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FINE STRUCTURAL AND BIOCHEMICAL STUDIES ON THE INFECTION  
OF VICIA FABA L. BY UROMYCES AND BOTRYTIS

By

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A thesis submitted to the University of Durham for the  
degree of Doctor of Philosophy

June 1971



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## ABSTRACT

(1) Fine structural and biochemical studies have been made on the infection of Vicia faba L. by Uromyces fabae (obligate parasite) and Botrytis fabae (facultative parasite).

(2) Fine structural observations of host cells infected with U. fabae showed that they could be divided into two categories, (a) cells with increased capacity for synthesis (2-8 days after infection), which are characterised by an increase in the volume of the cytoplasm and by an increase in the size of cytoplasmic organelles (i.e. rough endoplasmic reticulum (ER), nucleus, mitochondria); (b) cells with decreased metabolic activities (12-20 days after infection) and characterised by senescence of the cytoplasm and by the reduction in the size of cytoplasmic organelles, especially chloroplasts. Numerous vesicles were found to be associated with the inner membrane of chloroplasts in such infected cells.

(3) Electron microscopic observations showed that cells from leaves infected with B. fabae, can also be divided into two categories, (a) cells in the centre of the necrotic lesion which are characterised by blackening and the destruction of the tissues; (b) cells at the periphery of the necrotic lesion, which are



characterised by an increase in the volume of the cytoplasm, an increase in the size of cytoplasmic organelles, by the presence of lomasomes, and by the presence of vesicular inclusions in the cytoplasm and electron dense bodies in the extracellular spaces.

(4) The fine structure of U. fabae resembles that of other comparable obligate parasites, except for the presence of an electron dense ring in the middle part of the haustorial neck, and vesicular and tubular structures in the vacuoles of this fungus. The growth of B. fabae on the host tissues was found to be relatively limited as compared with its growth on artificial medium. Likewise, the cytoplasm and cytoplasmic organelles of B. fabae were found to be denser when the fungus was grown on artificial media. Lomasomes were found to be common in the cytoplasm of this fungus, and the relation of this structure to the hydrolysis of the host cell wall is discussed.

(5) Several biochemical tests were made to assess the significance of some of the structural changes taking place in the host as a result of infection (i.e. amino acid composition, protein synthesis and photochemical activities of isolated chloroplasts). Special emphasis was placed on the effect of both infections on the latter process. The results can be summarised as follows:-

(a) infection with U. fabae caused an increase in the rate of the Hill reaction, cyclic phosphorylation (2-10 days after

infection), followed by a decrease in such activity (12-20 days after infection). The infection also caused an alteration to ATP:e<sup>-</sup> ratio.

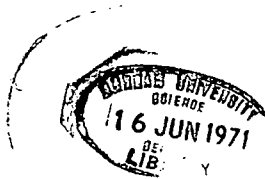
- (b) infection with B. fabae caused a marked reduction in all photochemical activities tested, (i.e. Hill reaction, cyclic and uncyclic phosphorylation).
- (c) The observed effects were found to be independent of the chlorophyll concentration and the reduction in the photosynthetic area.

Introduction

Many studies using light microscopy, histochemistry and biochemistry have been made to determine the sequence of events which take place between host plants and various of their parasites on infection. In most of these studies, the experimental methods and techniques used have been the limiting factors in discovering the type and sequence of reactions which occur, and consequently in our understanding of their significance.

Although a great deal of information on this subject has been obtained using the light microscope, such investigations are limited by the low resolving power of this instrument. The recent development of the electron microscope as a dependable instrument for studying biological material, has added therefore a new dimension to the study of fungi and their relationship to the host plants which they infect; as a result of this, our knowledge and understanding of the host-parasite complex are greatly improved.

Several electron microscopic studies dealing with various host parasite combinations have been made in recent years (Ehrlich & Ehrlich, 1963a; Peyton & Bowen, 1963; Berlin & Bowen, 1964; Ehrlich & Ehrlich, 1966; Bracker, 1967; Calonge & DeLa Torre, 1967; Calonge, 1969; Calonge et al., 1969a). However, the majority of such studies have been concentrated on obligate



parasitic fungi, with particular emphasis on the fine structure of the haustoria and the haustoria host-interface (e.g. Ehrlich & Ehrlich, 1963; Berlin & Bowen, 1964; Rubio-Huertos et al., 1965; Shaw and Manocha, 1965b; Bracker, 1967b). Although these studies were made on different host parasite combinations, a generalised concept for the structure of the haustorium can be formulated (Hawker, 1965; Bracker, 1967a). Thus, the haustorial protoplast, which is normally uninucleate, is surrounded by a well-defined wall. This wall is continuous around the neck of the haustorium and it extends through the host cell wall to the intercellular mycelium (Moore & McAlear, 1961b; Peyton & Bowen, 1963; Moore, 1965; Shaw & Manocha, 1965). As might be expected, there are a few exceptions to this condition, e.g. the haustorial protoplasm of Phytophthora infestans (Ehrlich & Ehrlich, 1966) and Albugo candida (Berlin & Bowen, 1964) has been found to be anucleate. With regard to the continuity of the wall, the haustoria of the latter organism was found to lack part of the wall in the distal end of the haustorial neck (Berlin & Bowen, 1964). Moreover, the haustorial neck of Erysiphe graminis was found to be septate (Bracker, 1967b). Most of the reports dealing with haustorial-forming obligate parasites agree that the haustoria are surrounded with an amorphous layer that is bounded by a membrane at the interface with the host protoplast (Moore & McAlear, 1961b; Shaw and Manocha, 1965; Bracker, 1967b; Calonge, 1969). The exact terminology for this layer differs

from one report to another, (e.g. sheath, sack, zone of apposition and incapsulation). Bracker (1967a), in his review, has stressed this problem and suggested that the term 'sheath' coined by Smith (1900) should be preserved. The origin of this layer is still uncertain, but some of the available evidence indicates its fungal origin (Ehrlich and Ehrlich, 1963); others indicate that it is derived from the host cytoplasm (Kojima and Hirata, 1961; Calonge, 1969). As far as the membrane surrounding the sheath, the early evidence of Thatcher (1942) and Fraymouth (1956) and some of the more recent reports (Peyton and Bowen, 1963; Bracker, 1967b; Littlefield and Bracker, 1970), indicate that the membrane is continuous with the host plasmalemma, which according to this view invaginates to allow the advancement of the developing haustorium.

As mentioned previously, most of the research into fine structure has been made on host-parasite combinations involving obligate parasitic fungi. Consequently, little attention was devoted to the host-facultative parasites. Some of the few reports concerning these organisms are those of Ehrlich and Ehrlich (1966) working with Phytophthora infestans, Calonge and DeLa Torre (1967) working with Exobasidium japonicum, and Calonge (1969) working with Phytophthora palmivora and Sclerotinia fructigena.

In view of the relatively many reports on haustorial structure and its relation to the host cytoplasm, very little has been done on the response of the host cell at the level of the fine structure of host organelles and cytoplasm. However, Shaw and Manocha (1965a,b) compared the changes in ultrastructure after infection with the degeneration of cells of detached leaves senescing in water. Their results indicated that the pattern of breakdown of the fine structure of cytoplasm is similar in both cases. Prior to this breakdown and in the case of infection their results indicated an initial increase in the amount of endoplasmic reticulum and the density of ribosomes.

Other reports indicated a marked increase in the electron density of the interchromatin region of the nuclei at the early stages of rust development (8 day). In the later stages of rust development (20-23 days old), both the interchromatin and the chromatin regions started to disintegrate (Manocha & Shaw, 1966). Harding et al. (1968) studied the changes in chloroplast ultrastructure in green island regions, induced by Albugo candida in detached Brassica juncea cotyledons. Their results indicated that chloroplast structure in the green islands was maintained much longer than in uninfected parts. However, the disruption of chloroplasts and mitochondria in infected tissues has been reported by several authors (Ehrlich & Ehrlich, 1963g; Berlin & Bowen, 1964; Manocha & Shaw, 1966).

Lomasomes were first reported to be present in disease-free mesophyll cells of 'Khapli' (a stem rust resistant variety of Emmer wheat). Whereas, the susceptible variety 'Little Club' was found not to contain such structures. From these results it was postulated that the presence of lomasomes in 'Khapli' and their absence in 'Little Club' may be related to the rust resistance of the former variety (Manocha & Shaw, 1964). On the other hand, Ehrlich et al., (1968) were not able to verify these observations. Their results indicated that regardless of the variety reaction (resistance or susceptibility), lomasomes occurred only in response to the presence of the pathogen. Their observations were made using infection with Puccinia graminis and P. recondita. Although designated as 'boundary formation', similar types of structure were also observed in vectorin treated oats, (Luke et al., 1966). Furthermore, such structures were also in a variety of other organisms (Barton, 1965; Crawley, 1965; Cronshaw, 1965a,b; Buvat, 1969). Their presence in fungi has been associated with several mechanisms, i.e. absorption (Berlin & Bowen, 1964; Peyton & Bowen, 1963), secretion (Calonge, et al., 1969a), and cell wall synthesis (Wilsenach & Kessel, 1965). In as much as their role and use are unknown, their contents are also a matter of some speculation (see review by Moore, 1965 and Bracker, 1967a).

Biochemical and physiological studies of the alteration in the metabolic activities of infected cells have been initiated relatively recently, and even now, such studies are largely confined to the respiration of diseased tissues. The majority of these studies indicate an increase in the respiration rate of infected tissues (Daly et al., 1957; Daly et al., 1961; Bushnell & Allen, 1962; Miller & Scott, 1962). As far as other metabolic processes are concerned, the available evidence indicates an increase in organic acids, soluble acids, and protein (Colatolo, 1961; Daly & Krupka, 1962), starch and other carbohydrates (Wang, 1960; Daly et al., 1962; Inman, 1962).

Work on photosynthesis is contradictory in that it sometimes indicates an increase, and sometimes a reduction in the photosynthetic rate following infection with fungal parasites. According to Yarwood (1966) ten out of twelve reports indicating increases in photosynthetic rate, are concerned with obligate parasitic fungi (rust and powdery mildew). As far as the duration of the increase, most reports agree that it is limited to the early stages of infection (Allen, 1942; Wang, 1960; Livne, 1964; Harding et al., 1968). On the other hand, the reduction in photosynthesis is sometimes assumed to be proportional to the reduction of the photosynthetic area which results from the infection (Allen, 1942; Chester, 1950).



Considerable progress has been made in the understanding of some of the enzymatic reactions involved in photosynthesis (Walker & Crofts, 1970), and convenient methods are now available for the measurement of the rate of several of these reactions in isolated chloroplasts. In the field of host-parasite relationships, little attention has been given to the effects of the infection on enzymatic reactions of photosynthesis. From the few studies made along these lines, the majority concern viruses as the causal organism. The results of such studies indicate that the two light reactions (namely Hill reaction and photosynthetic phosphorylation) are impaired in chloroplasts isolated from plants infected with either virus yellow or tomato mosaic virus (Spike, 1955; Zailten & Jagendorf, 1960). The former virus (virus yellow) was also found to have an adverse effect on the rate-limiting dark reaction (Spike, 1955). As far as could be ascertained, there are only two reports concerning the effect of fungal infection on the photochemical activities of isolated chloroplasts. Both of these reports were made on rust infection (Wynn, 1963; Pentzkaya & Kurprovesh, 1968). Wynn (1963) working with chloroplasts isolated from rust infected oat, showed that the photosynthetic phosphorylation of chloroplasts was not altered, when the results were expressed per unit of chlorophyll. However, Pentzkaya and Kurprovesh (1968) working with Puccinia dispersa on Winter Rye, showed that infection had an

adverse effect on the primary light reaction (oxygen evolution). This effect was evident from the first stage of disease development, and increased as infection progressed.

In view of the scarcity of electron microscope studies on the nature of facultative parasites, as compared with obligate parasites, more work is desirable. This will not only reveal the relationship between these types of pathogen and their respective host, but should also shed some light on how different they are from the obligate parasites reported on so amply.

Undoubtedly, useful information can be obtained if these fine structural studies were accompanied with biochemical investigations, orientated towards resolving the significance of changes taking place after infection.

The aim of this study was to observe and compare separately the behaviour and the fine structure of two pathogens of broad bean (Vicia faba), namely Uromyces fabae (obligate) and Botrytis fabae (facultative), within the tissues of the same host. In order to gain information on the nature of the interaction between the host and the second pathogen (Botrytis), studies were also made on the fine structure of this fungus growing outside the host tissues. Comparative observations were also made on the

fine structural changes taking place in the host tissues after infection with both pathogens. Biochemical studies were carried out on different aspects of host metabolism, i.e. protein synthesis, amino acid composition and photosynthesis, in order to elucidate the significance of structural changes taking place after infection. Special emphasis was directed towards the process of photosynthesis using chloroplasts isolated from both healthy and infected plants.

## Materials and Methods

### 1. General

#### 1. Biological Material

##### a. Host plants

Plants of Vicia faba var. 'Triple White' were grown in the greenhouse with temperatures ranging from 15-20°C. Supplementary illumination (1800-2000 F.C.) was used during the winter season, and this was provided by growing the plants under fluorescent tubes. Under these conditions the plants were receiving sixteen hours of light.

