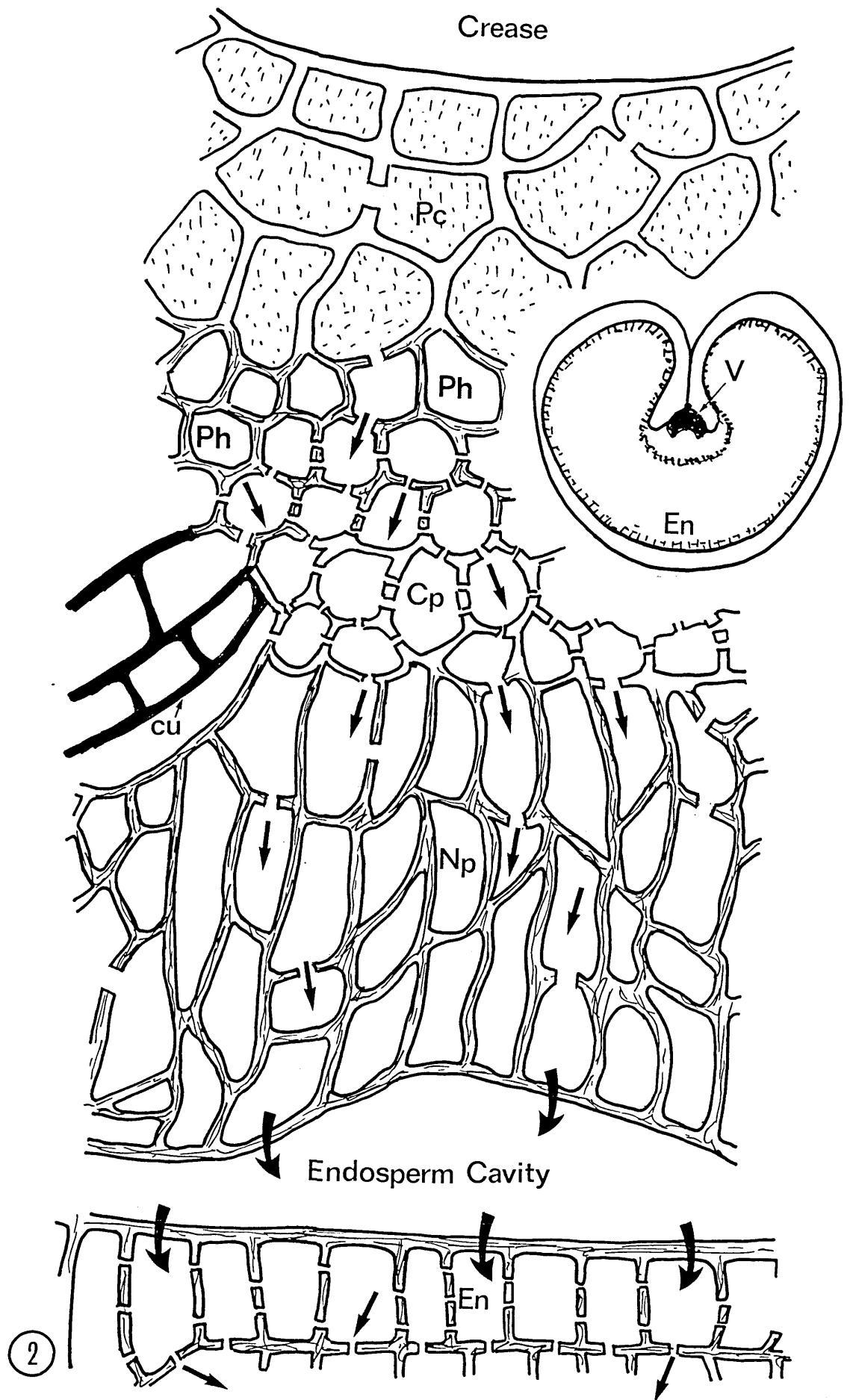
Solutes efflux from the phloem sieve tubes (Ph) in the crease region and are transported via the symplast through the chalazal pad (Cp) into the nucellar projection (Np). Solutes must pass into the endosperm cavity (apoplast) before they can be taken into the symplast of the seed via the endosperm cells (En).