##### b. Fungal material

The isolates of Botrytis fabae and Uromyces fabae used in this study were kindly provided by Dr. A. Carter of Wye College of the University of London. B.fabae was grown and maintained on Bean Leaf Malt Agar Medium. The composition of this medium is given elsewhere (see Growth Media and Solutions). U.fabae was maintained on the host plant and conidia spores collected from 15 day-old pustules were stored in small vials in an 8°C refrigerator.

### 2. Chemicals and Reagents

All chemicals used in connection with the electron microscope part of these studies were obtained from TAAB, 52 Kidmore Road, Emmer Green, Reading. With the exception of ADP (obtained from Koch Light Laboratories Ltd., Colnbrook) and DCMU 3-3,4 Dichlorophenyl-1-

diamethyl urea (obtained from K. and K. Laboratories, Inc., Plainview, N.Y. Hollywood, California) all chemicals used in the biochemical studies were obtained from British Drughouses Ltd., Poole.

### 3. Growth Media and Solutions

a. Composition of Bean Leaf Malt agar medium used for growing

#### B.fabae.

Young broad bean leaves	100 g
Malt extract	10 g
Agar	10 g
H <sub>2</sub> O	1000 ml

b. Composition of medium X (Last & Hamley, 1956)

KH <sub>2</sub> PO <sub>4</sub>	1.5 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.5 g
NaNO <sub>3</sub>	6.0 g
KCl	0.5 g
Dextrose	10 g
Peptone	2.0 g
Casein hydrolysate	3.0 g
Nucleic acid	3.0 g
Agar	20 g
H <sub>2</sub> O	1000 ml

c. Composition of the solution used for isolating Chloroplasts (Cockburn et al., 1968) pH 6.5 at 0°C.

Sucrose	0.33 M
MgCl <sub>2</sub>	5mM
Sodium isoascorbate	2mM
Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub> ·10H <sub>2</sub> O	10mM

d. Composition of solution used for Hill reaction assays pH 7.6 at 25°C.

Sucrose	0.33 M
MgCl <sub>2</sub>	1.0 mM
MnCl <sub>2</sub>	1.0 mM
EDTA	2.0 mM
HEPES (N-2 Hydroxyethylpiperazine-N-2 Ethanesulphonic acid)	50 mM
K <sub>3</sub> Fe(CN) <sub>6</sub>	0.8 mM

e. Solution used for the assays of coupled phosphorylation pH 8.0 at 25°C.

KH <sub>2</sub> PO <sub>4</sub>	20 M
MgCl <sub>2</sub>	10μM
K <sub>3</sub> Fe(CN) <sub>6</sub>	1.5μM
Tris-HCl Buffer	.05 M
NaCl	70μM
ADP	20μM

f. Solution used for the assay of cyclic phosphorylation pH 7.5.

Sucrose	0.03 M
$\text{KH}_2\text{PO}_4$	20 $\mu\text{M}$
ADP	1.5 $\mu\text{M}$
$\text{MgCl}_2$	0.1%
Tris-HCl Buffer	0.05 M
Ascarbate	10 $\mu\text{M}$
PMS (N-Methyphenazonium methosulphate)	$10^{-4}$ M

g. Reagent used for inorganic phosphate determination.

Solution A

Ammonium molybdate	25 g
Water	300 ml

Solution B

Ammonium metavanadate	1.25 g
$\text{HNO}_3$ (Conc.)	250 ml

Add Solution A to Solution B and make up to 1000 ml.

h. Solution used in the operation of Technicon Auto Analyser

(i) 0.05 M Sodium Citrate buffer at pH 2.875

Tri Sodium Citrate $2\text{H}_2\text{O}$	220.6 g
2.0 N NaOH	375.0 ml
Thiodiglycol	75.0 ml
Brig 35 Solution	150.0 ml
Deionised Water	126.000 ml

Adjusted to pH 2.875 with HCl, made up to 15 litres with deionised water and the solution stored at 2°.

(ii) 0.05 M Sodium Citrate Buffer at pH 2.875 with 10% (v/v) methanol.

0.05 M, pH 2.875 Buffer	9 parts
Methanol	1 part

(iii) 0.05 M Sodium Citrate Buffer at pH 3.8

Tri Sodium Citrate 2H <sub>2</sub> O	117.7 g
0.2 N NaOH	200.0 ml
Brig 35 Solution	150.0 ml
Deionised Water	13.500 ml

Adjusted to pH 5.0 with HCl, made up to 15 litres with deionised water stored at 2°.

(iv) Ninhydrin reagents

Ninhydrin	100.0 g
Hydrination	7.5 g
4.0 N Sodium acetate Buffer at pH 5.5	1.750 ml
Methyl cellosolve	11.500 ml
Deionised Water	6.750 ml

Ninhydrin and hydrination were dissolved in methyl cellosolve. Nitrogen was bubbled through as the remaining reagents were added and for 2 hours after the solution was made up. It was stored in the dark.



(v) Haemosol solution

Haemosol	35 ml
Hot deionised water	3.750 ml

(vi) Brig 35 solution

Brig 35 (melted)	40.0 ml
Deionised water	120.0 ml

4. Preparation of inocula and inoculation procedure

Plants were inoculated by spraying them with suspensions of either B. fabae or U. fabae. B. fabae inoculum was prepared from 10-12 day old cultures of the fungus grown on Bean Leaf Malt Agar. Spores were collected from cultures of B. fabae by submerging the aerial mycelium in water containing 0.1% (w/v) glucose and rubbing them with a fine brush. The inoculum of U. fabae was prepared by suspending 3-4 week old conidia spores in 0.01% nonyl alcohol. The inoculated plants were then incubated in a moist chamber overnight and removed to the greenhouse bench the next morning. The plants which served as control were sprayed with either 0.1% (w/v) glucose or 0.01% (v/v) nonyl alcohol and treated similarly to the inoculated plants.

## II. Microscopy

### 1. Sampling of infected material

Sampling of plants infected with B. fabae was made at 1, 2, 4, 8 and 12 day intervals after inoculation. In the case of material infected with the rust pathogen sampling was carried out at 4, 8, 10, 12, 14, 16 and 18 days after inoculation. In all cases 1 mm diameter disks were punched out of leaf lesions with circular punch fashioned from a Pasteur pipette. Care was taken that each of these disks contained both the diseased and sound tissues.

### 2. Light microscopy

Light microscope observations were carried out by either stripping off the infected epidermis or by sectioning (1-2 cm thick sections). These preparations were then mounted in water and stained with toluidine blue.

### 3. Electron microscopy

#### A. Preparation of material

B. fabae was grown on artificial substrates with both liquid and solid media. In the case of the solid media the agar surface was covered with pre-sterilized Visking tubing, and after

incubation the tubing with fungus attached was peeled off and cut into small pieces which were immediately placed in the fixative solution. The fungus was recovered from liquid media by centrifugation in  $\text{PO}_4$  buffer (pH 7.0). The fungal pellet was then fixed immediately.

In the studies relating to the nature of multivesicular bodies (M.V.B.) formed in both the host and the fungal cytoplasm, the fungus (B. fabae) was grown on several media with complex substrates. The basal composition of all these media was as follows:- asparagine 0.2 g.,  $\text{MgSO}_4$  0.075 g.,  $\text{K}_2\text{HPO}_4$  0.125 g. and traces of  $\text{FeCl}$ ,  $\text{ZnCl}$ ,  $\text{CuCl}$ ,  $\text{MoCl}$  and  $\text{CaCl}$ . After adding either pectin, sodium polypectate cellulose, starch or glucose, water was added to give a volume of 1000 ml.

#### B. Fixation

Unless otherwise mentioned, fixation of all the material was carried out by suspending it in 4% (v/w) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and post-fixed in 1.5% (v/w) osmic acid in 0.5 M sucrose. Tissues were dehydrated through an ethanol series and embedded either in araldite or Epon 812 accelerated with 2.0% D.M.P. 30. For the details about the processes of fixation, dehydration, infiltration and embedding, see Table 1.

#### C. Sectioning and staining

Sections were cut on an LKB ultratome using glass knives

TABLE 1

Schedule for the time and chemicals used  
in the process of fixation, dehydration and embedding

Process	Chemicals	Time
Fixation	Glutaraldehyde (4%)	4 hr.
	PO <sub>4</sub> buffer (pH 7.0)	2 x 10 min.
	Osmic acid (1.5%) (v/w) in 0.5 M sucrose	40 min.
Wash	Water	5 min.
Dehydration	Alcohol 25%	10 min.
	" 50%	10 min.
	" 75%	overnight
	" 100%	10 min.
Embedding	Propylene oxide	1 hr.
	Propylene oxide/Resin (50/50)	4 hr.
	Resin	overnight
	Resin (in dishes)	
	40 C	2 days
	60 C	3 days

and collected on formvar-coated grids. The sections were stained for 15-20 minutes in 1% uranyl acetate and 3-5 minutes in lead citrate. Between each of these stains sections were washed with distilled water for about one minute.

#### D. Electron Microscopic examination

Electron microscopic examinations were made on an AEI EM6B electron microscope. Examination was routinely carried out at 60 Kv. Ilford N50 plates were used throughout the present study.

### III. Biochemical

#### 1. Plants preconditioning

Plants which served as a source of chloroplasts were preconditioned by keeping them for 24 hours in reduced light intensities (approx. 500-700 FC). This was mainly to reduce the size of the starch grains in the chloroplast, as large grains usually ruptured the chloroplast membranes during the isolation procedures.

Plants were then transferred to the normal greenhouse light (1800-2000 FC) one hour before the chloroplasts are to be isolated. Just prior to the isolation procedures, plant shoots were cut 2-3 inches above the soil level and each group of plants (i.e. healthy and infected) were placed separately in polythene bags and kept in crushed ice.

## 2. Chloroplast isolation

For the purpose of comparing the fine structural differences of chloroplasts isolated from healthy and infected plants, it was necessary to obtain chloroplast preparation with a high degree of intactness. For this reason various methods and media of chloroplast isolation were employed (Honda, 1963; Nobel, 1967; Cockburn et al, 1968; Greenwood, personal communication 1970). The main differences between these methods and those which are normally used in isolating chloroplasts in most biochemical assays, were the addition of special protectives and the use of gentler methods of grinding the leaves. Unless it is otherwise mentioned chloroplasts used in most of the assays in this study were isolated by a modification of the method of Cockburn et al., (1968). By this method a higher yield of chloroplasts with about 50-65% intactness can be obtained. This isolation medium is usually prepared just prior to the chloroplast isolation by mixing equal volumes of two stock solutions each containing part of the constituents. All glassware, reagents and the stock solutions were normally stored at around 0°C. 10-15 g of V. faba leaves were homogenized in a pre-chilled domestic homogenizer containing 50 ml of semi-frozen isolation medium for 2-3 sec. at low speed and

2-4 sec. at the maximum speed. After homogenization the leaf macerates were squeezed through two layers of muslin followed by filtration through 8 layers. After filtration the supernatant was poured into prechilled 8 x 50 ml. plastic centrifuge tubes. Centrifugations were carried out in a MSE high speed 18 centrifuge at 0° by allowing the centrifuge to accelerate from rest to 6000 r.p.m. (approx. 400 G) and then to return rapidly to rest with a total centrifugation time of about 3 mins. The supernatant was then decanted and the pellet was resuspended in 2.0 ml. of an ice cold resuspending medium which will be specified in the appropriate place.

### 3. Microscopy of isolated chloroplasts

Light, (phase contrast) and electron microscopic tests were made on the chloroplast preparation. These two tests were carried out to examine the structure, the structural integrity and the purity of the preparation. For the light microscope examination a sample of each chloroplast preparation was diluted with a small volume of the resuspending medium and examined directly under phase contrast microscope. Samples for electron microscopic examination were taken with a microspatula from the chloroplast pellet immediately after centrifugation. These samples were then fixed at around 0°C in 20% (w/v) gluteraldehyde in the isolation medium for 4 hours followed by 40 mins. post

fixation in 1% (w/v)  $\text{OsO}_4$  in isolation medium. After washing in the isolation medium the fixed chloroplasts were dehydrated using a series of ethanol concentrations diluted with the isolation medium. This was followed by infiltration in propylene oxide and embedment in araldite. Thin sections were stained by lead citrate and uranyl acetate. Some preparations were negatively stained and examined directly under the electron microscope.

#### 4. Estimation of chlorophyll content of chloroplast preparations

Chlorophyll content of each preparation was estimated using a colorimetric method. In this method 0.5 ml. of the chloroplast suspension was shaken in glass stoppered volumetric flask with 45 ml. of distilled water and 20 ml. of absolute acetone. The suspension was then centrifuged for 5-10 mins. at  $2^\circ\text{C}$ . Pellet was discarded and the optical density value of the resulting supernatant was measured at 652 and 645 nm. using either Uvispec or SP500 Spectrophotometer. The chlorophyll concentration of each sample was then calculated using the formula derived by Arnon (1949) from the coefficient of Mackinney (1941).

#### 5. Relationship between chlorophyll concentration and chloroplast number

Chloroplast preparation of a known chlorophyll concentration was diluted with tris-HCl buffer to give the following concentration of chlorophyll: 0.03, 0.06, 0.09, 0.12 and 0.15 mg. chlorophyll/ml.



The number of chloroplasts in each of these dilutions were estimated by direct count under a phase contrast microscope using a 0.2 ml haemocytometer.