The small insert is a whole transverse section through a wheat caryopsis showing the position of the vascular tissues (V) in the crease region.

Solute movement →

Transfer of solute ↗

(from apoplast to symplast or symplast to apoplast)



Solute flux continues from the rachilla into the pericarp of the ovary via the symplast by transport mainly in the phloem. Continuity of solute flux in the symplast was established with ^{32}P and ^{14}C tracer experiments (Frazier et al, 1956; Sakri and Shannon, 1975). Movement of the tracers to the endosperm was restricted to the placenta-chalazal region, as observed with fluorescein, where unloading from the maternal symplast into the apoplast (endosperm cavity) is thought to be mediated by the presence of transfer cells in the nucellar projection (Smart and O'Brien, 1983).

Invertase and sucrose synthase, although present in the pericarp tissue and in the endosperm respectively, have been shown to be unnecessary for uptake of sucrose into the endosperm of wheat (Jenner, 1974; Sakri and Shannon, 1975; Chevalier and Lingle, 1983). Thus conversion of sucrose into another solute form is not the major driving force for continued sucrose transport. Unloading of nutrients into the endosperm cavity and loading into the endosperm is therefore either passive or facilitated by mechanisms other than hydrolysis and synthesis. Recent work using radio-isotopes (Jenner, 1985) showed that the unloading rate of carbon components was reduced by agents (heat and dinitrophenol) which inhibit cell metabolism. The author suggested that other mechanisms, apart from mass flow, operate within the stalk (Jenner, 1985). An energy-mediated unloading and loading (Lehninger, 1975) process across the endosperm cavity is also supported by the presence of ATPase activity in plasma membranes of the groove aleurone cells during grain fill and within certain cells of the pericarp and placenta chalaza at

different stages of maturity (Didehvar and Baker, 1986). The absence of transfer cell formation in the groove aleurone cells probably does not limit nutrient uptake into the endosperm tissue as it is suggested that the large endosperm cavity provides a free pathway for nutrient transfer as it does not restrict nutrient flow as observed in millet (Gunning and Pate, 1974). However, wall ingrowths have been detected in the groove aleurone cells of wheat 15 days post pollination. This may correlate with an intense transfer of nutrients occurring at this stage of grain fill (Ayres and Angold, 1979).

The possibility of a sucrose-proton co-transport system maintained by proton extrusion from either (or both) the nucellar projection and/or the groove aleurone cells, as proposed by Ho and Gifford (1984), would facilitate the short distance transfer of nutrients through the apoplast between the two successive host transport systems.

It is therefore concluded that in the maternal tissues of wheat solutes travel in the symplast beyond the xylem discontinuity. Efflux from the maternal symplast into the apoplast surrounding the seed occurs at the nucellar projection where mechanisms other than hydrolysis mediate the transport. The transfer of sucrose from one symplast to the other may be mediated by a carrier and a gradient favouring transport to the seed is maintained by metabolic activity and subsequent utilization of sucrose within the developing seed.

Having established the pathway for nutrient transport into the developing wheat ovary it is now possible to consider this

pathway in relation to the infection of the ovary by *Tilletia caries*.

10.10 TRANSPORT OF NUTRIENTS INTO THE WHEAT OVARY INFECTED

WITH *TILLETIA CARIES*.

In wheat ovaries colonised by *T. caries* the pathogen occupies the position within the ovary which would have accommodated the seed in pollinated tissue. The pathogen has effectively replaced the seed. The maternal tissues are hardly modified by the pathogen and they develop almost as if pollination had occurred. A mound of tissues similar in structure to that of the placenta-chalaza develops as does the vascular bundle which supplies these tissues. It was not possible to ascertain whether the tissues within the mound are symplastically inter-connected (as they are in the placenta-chalaza) but if so, then the pathogen may obtain its nutrients from the host in the same way as a developing seed. Nutrients which efflux from the maternal symplast of the mound tissue directly enter the apoplast, which contains the developing fungal spores. Uptake and utilization of the nutrients by the developing spores and the differentiating hyphae would produce a downhill concentration gradient towards the pathogen encouraging further unloading from the maternal symplast. The presence of intracellular hyphae within the placenta-mound may be significant in the maintenance of a nutrient supply to the developing spores. However very few intracellular hyphae were observed in this region and all were surrounded by both wall-like material and a plasma membrane indistinct from that of the normal host. Thus although they are intracellular their significance remains

unclear (as discussed above for *T. penicillariae*, section 10.8) and they appear to have no advantage over intercellular hyphae.

The pathway taken by solutes entering infected tissues could be tested using radio-labelling techniques or dyes as in pollinated tissue (Frazier et al, 1956; Cook and Oparka, 1983). Further detailed examination of the mound tissue may reveal whether it had differentiated to form the pigment strand, chalazal pad and nucellar projection and whether the cells of the nucellar projection possess wall ingrowths as they do in pollinated tissue.

In pollinated ovaries of both wheat and millet the developing seed is totally surrounded by cuticles except in the placenta-chalazal region. The apoplast surrounding the seed into which the solutes flow is thereby isolated. Solute entering this apoplast are directly controlled by the rate at which they enter and leave and the only route they can take is either back into the maternal symplast via the placenta-chalazal region or by uptake into the symplast of the seed. The terminal tissue of the placenta-chalazal region (chalazal pad in millet or the nucellar projection in wheat) therefore acts rather like a gate effectively controlling the flow of nutrients in and out of the developing seed. When colonised by either the smut or bunt the ovary is only slightly modified, the cuticles remain and it is very likely that they are still intact. Each pathogen grows within a cavity and the placenta-chalazal region (or undifferentiated placenta-chalaza in millet) remains the off-loading site and ultimately the controlling tissue for efflux into the cavity. In wheat infected with *T. caries* the efflux is

controlled by the maternal transport tissues whereas in millet infected with *T.penicillariae*, because the placenta-chalazal tissue does not develop, the presence of hyphae within the hilum region probably assists the regulation of efflux from this tissue into the cavity.

10.11 TRANSPORT OF NUTRIENTS INTO WHEAT AND MILLET OVARIES

INFECTED RESPECTIVELY BY *CLAVICEPS PURPUREA* OR *C. FUSIFORMIS*.

The colonisation of millet and wheat ovaries by *Claviceps fusiformis* and *C.purpurea* is more destructive than is observed in *T.penicillariae* or *T.caries* infections. *C.fusiformis* and *C.purpurea* displace the embryo-sac and nucellus into the upper part of the structure establishing a stromal interface at the base of the ovary. The nucellus, embryo-sac and ovary wall tissues above this stromal interface are displaced and eventually replaced by developing and differentiating hyphae. Consequently the placenta-chalaza cannot develop as its primordial tissues are displaced, and later replaced, by the invading fungus. Below the stromal interface the pathogen penetrates deeply forming an association with the vascular tissues. The vascular associated hyphae colonise inter- and intracellularly in the region of phloem sieve-tubes and xylem tracheids. The developing sclerotium therefore forms an alternative sink for the nutrients which would have supplied the pollinated ovary. A variety of mechanisms have been examined to establish how this alternative sink is maintained.

Since intracellular hyphae are formed it is necessary to consider the possibility of modification to the host plasma membrane, as discussed above in relation to infections by

T. penicillariae and *T. caries*. In the majority of cases the region surrounding each hypha was indistinguishable from the normal host plasma membrane. There were a few exceptions where there was no PACP staining of either the membrane surrounding the intracellular hyphae or the plasma membrane of neighbouring uninfected cells. This may be either regarded as a modification of all the plasma membranes in the region of the invading hypha or that the staining procedure produces variable results as has been found by Hickey and Coffey (1978). The attempt to localize the sites of ATPase did not clarify the possibility of membrane differentiation. The general phosphatase activity with ATPase and β -glycerophosphatase observed in the sphacelial tissue may be associated with enzyme activity involved in the utilization of incoming translocate. The absence of any enzyme reaction in the pedicel may be due to an alteration of the host's plasma membranes as discussed for PACP staining. If these are true results an enhanced leakage of nutrients from all the surfaces of the host's cells into the apoplast would be expected, as discussed in section 10.8. However the absence of staining with PACP has invariably been correlated with absence of ATPase activity (Gay and Woods, 1987). As the majority of plasma membranes stained intensely with PACP in the millet pedicel the negative reaction to ATPase activity suggests experimental problems with substrate penetration rather than an alteration of the host's plasma membrane. The only evidence of a distinctive part of the host plasma membrane is from the matrices surrounding the intracellular hyphae which are similar to those around haustoria. The difference between cell wall and sheathing

materials has been interpreted as indicating different activities of the adjacent parts of a host plasma membrane (Hardwick, Greenwood and Wood, 1971). In conclusion, it is uncertain whether or not intracellular hyphae are in a more favourable nutritive position than those between host cells.