#### 6. Measurement of oxygen evolution (Hill reaction)

Evolution of oxygen was measured either by the procedure of Jagendorf and Evans (1957) which involves measuring the change in optical density at 620 nm or polarographically in perspex water jacket cell containing a basal clark type electrode separated from the reaction mixture by a teflon membrane. Calibration of the system was made by observing the output of the electrode with 3 ml distilled water which was left overnight in the reaction vessel to equilibrate with air at  $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ . The output of the electrode in such a case usually results in a chart reading of 35 units (100 units a full scale deflection). Adding sodium dithionite to such air saturated water results in a rapid shift in the output reaching, chart reading of zero in about 50 secs. The difference between the output of the electrode with air saturated water at  $25^{\circ}\text{C}$  and with water containing sodium dithionite was taken to represent  $0.28\mu$  moles of oxygen per ml. Such a value was found to agree with calibration using standardised  $\text{H}_2\text{O}_2$  and catalase. The reaction mixture generally used in this assay was similar to that used by Cockburn et al (1968) except in the addition of 0.8 mM of  $\text{K}_3\text{Fe}(\text{CN})_6$  as an external acceptor and the chlorophyll concentration was 100  $\mu\text{g}/\text{ml}$ .

Illumination - The reaction vessel was illuminated by 150W spot light fitted with a projector lamp, the light from which was passed through 7 cm. water. The light beam was filtered using a red colour gelatin plate. The temperature of the assay mixture was kept at about  $24^{\circ}\text{C}$  ( $\pm 0.5^{\circ}\text{C}$ ) by circulating temperature controlled water through the jacket of the cell.

## 7. Assays of phosphorylation

### a. Coupled (non cyclic) phosphorylation

Chloroplasts which were isolated as described previously, were washed twice with  $40\ \mu\text{M}$  tris buffer and resuspended in a medium containing tris buffer,  $40\ \mu\text{M}$  pH 7.4, NaCl  $70\ \mu\text{M}$   $\text{MgCl}_2$   $10\ \mu\text{M}$ . After resuspending the chloroplasts were immediately used to avoid any loss of activity due to ageing. The reaction was carried out in a Clark oxygen electrode, the cell of which contained 3 ml. of the reaction solution. After 2 minutes of equilibration with air in the dark an aliquot of chloroplast suspension containing about 0.5 mg. chl/ml. were added. Reaction was started by turning on the light from the 150W spot light projector lamp. The reaction was allowed to continue for 2 mins. after which it was stopped by adding 0.1 ml of 6% (v/v) perchloric acid. Two 0.1 ml. aliquots of the reaction solution were pipetted out from the electrode reaction vessel and each placed in 10 ml tapered centrifuge tubes.

A sufficient amount of distilled water was added to each tube to allow centrifugation. Centrifugation was carried out in the cold room ( $5^{\circ}\text{C}$ ) using an MSE minor centrifuge at full speed setting for about 5 mins. after which the precipitated protein was discarded and the supernatant of each tube was poured into 10 ml. volumetric flasks. 2 ml. of phosphate reagent was added to one of the flasks and the volume of both was made to 10 ml. by distilled water. The contents in the flask containing no phosphate reagent was used as the blank solution to correct any possible interference in the optical density measurement due to the presence of ferricyanide. Phosphorylation was measured spectrophotometrically at 420 nm by following the esterification of inorganic phosphate by chloroplast. The activities were expressed as  $\mu\text{M PO}_4$  esterified/mg. chlorophyll/ml. Coupled oxygen evolution was measured polarographically as described earlier.

b. Cyclic phosphorylation (PMS)

Cyclic phosphorylation was measured using  $10^{-4}\text{ M}$  phenazonium methsulphate as a co-factor and the reaction was carried out in  $1.5 \times 10\text{ cm}^2$  glass centrifuge tubes with round ends. Each tube contained 0.3 ml of reaction solution (see Growth Media and Solutions). The final chlorophyll concentration in each tube was 0.01 mg. chl/ml.

Illumination - All the tubes containing the phosphorylation reactant were attached with terry clips to the outer rim of a 9 cm<sup>2</sup> perspex disk. This disk was fastened at its centre to the end of a motor-driven overhead stirrer, which was adjusted so that the tubes were held at an angle of about 20° to the surface of the water in a glass bath. The lower ends of the tubes were immersed in the bath whose temperature was about 23 ± 2. The source of light used in this assay was similar to that described in earlier assays. Before turning on the light the disk with the reaction tubes was rotated for a few seconds, at maximum speed, to ensure the mixing of all reactants in the tubes. The speed of the stirrer was then adjusted so that the contents of the tubes swirled around in a thin film facilitating light-saturation. Tubes containing the control reaction were treated similarly but painted black to exclude light. After illumination the reaction was stopped by the addition of small volume of 60% (v/v) perchloric acid. Tubes were then centrifuged to remove the protein precipitated by the acid and the supernatant was then taken to a known volume (8 ml.).

### 8. Estimation of Inorganic phosphate

Two colorimetric methods were used to determine the concentration of inorganic phosphate. In the first method (Allen, 1940 ) a blue coloration developed shortly after the addition of the following reagents: 0.15 ml. of 20% (w/v) sodium bisulphate, 0.15 ml. of 1% amidol (2,4-diaminophenylhydrochloride) and 0.15 ml. of 8.3% (w/v) ammonium molybdate. The intensity of the blue colour developed was then determined by reading at 600nm. In the second method inorganic phosphate was determined by measuring the optical density at 430nm of a solution containing 2 ml. of the yellow phosphovando-molybdate complex (Unican Instruments, method sheet No.53). The quantity of the phosphate in the samples was obtained in both methods by reference to a standard curve (Fig.1). At lower phosphate concentration the latter method seems to be more sensitive and was usually used in most of this work.

### 9. Amino acid incorporation by isolated chloroplasts

Chloroplasts isolated from hypochlorite surface sterilized leaves were suspended in sterilized tris-Mg medium (25 mM tris-HCl, 10 mM MgSO<sub>4</sub>, 5mM 2-mercaptoethanol, pH 7.8 at 20°). The incorporation of (C<sup>14</sup>) leucine into protein was measured after incubating chloroplasts (0.025 mg chl.) with ATP 0.5 mM, 0.05 mM GTP, 30 mM KCl 20 M C<sup>14</sup> leucine (sp.act. 55.2 mC) a final volume of 1 ml. After 60 minutes of incubation at 25°C,

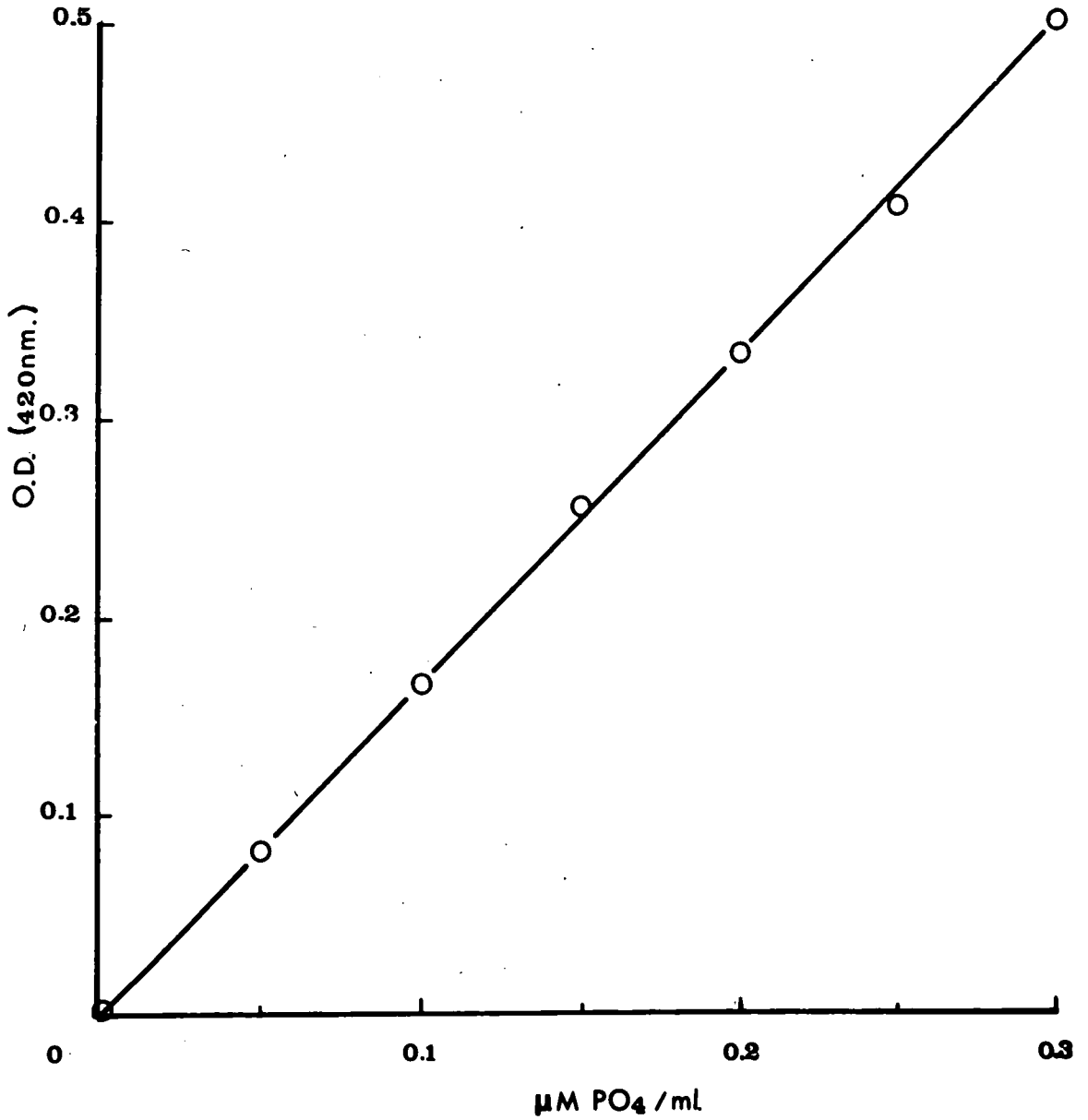


Fig. 1

Calibration graph determined using standard solution of Potassium dihydrogen phosphate

2 ml. of 5% (w/v) TCA containing 0.1M ( $C^{12}$ ) leucine was added to one of the incubation tubes. To another tube 1 ml. of (v/v) 5% triton detergent was added and the tube was kept at  $0^{\circ}C$  for 20 minutes, after which the content was centrifuged for 20 mins. 1 ml. of 5% TCA was added to the resulting pellets and supernatant. Tubes containing the zero time (control) incubation was also treated in a similar manner. All tubes were then kept in the cold room ( $4^{\circ}C$ ) overnight after which the precipitates were collected by centrifugation and washed four times with 5% TCA. All supernatants were discarded. The washed pellets were resuspended in 5% TCA and heated in  $90^{\circ}C$  water bath for 20 mins., after which they were cooled and centrifuged twice. Finally the hot TCA insoluble material was collected on Millipore filters. These Millipore filters were then dried under two infra red lamps and placed in the scintillation vials with 10 ml. of scintillation fluid. The radioactivity present on the filter was measured using a Beckman liquid scintillation spectrometer, model LS-200b at an average counting efficiency of approximately 90%. A vial containing a blank Millipore disk with 10 ml. scintillation fluid was always included among the samples counted to correct for counts due to background radiation and other sources. Results are expressed as counts/mins/mg. chlorophyll and are fully corrected for background radiation and zero time incorporation.

#### 10. Extraction of Free Amino Acids

Free amino acids were extracted from infected leaves at various intervals (i.e. 4, 8, 16 and 20 days after infection). Extracts from healthy leaves were also included each time. In each assay, three leaf samples (each = 1g/fresh wgt) were obtained from the second set of leaves of three different plants (see Plate 1). Amino acid extraction was carried out by grinding the leaf material in 60% (v/v) ethanol using acid washed sand. Extraction was continued until no further amino acids can be detected when extracts were tested using ninhydrin method (Yemm & Cocking, 1955). This usually takes place after the fourth extraction. Cell debris was then centrifuged out and the extracted amino acids from the three samples (replica) were combined and concentrated in a rotary evaporator. Total N amino acids in the various extracts were estimated by the Yemm and Cocking method (1955) with reference to a standard calibration curve (Fig. 2 ).

#### 11. Amino Acid Composition

Quantitative analysis was made on the various samples using a Technicon Automatic Analyser with a 133 cm. column of chromobead type A resin. The nine chamber autograd 18h elution system was used and the recorder had a scale expander fitted.