The osmiophilic bodies in host cells surrounding the core of vascular tissue are present in both the pedicel of infected and pollinated millet tissue and therefore do not form in response to fungal colonisation. The accumulation of polyphenolic materials, detected by chemical and cytological methods, in pollinated millet tissue, is thought to be related to the cessation of assimilate transport into the ovary as crushing of the maternal transport tissues releases these bodies from their vesicles (Fussell and Dwarthe, 1980). In the infected millet ovaries the osmiophilic bodies remain intact in their vesicles. This suggests that the fungus prevents their release and it seems likely that this is instrumental in maintaining a flow of nutrients into the developing sclerotium for a longer period of time.

Certain other features also probably enhance the availability of nutrients to the pathogen. It is notable that the hyphae within the pedicel remained undifferentiated throughout the development of the sclerotium. This was also observed by Bacon and Luttrell (1982) who also found that ^{14}C assimilates were translocated to the stroma throughout the period of development. It may be expected that excessive and prolonged fluxes through the host tissues might cause the phloem sieve tubes to plug. However the absence of aniline blue fluorescence

at both the stromal and vascular interfaces indicates that the fungal hyphae are not disadvantaged by the formation of callose. The possession of a β -glucanase capable of hydrolysing the callose most probably prevents its accumulation and is therefore instrumental in the maintenance of nutrient flow to the fungal stroma (Dickerson et al, 1973; Dickerson and Pollard, 1982).

Ergot infected inflorescences exhibit a greater capacity and efficiency of assimilate uptake than healthy inflorescences (Dickerson et al, 1976; Bacon and Luttrell, 1982). Seeds on a diseased inflorescence continue to develop beyond the normal 5-6 week maturation period presumably because of the strong competition for assimilates demanded by the hyphal sinks (Bacon and Luttrell, 1982). This strong assimilate sink was demonstrated by Mower and Hancock (1975) who studied the translocation of ^{14}C -sucrose into *C. purpurea* stroma on rye. The authors proposed that the increased influx of solutes into the fungal stroma is due to a decrease in the water potential as sucrose-derived products accumulate in the stroma. Evaporation only occurs primarily through the surface of honey-dew, thus furthering a decrease of the water potential in the floral cavity. The flow of water towards the more negative potential is accompanied by translocate as transport beyond the xylem discontinuity in wheat and the xylem terminus in millet must occur in the phloem sieve tubes. This maintains a supply of sucrose for transport into the fungal stroma. Hydrolysis of incoming sucrose could occur within the fungal stroma as a β -fructofuranosidase has been located, as a cell bound (apoplastic) enzyme, in all tissues of the developing sclerotium

(Dickerson, 1972; Pollard and Dickerson, 1984). The conversion of the host assimilates to fungal products further increases the efflux of sucrose from the host tissues, as a concentration gradient towards the fungus is established.

In the pollinated tissue of both wheat and millet the uptake of solutes into the developing seed does not appear to be accompanied by a net influx of water, as no change in the amount of water in the developing caryopsis is found (Jenner, 1982). If water accompanies solutes into the developing grain then it must in some way be exported by a mechanism not subject to the water potential gradient prevailing in the grain's immediate surroundings (Jenner, 1985). Furthermore it is most likely that this function is achieved within the maternal transport tissues. Following this argument *Claviceps* is at an advantage as it displaces the maternal transport tissues (i.e. undeveloped placenta-chalaza) and thereby is able to take over control of the flow of solvent and solute into the developing stroma. If this is the case the fungus may be able to control the import of assimilates into the stroma by coupling the sucrose concentration and the water potential in the pedicel thus effectively shortening the distance of, and decreasing the resistance to solute flow into the fungal stroma. The positioning of fungal hyphae in the xylem tracheids (and the xylem discontinuity) and in the cells adjacent to the phloem sieve tubes may be an important factor in controlling assimilate uptake by the stroma.