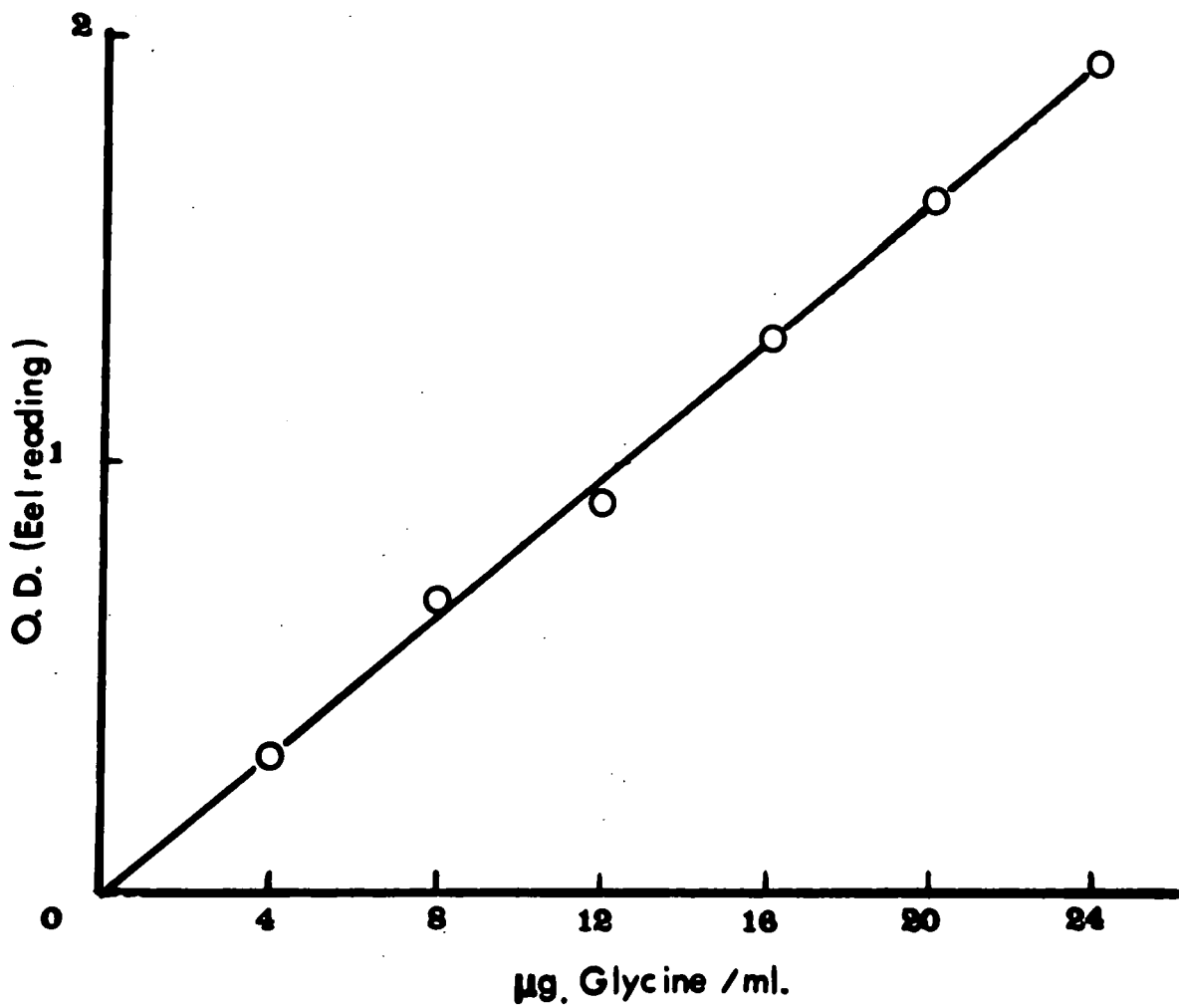


Fig. 2

Calibration graph determined using different concentrations of glycine (using Yemm and Cocking method, 1955).

## Results

### I. General light microscopic observations

#### 1. Infection of bean with *Uromyces fabae*

Infection of bean with *U. fabae* under normal conditions took place within 6-8 days after inoculation. The first visible symptoms are marked by the appearance of small white pustules which may later become larger and produce brown uredispores (Plate 2). Leaf strips or thin sections from 6-20 day-old infected leaves show an extensive fungal growth of intercellular mycelium (Plate 3). These mycelia can penetrate the host cells by means of a specialised branch, or haustorium. Plate 3 shows the form and the distribution of the mycelia and haustoria in the mesophyll regions and the infected cell may contain more than one haustorium, which is most embedded in the host cytoplasmic matrix. The light microscopic examination did not reveal any fine structural organisation of either the host or the fungus, but simply indicated that the haustorium consists of a swollen body.

#### 2. Infection of beans with *Botrytis fabae*

In contrast to the infection of bean with *U. fabae*, the visible signs of infection with *B. fabae* are comparatively fast and appear about 24 hr. after inoculation. The symptoms of *B. fabae* infections are expressed by the appearance of brown 'chocolate' spots, which

under favourable conditions spread to cover the entire leaf (Plate 2). Fungal growth was enhanced and could be detected by the naked eye if leaves were incubated for a further 4-5 days in a moist petri dish. Light microscope observations indicate <sup>that</sup> the necrosis, and the coloration of leaf cells, are restricted to the cells in the necrotic lesion (Plate 4a). Fungal growth was found to have been initiated but only to a limited extent in 1-2 day old infections. Plate 4b shows some of the extracellular (dense) bodies found attached to the outer wall of the mesophyll cells. Detailed discussion about these bodies will be given elsewhere.

## II. Electron microscopic observations

### 1. Fine structure of healthy mesophyll cells of Vicia faba

Typically, healthy mesophyll cells of V. faba contain a single, very large central vacuole. The chloroplasts and other organelles normally surround the vacuole and are embedded in a very thin layer (0.87 $\mu$ ) of ground cytoplasm; characteristically the cytoplasm is separated from the central vacuole by a unit membrane (tonoplast) (Plate 5). The chloroplasts in the uninfected cytoplasm are typically ellipsoid in shape and vary in size, ranging from 0.22-7.7 $\mu$ . The fine structural features and shape of the chloroplasts are basically similar to those described by Weintraub and Ragetli (1966) (Plates 6 and 7).

Mitochondria in the uninfected cells, are frequently situated between the end of two chloroplasts and were found to be both spherical and the rod-shaped, with sizes ranging from 0.6 to 1.2  $\mu$  (see Table 2).

Observation of healthy control cells of V. faba indicate that the amount of endoplasmic reticulum (ER) in these preparations was comparatively low. The measurement on the observed ER showed that their diameters range from 0.04-0.029  $\mu$ .

Both smooth and rough ER was present. The nuclei in healthy cells of V. faba appeared to have either a circular or irregular ellipsoid shape and varied in size ranging from 3.2 to 4.7  $\mu$ . Inside the nucleus there was one or two circular electron opaque nucleoli (0.6-1.6  $\mu$ ) surrounded by irregular areas of granular and fibrillar regions (Plate 6 and 8).

Occasionally in such preparations vesicle, or lomasome-like bodies were found in either pockets between the cell wall and the plasma-membrane or in pockets forming multivesicular bodies (MVB) in the cytoplasm. In some instances these vesicles were seen in a position to suggest that they might migrate from one cell to another through the plasma<sup>2</sup>desmata (Plate 9). The vesicles appeared to be circular in shape with electron transparent appearance. Their size varied from 0.04 to 0.34  $\mu$  in diameter.

So far as the origin and function of these vesicles in healthy leaves is concerned, no attempt was made to evaluate their function. However, there was some evidence to indicate their association with the ER (Plate 10).

Frequently, inclusions were found attached to the tonoplast and intruding into the vacuole. The shape of these inclusions were usually spherical or hemispherical 16-34 in diameter with electron dense contents and unit membrane outer layer. The inner part of these inclusions has a net-like appearance. These bodies may be similar in nature to those identified by Weintraub and Ragetli (1966), as tannin globules (Plate 6a).

## 2. Fine structure of the 'interface' of *V. faba* and *U. fabae*

### A. Fine structural investigation of the method of fungal penetration of cells

As *U. fabae* infects its host leaves, it grows and branches throughout the leaf tissues (Plate 11) and as a consequence of this, some of the proliferating intercellular mycelium contacts the outer part of the cell walls of the host and attaches itself. The attachment of the parasite to the host cell wall is made firmer by the invagination of the host cell wall at the point of attachment, and possibly by the production of adhesive substances, since electron dense materials can be seen at these sites (Plate 12a,b).

As the infection progresses some of the intercellular hyphae attached to the host cell wall starts to produce specialised branches, haustoria, which penetrate the host cells at this point. At this stage the host cell wall and plasma membrane undergo a considerable invagination under the action of the penetrating haustorium (Plate 13). As this continues, a hole is made in the cell wall of the host from which the developing haustorium penetrates the cell wall (Plate 14).

By this time a well-developed haustorium is formed surrounded by intact host plasma membrane (Plate 15) and normally, there is no evidence of the presence of the host cell wall around a well-developed haustorium (Plate 15 and 17). However, occasionally haustoria were observed which were completely walled off from the host cytoplasm by a thick covering, continuous with the host cell wall (Plate 18a,b). The penetration and complete formation of well developed haustoria takes place over a period of 2-8 days after the inoculation of the host with the parasites.

#### B. Fine structural changes in the infected host cells

The haustoria of U. fabae invade the region of the large vacuole of the host mesophyll cell, but continue to be surrounded by intact plasma membrane and layers of the host cytoplasm.

The volume of the cytoplasm of the host in the area of penetration was found to be considerably larger when compared with that of the cytoplasm of uninfected cells (Table 2 Plate 19.) Thus, in relatively advanced stages of infection (6-10 days after infection) the increased volume of the cytoplasm fills most of the region surrounding the haustoria.

The ER which is mainly rough increases both in concentration and in diameter (Plate 20a,21).

Frequently, the host plasma membrane around the haustoria exhibits a wide range of invaginations varying from small folds to large pockets which penetrate the ground cytoplasm (Plate 21, 22). Invaginations similar to these have been previously described by various authors working with different parasites, (Ehrlich & Ehrlich, 1963b; Peyton & Bowen, 1963; Berlin & Bowen, 1964). The host cytoplasm in the vicinity of the haustoria has many vesicles and MVB, which were not present in healthy cells (Plate 20,22). Further examination of these vesicles showed their association with the host Golgi bodies which are now also much more frequent (Plate 20b). Although, whether the origin of these vesicles among the haustorium body is the host Golgi bodies, still awaits further confirmation.

The chloroplasts in the infected mesophyll cells (2-6 days after infection) show very little change in shape or ultra-structure (Plate 23). At the time of flasking (6-10 days after infection) chloroplasts were found to be reduced in size, and contained many vesicles apparently originating from the inner chloroplastic membrane (Plate 24). Such vesicles have never been observed in chloroplasts of healthy leaves. In advanced stages of infection (10-20 days after infection) the chloroplasts continued to be reduced in size and the amount of photosynthetic lamellae were relatively reduced (Plate 25). At this stage of infection, the chloroplasts showed some deformation and aggregate of vesicular structure were frequently observed (Plate 25). Also Plate 27 shows that some chloroplasts occasionally produced curved extensions into the cytoplasm.

The cytoplasm in material infected with the rust pathogen, frequently shows higher concentrations of ribosomes than found in healthy tissues (Plate 26). This high concentration of ribosomes, in addition to the increased proliferation of rough ER, indicates an increase in the level of RNA in the diseased leaves. This accords with the findings made by Rash, Swift and Klien (1959) and Person (1960).



In most of the studies relating to the fine structure of the host parasite relationship, little or no attention has been devoted to the ultrastructural changes brought about by the infection of tissues with the parasite (Ehrlich & Ehrlich, 1962; 1963a,b; Peyton & Bowen, 1963; Berlin and Bowen, 1964; and Shaw and Manocha, 1965a,b). However, more recently the latter authors (Manocha and Shaw, 1966) have published an account of the fine structural changes in rust infected wheat nucleus. They indicated a marked increase in the electron density of the diffuse 'interchromatin' region of the nuclei at more or less earlier stages of infection (8 days after inoculation).

The results of the present study, indicated that the nucleus of uninfected mesophyll cells of bean is surrounded by a well-defined double unit membrane, and contain well differentiated interchromatin and chromatin regions (Plate 5,6b). The distribution of these two areas appeared to be in equal proportions (Plate 8).

In rust infected bean cells (6-8 days after inoculation) there are marked increases in the size of both the nuclei and the nucleoli (Plate 28, Table 2 ). An equally considerable increase in both the density and the concentration of the interchromatin region were also noted (Plate 29). Another difference in the nucleus of the infected cell over the controls indicated that the

chromatin region are more or less scattered and less aggregated. These results agree with some of the findings made by Manocha and Shaw (1966) working with Puccinia graminis tritici.

In this connection, a comparative biochemical study of the protein and the nucleic acid synthesis might cast light on some aspects of the relationship between the fine structural and biochemical changes of the host infected cells.

In occasional sections of the same material haustoria, similar to those commonly described as 'necrotic' haustoria, were observed. Whether the necrosis or the death of these haustoria are due to specific host reaction is not yet clear. However, some evidence suggested that these haustoria are walled off by the complication of the host cell wall and electron dense vesicles (Lomasomes) (Plate 18a,b). At the same time, a similar type of haustoria were also found which were free of the host cell wall and embedded in apparently healthy cytoplasm (Plate 30). It is also worth mentioning that both the necrotic and the apparently normal haustoria can be found in the cytoplasm of one cell.

Studies relating to the cause of haustorial necrosis should prove interesting and may reveal some aspects of the nature of resistance of V. faba to U. fabae.

C. Fine Structure of *U. fabae*

(a) Fine structure of the haustorial apparatus

Following the penetration into the host cell, the developing haustoria elongate forming a neck (Plates 13,14,15). The plasma-membrane and the cell wall of the haustorial neck appear to be continuous with the plasma-membrane and the cell wall of the intercellular hyphae 'the haustorium mother cell' (Plates 13-15). Eventually the haustorial neck ceases to elongate and the apical portion swells to form either one single haustorial head or branched body. Although a complete set of serial sections through a parasitized host cell was not obtained, it is apparent, that in one single mesophyll cell of infected leaves of *V. faba*, <sup>containing</sup> more than one haustorial head varies considerably from spherical to flattened cylindrical or branched lobes (Plates 15,16,32). Their size also varies from 0.24-0.5  $\mu$  averaging about 0.39  $\mu$  in diameter. The haustorial heads are connected to the intercellular hyphae by a neck about 0.33  $\mu$  in length and 0.083  $\mu$  in diameter.