In summary, a number of different mechanisms contribute to the sink efficiency of the pathogen. All are consistent with normal efflux mechanisms in the supply of assimilates to a

natural sink region. The fungus has effectively tapped the primary source of nutrients and has utilized them to its own advantage.

10.12 THE TRANSPORT OF NUTRIENTS INTO A WHEAT OVARY INFECTED

BY *T. CARIES* AND *C. PURPUREA*.

The observation of wheat ovaries infected with both pathogens confirms the suggestion that the bunt replaces the seed whereas the ergot displaces it. Within the floret the two pathogens remain separated, a phenomenon demonstrated by Willingale and Mantle (1987b) who used a fluorescent probe to distinguish the hyphae of *C. purpurea* from those of *T. caries*. The *C. purpurea* hyphae not only displace *T. caries* but also the terminal maternal transport tissues, severing them from the vascular tissues within the pedicel. *C. purpurea* hyphae associate directly with the vascular tissues in the pedicel thus intercepting solutes prior to their entry into the ovary tissue, thereby controlling the flow of assimilates into the floret. Any further development of *T. caries* is thus dependent on the leakage (off-loading) of nutrients from or by *C. purpurea* hyphae. Substantial quantities of nutrients are available within the *C. purpurea* stroma as the development of an ovule into a mature caryopsis after displacement by the *C. purpurea* hyphae has been observed (Mantle, 1972). These experiments confirm that *T. caries* normally obtains its nutrients from the apoplast adjacent to the terminal region of the maternal symplast and *Claviceps purpurea* intercepts them from the subterminal vascular region.

SUMMARY

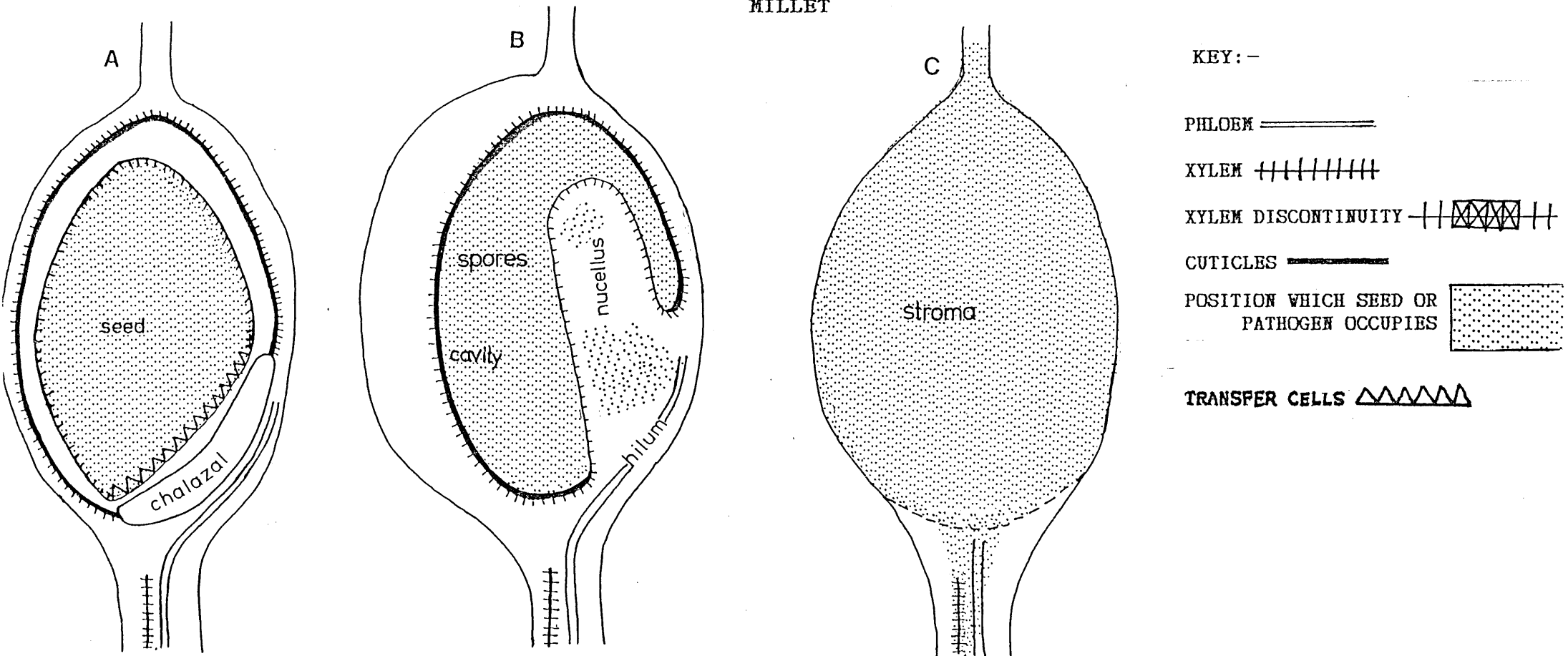
The four pathogens investigated colonise a region which potentially has a substantial nutrient supply (FIGURE 10.3). The developing seed in pollinated tissues is essentially a separate organism nourished by solutes from a special maternal transport tissues formed beyond the terminus of the vascular supply. Hyphae of *T.penicillariae* and *T.caries* associate with the terminal tissues in a mainly intercellular position into which solutes are normally off loaded before being absorbed by the seed. The intracellular hyphae which develop do not kill the host cells or breach the host plasma membranes nor cause those membranes to differentiate as is common around most fungal haustoria. Thus it is likely that the fungus relies on solutes which are released by the mechanism which operates in uninfected pollinated flowers.