In the early stages of haustorial formation, the haustorial neck was found to be bound by a very electron dense wall, which is continuous with the wall of the haustorial mother cell (Plate 13). Similar electron dense walls were also found bounding a well developed haustorial neck. Less frequently, the electron dense wall surrounding the haustorial neck (in the early stages of development) ceases to elongate and a new less electron dense wall

evolves to surround the entire haustorial head and the upper and lower parts of the haustorial neck. The old electron dense wall now forms a ring in about the middle part of the haustorial neck (Plates 14, 33a). In this respect, the haustorium neck differs in its wall structure from other fungi reported on, and is consistent with the structure of the wall of the haustorial neck of U. phaseoli (Greenwood, et al., personal communication). Haustorial neck cytoplasm contained few if any organelles (Plates 13, 14, 15, 33). On the other hand, the cytoplasm of the haustorial head was filled with granular cytoplasm, with many organelles. The mitochondria appeared to have a structure similar to plant mitochondria (Plate 15, 16, 19, 21, 33). They are bound by a double unit membrane. The inner mitochondrial membrane invaginates to form typical tubular cristae. In sectioned profiles of haustorial heads, mitochondria appear to vary in shape from spherical to nearly cylindrical, with average size about  $0.08 \mu$  in diameter.

Nuclei and ER along with other unidentified organelles were observed in the cytoplasm of the haustorial head (Plate 15, 16, 19, 21). Usually each haustorium has one or two nuclei. These nuclei are bound with an inner and an outer membrane and contain one or two nucleoli surrounded by masses of well differentiated chromatin and interchromatin regions (Plates 21, 31, 32). Numerous

ribosomes were found in the cytoplasm of the haustorial head.

The plasma-membrane appear to be continuous on the entire haustorium cytoplasm and no evidence of lomasomes or lomasome-like bodies, similar to those reported by various authors, could be found in the haustoria of U. fabae. Similarly, the haustorium cytoplasm appear to lack any evidence of Golgi dictyosomes similar to those reported to be found in various obligate and facultative parasites. Bodies which could be identified as lipid bodies were only found in some haustoria (Plate 19) and Ehrlich and Ehrlich (1966) suggest a possible relationship between the presence of such bodies and the age of the haustoria.

Frequently, one or more vacuoles were found in the haustorium of U. fabae. These vacuoles appeared to contain numerous vesicular and tubular elements (Plate 21, 22, 32, 34). The vesicular elements frequently aggregate together to form M.V.B. The outer membranes of these bodies were normally masked by a very electron dense coating with irregular appendages. Similarly, the tubular elements in the vacuoles interwove to form coil-like shapes. The outer part of these interwoven tubules were equally coated by electron dense material (Plate 34c).

Such elements in vacuoles of haustoria have not been reported before nor could they be traced in photographs obtained in related

studies (Ehrlich and Ehrlich, 1963b; Peyton and Bowen, 1963). It is also worth mentioning here that bodies similar to the vesicular types were also found in the host cytoplasm in the vicinity of the haustoria (Plate 21). Whether these vesicles have originally passed from the haustorium to the host cytoplasm is not completely clear. However, the fact that these elements are always found in the vacuole of the fungus (and not in the cytoplasm), suggests its possible fungal origin. On this basis its function could either be secretory or to stimulate synthetic activity. The entire haustorium is normally surrounded by a 'sheath' of unknown material. In sectioned profiles, this encapsulation appeared to vary greatly in both thickness and distinctness. While in some sections they are clearly differentiated from the haustorial cell wall (Plates 25, 31) in other sections, such a layer is very difficult to trace (Plates 19, 21). No channels or 'plasmadesmata like' connections similar to those reported in other fungi (Calonge, 1969) were found in the haustoria. Thus, the cytoplasm of the fungus and the host appear to be discontinuous.

(b) Fine structure of the intercellular hyphae

As the uredospores of U. fabae contact the susceptible host, they produced germ tubes which penetrated the leaves and start to branch in the intercellular spaces. The intercellular hyphae which are on average about  $0.33\mu$  in diameter, continued to proliferate

producing a branched growth (Plate 11). These hyphae appeared to have a relatively thick (0.064-0.1 $\mu$ ) electron dense wall. The plasma membrane which is a unit membrane, invaginates in various areas to give numerous moderately electron dense lomasomes (Plate 35). The average size of such vesicles is about 0.07 $\mu$  in diameter. Similar structures in the intercellular mycelia and haustoria have been observed by various authors working with various pathogens (Ehrlich and Ehrlich, 1963a; Peyton and Bowen, 1963; Berlin and Bowen, 1964). In the present studies such structures were not found in the haustorium (Plate 14-16). Little work has been done on the nature of these structures, and consequently various roles have been assigned to them such as secretion wall formation (Wilsenach et al., 1965), absorption by haustoria (Peyton and Bowen 1963, micropinocytosis (Carroll, 1964). My observations on this fungus as well as on B. fabae suggest their possible secretory nature and detailed discussion on this point will be given elsewhere.

Various M.V.B. and tubular bodies were also observed in the cytoplasm of the intercellular mycelium. The content of the vesicles of these bodies react to electron stain in a way similar to the materials of the vesicles found between the plasma membrane and the cell wall. This tends to suggest that both types of vesicles are of similar nature.

3. Fine structure of the interface of *V. faba* and *B. fabae*

(a) Fine structural investigation of the method of fungal penetration of the host cells

In contrast to the infection of bean by the rust pathogen (*U. fabae*) the infection by *B. fabae* can take place within 24 hours after inoculation. Because of the rapidity in symptom expression and tissue breakdown, no serial section of the method of penetration was possible to obtain. However, occasional sections of leaves at various stages of infection tend to suggest that host cell penetration is accomplished by hydrolysis of the host cell wall. Prior to this the invading fungus attaches itself to the host cell wall which invaginates at the point of attachment (Plate 37). The fungal mycelium then produced a hydrolytic enzyme which dissolve the part of the host cell to which it is attached (Plate 38). Evidence in this study suggest that the hydrolytic enzymes is probably contained in the vesicle component of the lomasome structure which were frequently encountered in the fungal mycelium (Plates 39,40,41). Plate 38 shows the association of vesicles with the breakdown of host cell wall. Accordingly, this fungus does not produce a haustorium as such but by virtue of the disintegration of the host cell the intercellular mycelium became intracellular (Plate 41,42,43). Plate 44 represents a diagrammatic drawing for the method of host cell penetration by *B. fabae*.



(b) Fine structure of the infected host cell

The most early features of this infection which could be revealed by both light and electron microscope is the necrosis and the breakdown of the tissues in the immediate vicinity of the necrotic lesion (Plate 41-44). Under favourable conditions, the necrotic lesions could spread in a period of 2-10 days to cover the entire leaf.

The early death and necrosis of the tissues under the direct effect of the infection droplets could be attributed to the reported production of enzymes by the parasite (Deverall and Wood, 1961) and the host's response to such a rapid infection is thought to be the production of anti fungal substances (Purkaystha and Deverall, 1965b).

Electron microscope examination of the necrotic tissues indicates that the early changes in such areas is the loosening of the cells connection. This was followed by various effects, some of which are the breakdown of cell organelles and the hydrolysis of the cytoplasmic membranes (Plate 45). Another effect which could be seen in the necrotic lesion is the blackening of the cytoplasm followed by the disorganisation of the cell wall (Plate 46, 47). Fungal development in such lesions seems to be very limited and consequently sectioned profiles of fungal mycelium were only occasionally seen.

Cells at various distances from the centre of the necrotic lesion appeared to show gradually less necrosis and blackening (Plate 48); and the periphery of such lesion contained cells with organisation completely different from those within the necrotic lesion and also that of healthy cells (Plates 48-53). In addition to the increased volume of their cytoplasm, the reaction of these cells to the infection was marked by the increase in both the quantity and the diameter of the ER and also by an increase in the size of the mitochondria (Plates 48-49 and Table 2). The concentration of ribosomes in the cytoplasm and on the surface of the ER was also found to be increased (Plate 52). However, the size of the chloroplasts of these cells appeared to have been reduced in comparison to healthy ones, although no changes in chloroplast ultrastructure could be detected. Golgi bodies have frequently been observed in such cells, such elements could rarely be found in the uninfected tissues. Although no clear-cut changes could be observed in the fine structure of the nucleus of these cells, the size of both the nucleus and nucleolus were found to be increased over the control (Plate 48,49,51 and Table 2). Other changes which seem to be a characteristic feature of such cells in relatively later stages of the disease (6-9 days after inoculation), is the presence of various extracellular electron-dense bodies attached to the outer part of the host cell wall (Plates 53-55). These bodies appear to

vary in organisation, shape and electron density. According to these characters they can be divided to either 'electron dense extracellular bodies' (E.B) or 'electron dense extracellular bladders' (E.L.). The first type is characterised by high electron density and by the absence of any outer covering. Frequently such bodies appear to be stratified to two layers with different electron density (Plate 54). The second type is characterised by a layer of similar electron density bounded by equally dense ornamented walling (Plate 55a,b,c). Because of the high electron density of both bodies, no resolution of any organelles was possible.

Frequently the cytoplasm underneath the cell wall, to which these bodies are attached, produces an electron dense band corresponding in its density to that of the content of these bodies (Plate 54, 55). On the light microscopic level these bodies appeared to be similar in appearance to cross sections of fungal mycelium attached to the host cell wall. Structures similar to these bodies were frequently seen in the free extracellular spaces. More frequently, vesicles with diameter averaging about  $0.109\mu$  were found in the cells at the periphery of the necrotic lesion. These vesicles are found either in cells neighbouring a fungal mycelium or in tissues which lack any evidence of fungal presence (Plate 56, 57). Occasionally similar vesicles, but surrounded by an incapsulation of unknown material,

were found in the extracellular spaces (Plate 58).

In relatively later stages of the disease development (12 days after infection) the vesicles in the cytoplasm of such cells appear to aggregate together forming a vesicular cytoplasmic inclusion. These inclusions are found to be either embedded in the ground cytoplasm or to be bound by the tonoplast and intruding into the vacuole (Plate 59a,b,c). In any of these cases these inclusions appear to have an outer bounding membrane. Evidence suggests that such inclusions may eventually breakout from the cytoplasm and discharge to the vacuole. The content of these inclusions seems to be composed of masses of the host cytoplasm with numerous vesicles. Other than these vesicles no other cytoplasmic organelles were found in such inclusions.

(c) Fine structure of *B. fabae* growing in the host tissues

Although fungal development in 2-6 days old necrotic lesions is very limited, sectioned profiles of *B. fabae* mycelium could occasionally be found (Plate 39,43, 46-47). In such profiles the mycelium is characterised by an electron dense cytoplasm with very few cytoplasmic organelles. Sometimes fungal mycelium appears to be black, with no evidence of any cytoplasmic organelles. This effect could have been brought about by the action of the parasitised host cells. At points where the mycelium has contacted the host cells, the cell walls of both organisms look undefined, and the

cytoplasm at the points of the contact appear to have many vesicles (Plates 37, 56). Indirect evidence suggested that these vesicles may contain some hydrolytic substances which act on both the host and the fungus wall. This point was investigated further, the results of which are described in Results 2.C.

As mentioned previously, under the normal conditions, fungal growth in host cells was relatively limited. This would consequently limit detailed examination of the structure of the fungus. To overcome this problem, fungal development was enhanced by incubating leaves which showed typical infection symptoms in moist Petri-dishes at room temperature. Under these conditions growth and the sporulation of the pathogens were enhanced as indicated by the appearance of fluffy fungal growth on the surface of the incubated leaves. After 3-4 days of incubation in the moist chamber, leaves were then processed and sectioned for electron microscopy in the usual way (see Methods). Electron microscope examination of such leaves showed that the host tissues were completely blackened, and most of the host cytoplasmic organelles have been broken down. In the electron microscope some of the cytoplasmic membranes appeared to be destroyed, and membranes and the grana of the chloroplast were electron transparent (Plates 42, 50). The host cell wall in these preparations appeared to be dissociated and granular in appearance (Plate 47).

Although the fungal development was enhanced, the cytoplasm of the mycelium appeared to be vacuolated, and consequently cytoplasmic organelles were much reduced in number (Plate 39, 43). Fungal cell wall appeared to have inner (electron transparent) and outer (electron opaque) layers. Sometimes these two layers are ill-defined and the outer (electron opaque) layer was frequently lacking. The plasmalemma was usually well-defined and continuous around the cytoplasm, except at some points where it invaginated to contain lomasome-like vesicles (Plate 39, 43). Mitochondria appeared to be relatively small and varied greatly in shape, ranging from nearly ovule to short rods (Plate 39, 43); nuclei with diameters ranging from 0.153-0.46  $\mu$  having a single electron dense nucleoli were found in the fungal cytoplasm. The nucleus was filled by irregular fibular and granular regions and surrounded by a double unit membrane. In the cytoplasm, scattered double unit membranes ER were usually found in very low concentration (Plate 40, 43).

Dense bodies with diameters ranging from 0.09-0.99, and similar to those reported and described by Buckley et al., (1966) as 'less dense inclusion' in B. cinerea, were commonly found in the cytoplasm of B. fabae (Plate 39).

(d) Fine structure of *B. fabae* growing on bean extract agar medium

The usual medium used for growing and maintaining *B. fabae* was bean leaf malt agar medium. This medium is thought to enhance the growth and the sporulation of the pathogen.

Electron microscopic examination of the fungal mycelium growing on this artificial medium, indicated that the mycelia have a double layered cell wall differentiated to an inner (electron transparent) layer about 0.022-0.032 $\mu$  and an outer (electron dense) layer about 0.012 in thickness. The septa of the mycelium were continuous with the inner layers of the cell wall, which taper towards the centre of the mycelium to form septal pores (Plate 60, 61). Electron dense membrane-bound bodies were usually associated with the septal pore, and sometimes these bodies appeared to plug the opening (Plate 60). These structures are probably homologous with 'Wormin bodies' described by Reichle and Alexander (1965). The plasmalemma is closely appressed to the cell wall except when lomasomes are present. These are few and similar to those described by Moore and McAlear (1961a). The cytoplasm enclosed within the plasmalemma is of complex nature with irregularly distributed ER. The ER in the cytoplasm of the fungus growing on artificial medium (Plate 60) is more abundant than that in the fungus growing in the host tissues.

The cytoplasm is mostly granular with a relatively higher electron density. In contrast to those of mycelium growing in vivo the mitochondria were numerous and mostly long rod shaped about  $0.16-0.47\mu$  in length and of an average width of  $0.18\mu$ . Their cristae appeared to be more or less tubular shaped (Plate 62). The nuclei contained a relatively dense nuclear material and were bounded by double layered nuclear envelope usually interrupted by some pores (Plate 60). Relatively mature hyphae contained several vacuoles which frequently seemed to coalesce into larger vacuoles.

In contrast to the mycelium of B. fabae growing on its host, lipid globules appeared to be numerous in cytoplasm of the fungus growing on artificial media (Plate 62). No evidence of Golgi bodies could be seen in the cytoplasm of the fungus growing on any substrate.

(e) Relationship of Vesicular structures to the hydrolysis of complex substrates by B. fabae.

Reports on vesicular structures in fungi are numerous, but the first observation of such structures was made by Girbardt working on Polystictus versicolor (1958). Since then various reports on the presence of these structures in different fungi were published (Glauret and Hopwood, 1959; Moor and McAlear, 1961a; Hawker and Hendy, 1963; Peyton and Bowen, 1963; Berlin and Bowen, 1964; Pitt, 1968). In 1968 Pitt working with B. cinerea demonstrated



the location of several acid and neutral hydrolyses in cytoplasmic particles by means of histochemical methods. Other authors working with different fungi expressed the view that the function of these structures was to be involved in the synthesis of cell wall. (Glauert and Hopwood, 1959; Crawly, 1965; Esau et al., 1966). Electron microscopic examination of B. fabae growing on its host tissues, showed that similar vesicular structures are common in both the fungus and the host cytoplasm.

To examine this possibility further, electron microscopic preparations were made from the mycelia of the fungus growing on various complex substrates (pectin, sodium poylpectate, cellulose and starch). For comparison, preparation was also made from mycelia of the fungus grown on bean leaf malt agar medium, glucose medium and medium X). No changes were found in the fine structure of the fungus growing on any of these medium (Plates 63-66). As far as vesicle formation in the cytoplasm of the fungus, it appears that such structures are found in the cytoplasm of the fungus growing on all the above-mentioned substrates, with the exception of medium X and medium containing glucose as the only source of carbon (Table 3 ).

As is seen from the results in Table 2 and from electron photographs (Plates 63-66), these vesicular structures are more

numerous in the cytoplasm of the fungus growing on bean extract medium. Although these vesicles are usually found between the plasmalemma sometimes they are found in the cytoplasm matrix near the cell wall.

TABLE 2

Changes in the organelles of Uromyces and Botrytis fabae  
infected mesophyll cells of bean\*

System	ER quant.	diameter	Mitochondria diameter	Cytoplasm thick	Vesicles dense trans.	Chloroplast size	Nucleus diam.	
Cells infected with <u>B. fabae</u>	++	.04-0.46 0.067	0.4-1.4 0.79	1.2-6.8 3.05	- none	.063-.26 0.109	1.4-5.6 3.23	3.3-8.8 6.53
Cells infected with <u>U. fabae</u>	+++	.04-.08 0.059	0.31-1.2 0.74	0.31 3.3	0.11-0.22.09-0.2 0.16	0.11 0.11	1.9-5.5 3.3	3.4-9.2 6.9
Healthy cells	+	.04-0.29 0.06	0.6-1.2 0.7	0.18-3.7 0.87	.04-.34 0.11	2.22-7.7 4.8	3.2-4.7 4.6	

Note: First row represents the spread of observations

\* All measurements are in  $\mu$  and based on average size of 20 organelles (second row)

TABLE 3

Effect of the composition of the medium on the growth  
and the production of vesicles in the cytoplasm of the mycelia

Complex substrate in medium *	Amount of growth cm.	production of vesicles †
Bean extract medium	8	+++
Starch	4	++
Cellulose	3	++
Pectin	2.5	++
Sodium polypectate	3.5	++
Glucose	6	†
Medium X	6	†

\* Basal medium (Asparagine 0.2 g.,  $MgSO_4 \cdot 7H_2O$  0.075,  
 $K_2HPO_4$  0.125 g. and traces of FeCl, ZnCl, CuCl,  
MoCl and CaCl + 1000 water).

† No vesicles

TABLE 4

Comparison between the fine structure of the cytoplasm  
of *Botrytis fabae* and *Uromyces fabae* intercellular mycelium

Organelles	<u>U. fabae</u>	<u>B. fabae</u>
Cell wall	One electron dense	Two inner electron transparent and outer electron dense layer
M.V.B.	Present	Present
Lomasomes and vesicles in vacuoles	Present	Absent
Protein bodies (dense bodies)	Absent	Present
Mitochondria	Rod and spherical shaped	Mostly rod shape, few spherical
Nucleus	1.1-1.9 m with double unit membrane	0.7 m-1.0 with double membrane
Ribosomes	Present	Present

### III. Microscopy of isolated chloroplasts

As judged by both phase contrast and electron microscopy, the preparation appeared to consist mainly of chloroplasts. Very few mitochondria, nuclei and cell debris were seen frequently and little or no bacterial contamination could be seen in such preparations.

Phase contrast microscopy using dark ground illumination showed the presence of two types of chloroplast in the preparations (Plate 68). The first type appeared very shiny and opaque with surrounding haloes, and were clearly differentiated from the other group of chloroplasts, which were characterised by their flattened appearance and relatively large size. The latter group were granulated and their grana could be easily distinguished (Plate 68).

According to Khan and Von Wettstien (1961) the shiny opaque chloroplasts are intact, while the flattened and granulated ones are membraneless. Electron microscopic examination in the present study confirmed these suggestions (Plates 69-72). The ratio of intact, to membraneless, chloroplasts in preparations obtained using different conditions and methods of preparation, is given in Table 5. The discrepancies in the estimated ratio of the two types of chloroplast in preparation viewed by phase contrast and electron microscopy, is probable due to some loss of chloroplast

TABLE 5

Effect of the method of isolation and the presence or  
absence of protective chemicals on the percentage  
of intact isolated chloroplasts

Method of isolation	Protective chemical added	% Intact	
		Phase Cont.	E. Microscope
Mortar and Pestle	Ficol and Dextran	60-70	55-60
"	none	55-65	50-55
Domestic Mixer	Ficol and Dextran	50-55	45-50
"	none	40-50	30-40

membranes, during sample preparation for the electron microscope. As can be seen from the table, preparations obtained by grinding the leaves in a mortar and pestle contained relatively higher proportions of chloroplast with their outer membrane still intact. The addition of protective chemicals such as ficol or dextran also led to the recovery of more intact chloroplasts.

Comparing chloroplasts isolated from leaves infected either by U. fabae or B. fabae with those from healthy leaves indicated few fine structural differences. Vesicles were found in chloroplast preparations obtained from both healthy and infected leaves, although the number and frequency of vesicles was greater in chloroplasts from rust infected leaves (Plate 71). Little or no change was observed in the case of preparations from B. fabae infected leaves (Plate 72).

#### IV. Influence of Infection on Chloroplast number and chlorophyll content.

##### (1) Effect of the infection of V. faba on the number of chloroplasts

The number of chloroplasts was estimated at two different stages of disease development (6 and 16 days after infection). Only the results concerning the latter stages (16 days after infection) are included in Fig.3. As far as the earlier stages are concerned, the results indicated that the number of chloroplasts in rust



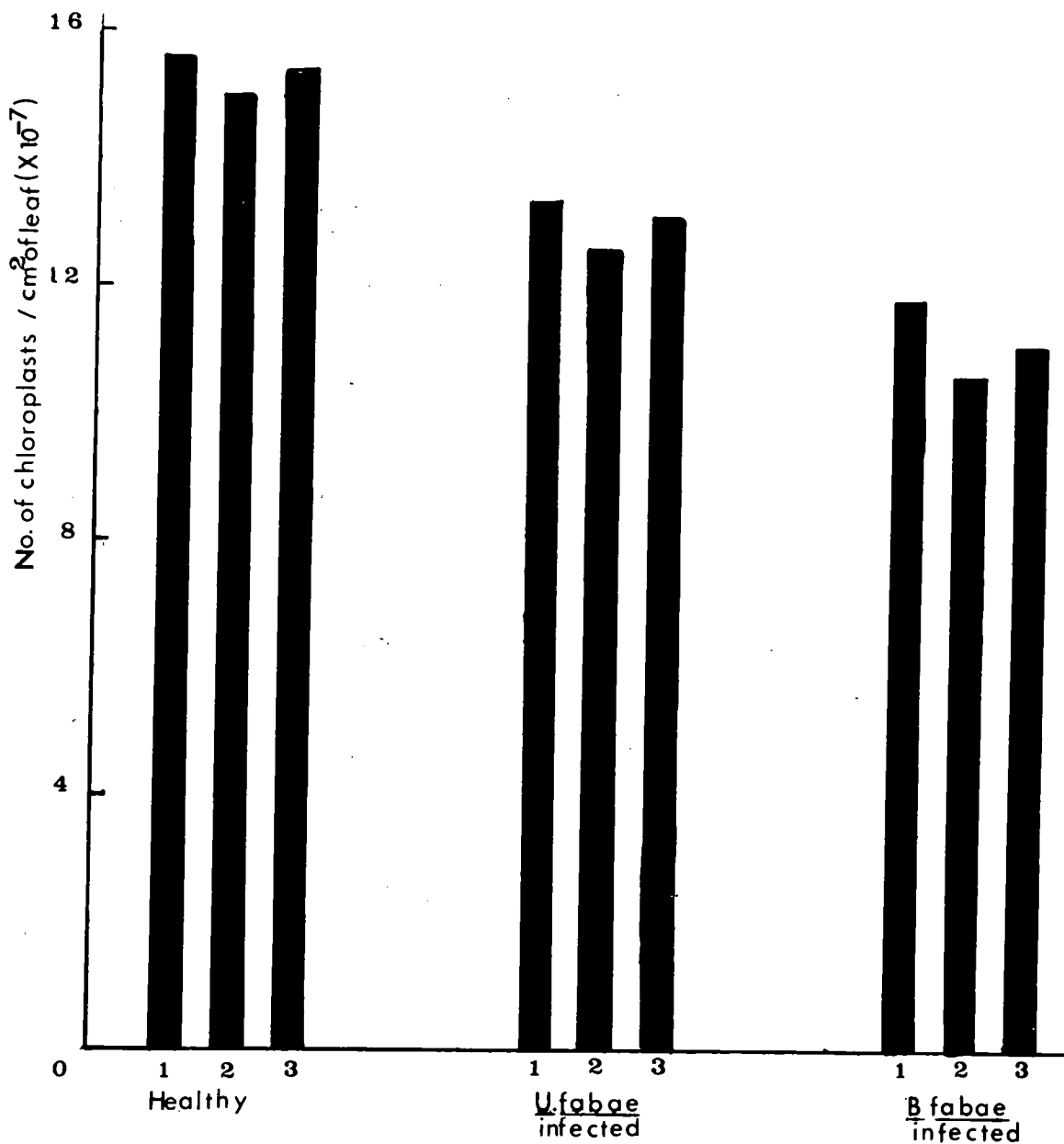


Fig. 3

Influence of V. faba infection by U. fabae and B. fabae on the number of chloroplasts per cm. of leaf area. Bars 1 and 2 are the results of duplicate experiments, bar 3 average of the two.

infected leaves (6 days after infection) remain more or less equal to that of healthy leaves (i.e.  $16.8 \times 10^7$  - Average of three replicates). However, the number of chloroplasts in leaves infected with B. fabae was found to be reduced ( $1.2 \times 10^7$  chloroplasts per  $1 \text{ cm}^2$  of leaves) as early as 6 days after infection. This was probably due to the early development of the disease and the production of the necrotic lesion. As both infections progressed and particularly following the sporulation of the rust pathogen (16 days after infection), the number of chloroplasts were found to drop in comparison with those of healthy leaves. The number of chloroplasts in Botrytis infected leaves was found to be considerably reduced in relation to the number of chloroplasts in Uromyces infected leaves (Fig. 3)

(2) Relationship between the number of chloroplasts and chlorophyll concentration in chloroplast preparations from healthy Uromyces infected and Botrytis infected leaves.