Hyphae of *Claviceps* sp. penetrate more deeply and associate with the terminal vascular tissues in the pedicel, especially with the phloem sieve tubes and xylem tracheids. The special maternal transport tissue does not develop as it's primordial tissues are displaced and destroyed. Nutrient flow is probably assisted by the absence of the disorganisation of host cells and sub cellular vesicles, which release polyphenolics in uninfected pollinated flowers, and by enzymes preventing glucan deposition. The simultaneous development of both *T.caries* and *C.purpurea* in one flower is consistent with the conclusion of nourishment from terminal or subterminal tissues respectively.

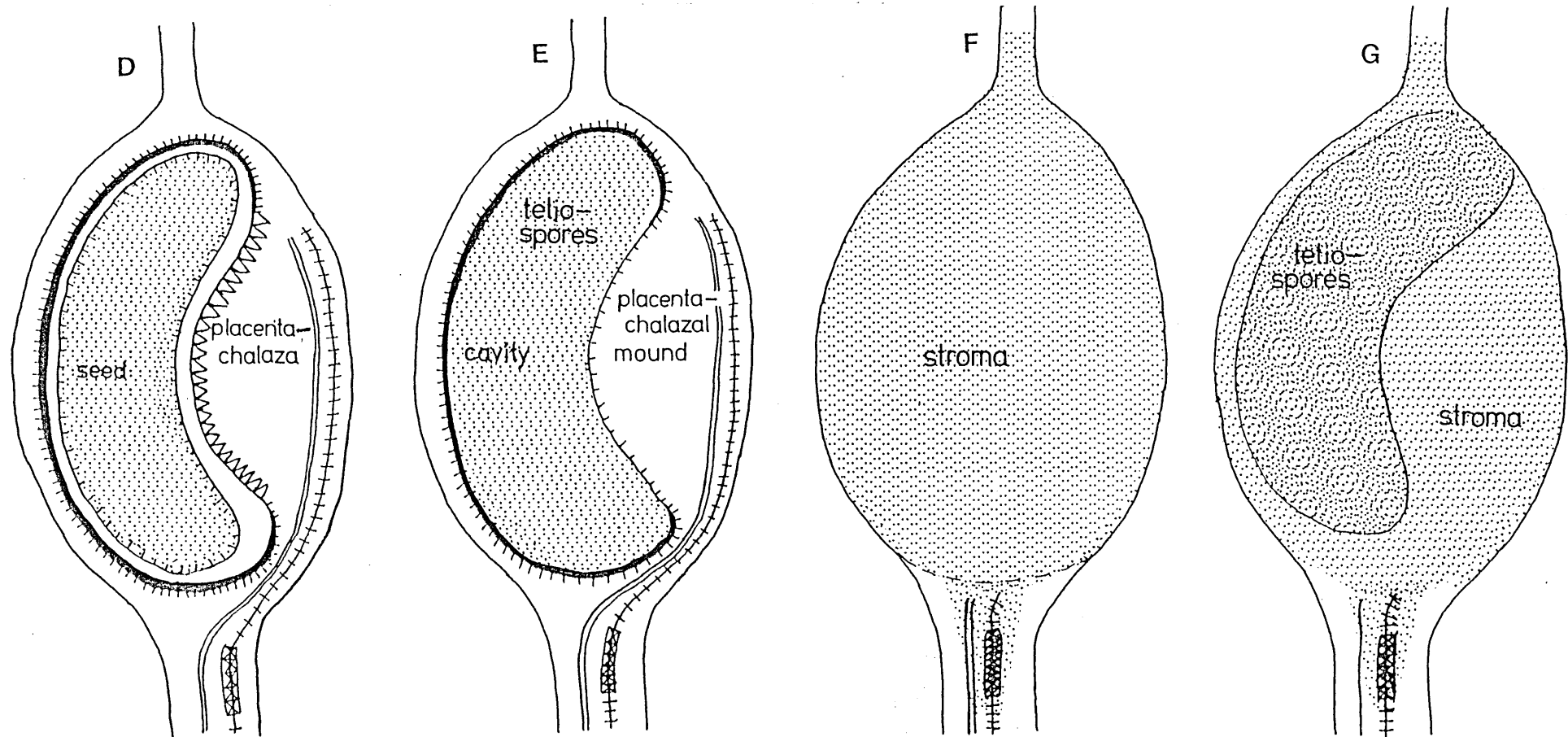
FIGURE 10.3

A SERIES OF DIAGRAMS SHOWING THE DIFFERENT POSITIONS
WHICH THE SEED OR PATHOGEN OCCUPIES WITHIN THE CEREAL OVARY.

MILLET



WHEAT



- A - A pollinated millet ovary containing the developing seed.
- B - A millet ovary infected with *Tolyposporium penicillariae*. The spores develop within the cavity.
- C - A millet ovary infected with *Claviceps fusiformis*. The whole ovary is occupied by the fungal stroma.
- D - A pollinated wheat ovary containing the developing seed.

- E - A wheat ovary infected with *Tilletia caries*. The teliospores develop within the cavity.
- F - A wheat ovary infected with *Claviceps purpurea*. The whole ovary is occupied by the fungal stroma.
- G - A wheat ovary infected with both *T. caries* and *C. purpurea*. The teliospores are displaced into the upper region of the ovary and enclosed by the *Claviceps* fungal stroma.

APPENDIX A

Table to show the enviromental conditions in Hyderabad,

India during 1972.

Month	Rainfall (in cm)	$\bar{x}$ Temp. (in°C)	Max. Temp.	Min. Temp.	$\bar{x}$ Humidity Percent. (%)	HUMIDITY AT	
						Min	Max
						Temp.	Temp.
Jan.	0	21.4	31.6	10.6	58	24	87
Feb.	0	24.6	33.9	13.9	46	24	74
Mar.	0.45	28.0	37.8	17.3	38	22	69
Apr.	0.8	30.0	39.4	20.6	43	21	41
May	1.5	30.7	39.4	20.6	47	29	93
June	18.7	27.2	38.9	21.2	70	31	69
July	19.2	24.7	36.7	21.2	81	41	93
Aug.	29.2	23.9	29.5	20.6	85	59	93
Sept.	24.9	25.2	31.6	20.6	82	50	93
Oct.	9.4	24.0	30.6	16.2	84	38	85
Nov.	0	20.3	28.9	10.6	68	54	96
Dec.	0	19.8	27.8	11.6	62	31	91

Anon (1955)

Data obtained from: Government of India Meterological Department

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