The results in Figure 4 illustrate the relationship between the number of chloroplasts and the chlorophyll concentration in preparations from healthy and infected leaves. As shown in the graph, there was a linear relationship between the chlorophyll concentration and the number of chloroplasts in all preparations. However, preparations from infected leaves (both Uromyces and Botrytis infected) were found to contain more chloroplasts per unit chlorophyll concentration. This suggests that the chlorophyll content per

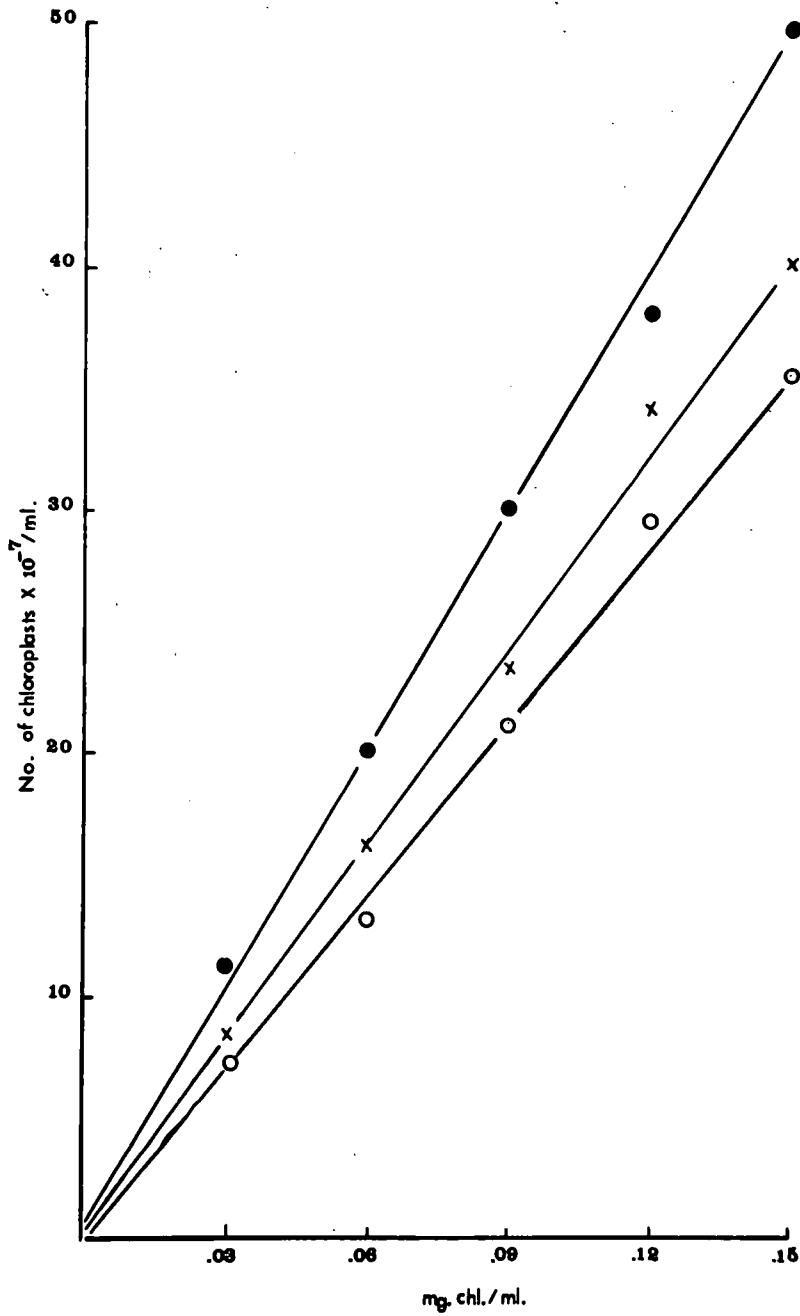


Fig. 4

Relationship between the number of chloroplasts and the chlorophyll concentration in chloroplasts preparations from healthy (○ ○), U. fabae infected (X X) and B. fabae infected leaves (● ●) (12 days after infection). Values are average of three replicates.

chloroplast is probably reduced as a result of the infection. This reduction in chlorophyll content was found to be greatest in the case of preparations from leaves infected with B. fabae (Fig. 4 )

V. Photochemical activities of isolated chloroplasts

1. Factors affecting Hill reaction of chloroplasts isolated from healthy leaves of V. faba

Preliminary experiments in the present study and those of others (R. Leach and D. Walker et al., personal communication) on chloroplasts isolated from leaves of Vicia faba, suggest that they possess little photochemical activity. In fact, under conditions known to stimulate active oxygen evolution with other plants (Cockburn et al., 1963), these chloroplasts had a rapid oxygen uptake. In view of this and in order to evaluate the effect of infection, a study of some factors known to affect oxygen evolution by isolated chloroplasts from healthy leaves, was made.

A. Effect of plant age on the evolution of oxygen by isolated chloroplasts

Chloroplasts isolated by a method similar to that of Cockburn et al., (1968) were obtained from plants of different ages. In the absence of added electron acceptor, there was no oxygen evolution. On the addition of 0.8 mM potassium ferricyanide to the reaction mixture, an appreciable amount of oxygen output was recorded as can be seen from Figure 5. The rate of oxygen

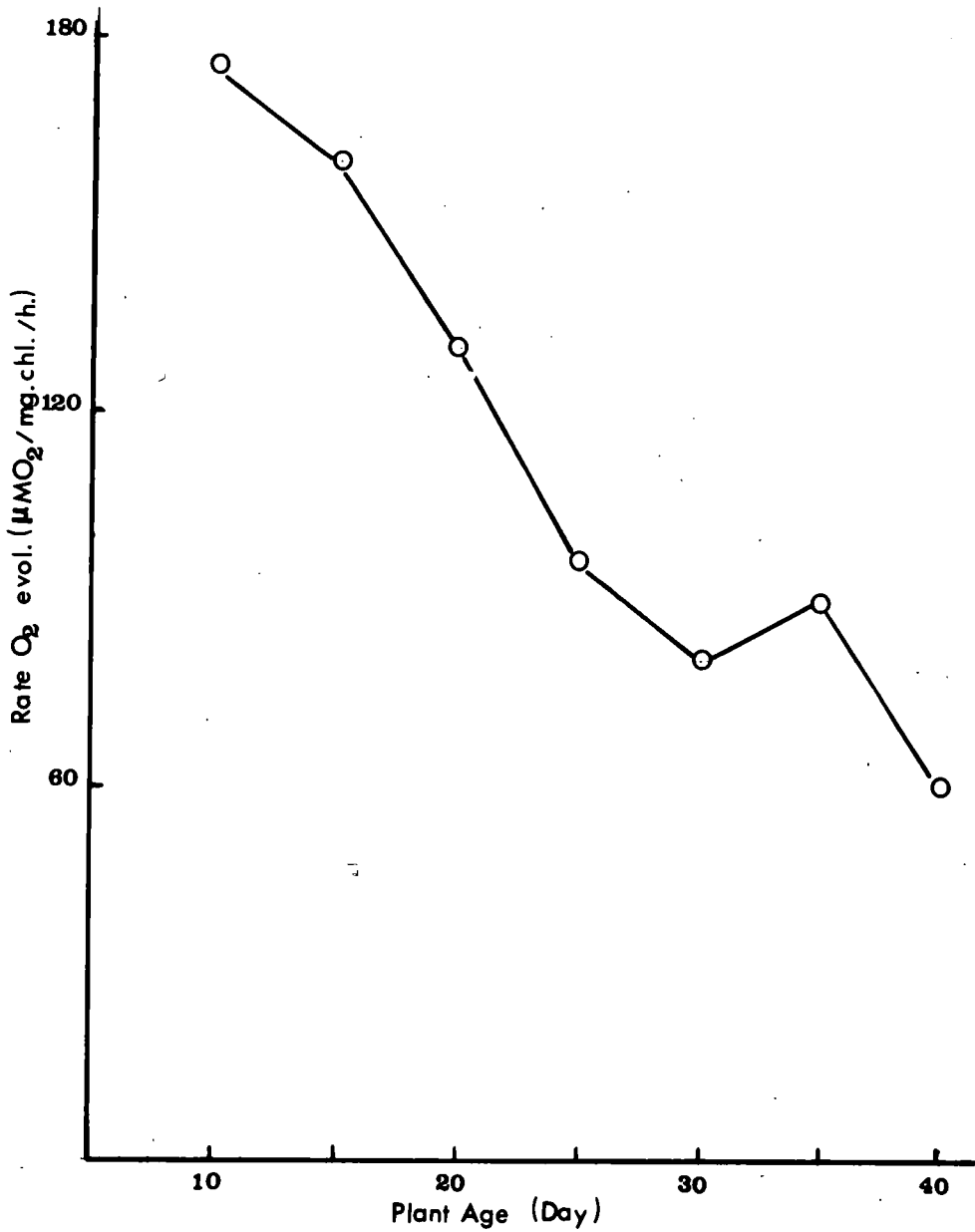


Fig.5

Effect of the age of plants on the rate of oxygen evolution of isolated chloroplasts. (Reaction mixture contained  $K_3Fe(CN)_6$  when no oxidant present, there was oxygen up to  $K_2$ ).

TABLE 6

Effect of the type of oxidants on the Hill reaction  
by isolated *V. faba* chloroplasts

Type of Hill Oxidant	Rate of Hill reaction $\mu\text{MO}_2/\text{mg.chl/h}$
Potassium ferricyanide	125.0
Phenoindo-2,6-dichlorophenol (DPIP)	-25
2,6-DPIP + 0.1 ml. of Potassium Ferricyanide	-0.63
Nicotinamide adenine dinucleotide (NADP)	0*
No electron acceptor	-25

- negative values = uptake

\* small initial  $\text{O}_2$  evolution

evolution (Hill reaction) by chloroplasts was found to decrease with the age of the plants from which chloroplasts were isolated.

B. Effect of the type of added oxidant on the rate of oxygen evolution

Various reaction mixtures containing either no added oxidant or potassium ferricyanide, Phenolindo-2,6-dichlorophenol (DPIP) or nicotnamide dinucleotide phosphate (NADP), were investigated. The results of such experiments indicated that potassium ferricyanide was the only external oxidant which stimulated the evolution of oxygen. Oxygen uptake rather than evolution resulted from when the reaction mixture contained either no added oxidant or when DPIP or NADP were added. However, it is worth mentioning that when NADP was used a small initial oxygen evolution was obtained, followed by some oxygen uptake (Table 7).

C. Effect of the addition of carbon dioxide on the rate of oxygen evolution

The influence of carbon dioxide on the rate of oxygen evolution was studied under different experimental conditions (Table 7). The results indicate that the addition of carbon dioxide had no effect on the rate of Hill reaction catalysed by any of the oxidants used. Also, the rate of oxygen uptake, previously discussed, seemed to remain unaffected by the presence or absence of carbon dioxide in the reaction mixture (Table 7).

TABLE 7

Effect of the addition of CO<sub>2</sub> on the rate of oxygen evolution catalysed by various Hill oxidants

Type of Hill Oxidant	Rate of Hill reaction $\mu\text{MO}_2/\text{mg.Ch/h}$	
	-CO <sub>2</sub>	+CO <sub>2</sub> *
Potassium Ferricyanide	138.6	138.6
DPIP	-0.63	0.63
NADP	0	0
No electron acceptor	-25	-25

\* CO<sub>2</sub> was introduced by adding 8 mM of NaHCO<sub>3</sub>

(8 mM NaHCO<sub>3</sub> = 1.6 mM of CO<sub>2</sub>)

negative values = uptake



D. Effect of the concentration of potassium ferricyanide on the rate of Hill reaction

Since potassium ferricyanide was the only oxidant which catalysed oxygen evolution, an experiment was made to determine the effect of varying its concentration. The results of this experiment indicated that the Hill reaction is enhanced by increasing the concentration of potassium ferricyanide from 0-.08 mM, when it reached a maximum (Fig.6 ).

E. Effect of varying the chlorophyll concentration per preparation on the rate of Hill reaction

Varying the chlorophyll concentration from 0-0.2 mg chlorophyll/1 ml resulted in an increase in oxygen production. As shown in Fig.7 this increase was proportional to the concentration of chlorophyll in the reaction mixture.

F. Influence of light on Hill reaction

In order to obtain some information as to what extent the colour of light affected the rate of Hill reaction, a number of experiments were made using gelatin filter sheets, obtained from Griffin and George. When the reaction mixtures were illuminated with blue (4000-5000nm) or green light (5200-6200nm) no oxygen evolution was observed; the rate of Hill reaction was the same when illuminated by red (5800-7000nm) or white light (Fig. 8 ).

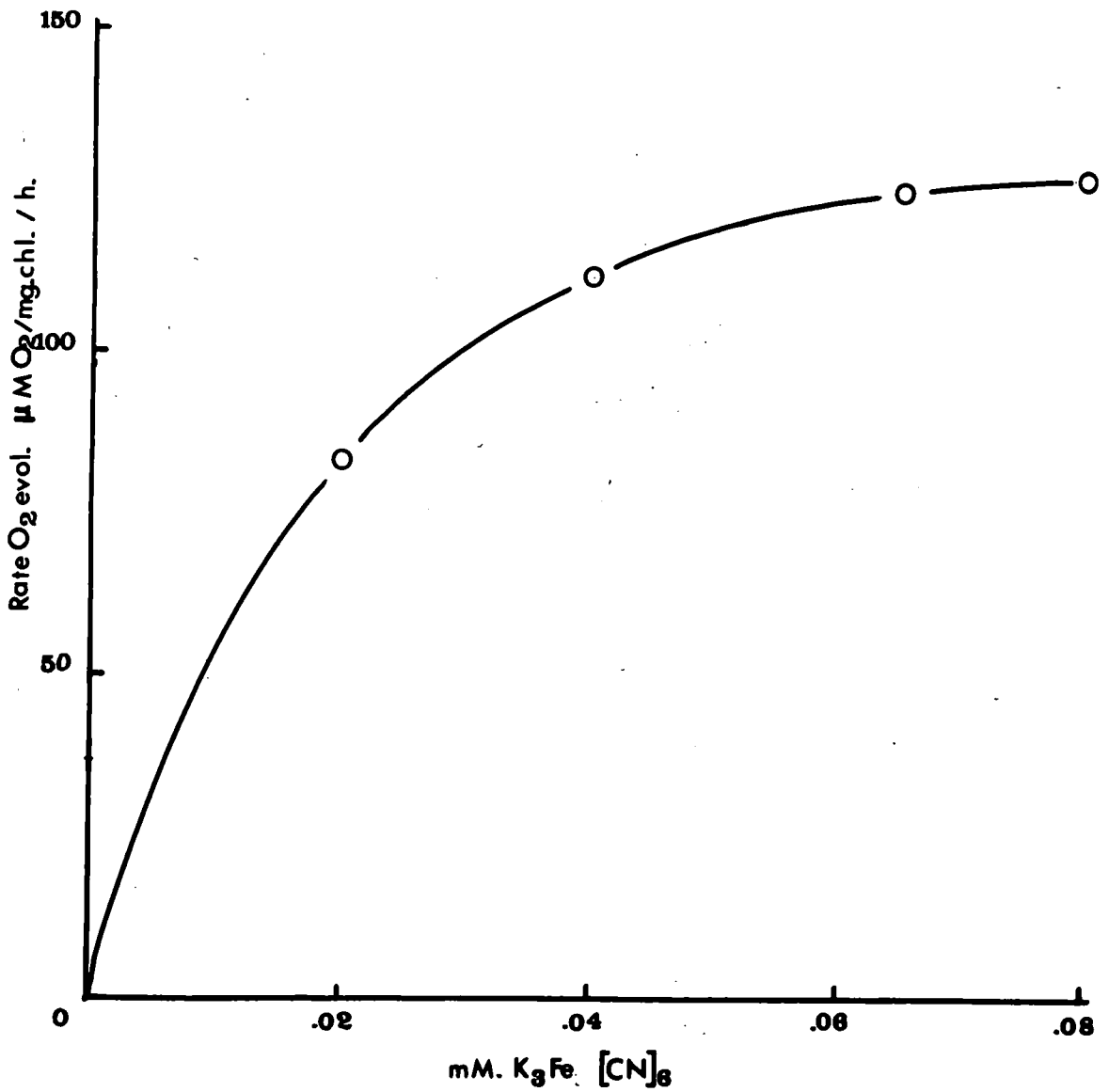


Fig. 6

Effect of varying the concentration of  $\text{K}_3\text{Fe(CN)}_6$  on the rate of oxygen evolution. Values are average of three determination using a combined chloroplasts preparation from three plants.

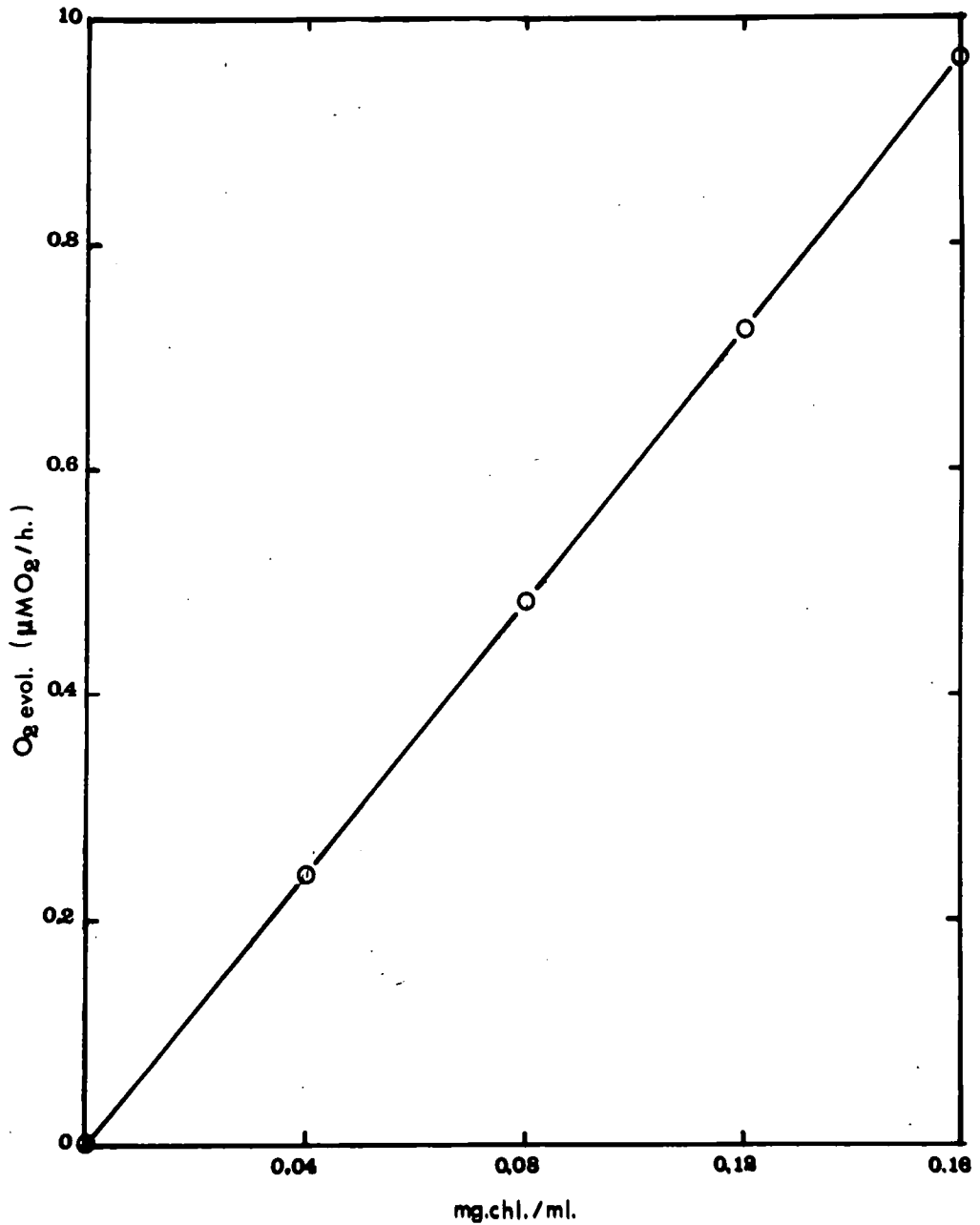


Fig. 7

Effect of varying the chlorophyll concentration on the rate of oxygen evolution of chloroplasts preparations of healthy *V. faba* leaves. Values are average of three determination using a combined chloroplast preparation from three plants.

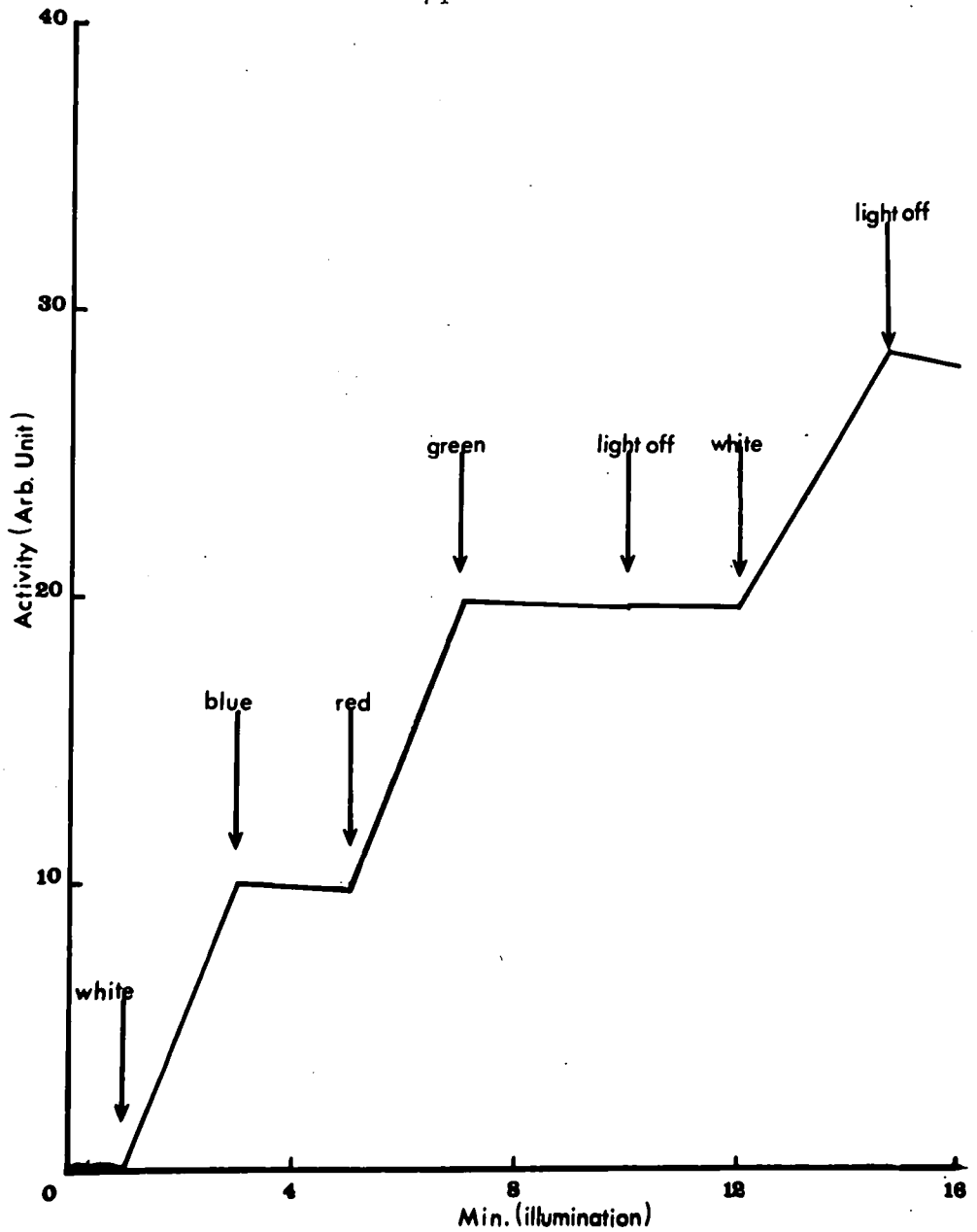


Fig. 8

Oxygen evolution tracing showing the effect of the colour of light on the oxygen evolution of chloroplasts from healthy *V. faba* (Chloroplasts in preparation were obtained from three plants). See text for the approximate wave length of the different colour of light used.

VI The Influence of infection on the rate of Hill reaction of isolated chloroplasts

A. Effect of *U. fabae* infection

A series of experiments comparing the rate of Hill reaction (oxygen evolution) using potassium ferricyanide as an oxidant in the absence of any phosphorylating reagent, were carried out over a period of 20 days after infection. As it will be seen from Table 8 and 9 the rate of oxygen evolution of chloroplasts isolated from the *U. fabae* infected leaves, decreased slightly in comparison with those isolated from healthy leaves, over the period 2-4 days after infection. By the sixth day the reaction rate of isolated chloroplasts was higher than those of healthy leaves of comparable age. This increase usually continued to the eighth day and sometimes to the tenth day after inoculation. After such periods, the chloroplasts isolated from rusted leaves were characterised by a lower rate of Hill reaction when compared with healthy chloroplasts. This drop in the rate normally continued up to the twentieth day after inoculation when the experiment was usually terminated.

The results of oxygen evolution by chloroplasts isolated at the two stages (stage of increased and decreased activity) were plotted against duration of illumination time (see Figures 9 and 10). As it can be seen, oxygen evolution by chloroplasts from rusted leaves (6 days after infection) is higher than that produced by the healthy chloroplasts (Fig.9). However, the difference between

TABLE 8

Effect of *U. fabae* infection on the rate of Hill reaction  
by isolated chloroplasts

Days after inoculation	Rate of O <sub>2</sub> evolution* ( $\mu\text{MO}_2/\text{mg. chl/h}$ )		% stimulation or inhibition
	chloroplasts from healthy leaves	chloroplasts from infected leaves	
2	176.0	160.0	9.1
4	168.0	142.0	15.5
6	100.0	116.0	16.0
8	80.0	108.0	35.0
10	70.0	67.0	4.3
12	79.0	48.0	39.0
14	64.0	48.0	25.0
16	96.0	68.0	29.6
18	72.0	53.0	26.4
20	54.0	24.0	55.5

\* Values are average of three determination ( $\pm 5 \mu\text{MO}_2$ ) using a combined chloroplast preparation from three plants.

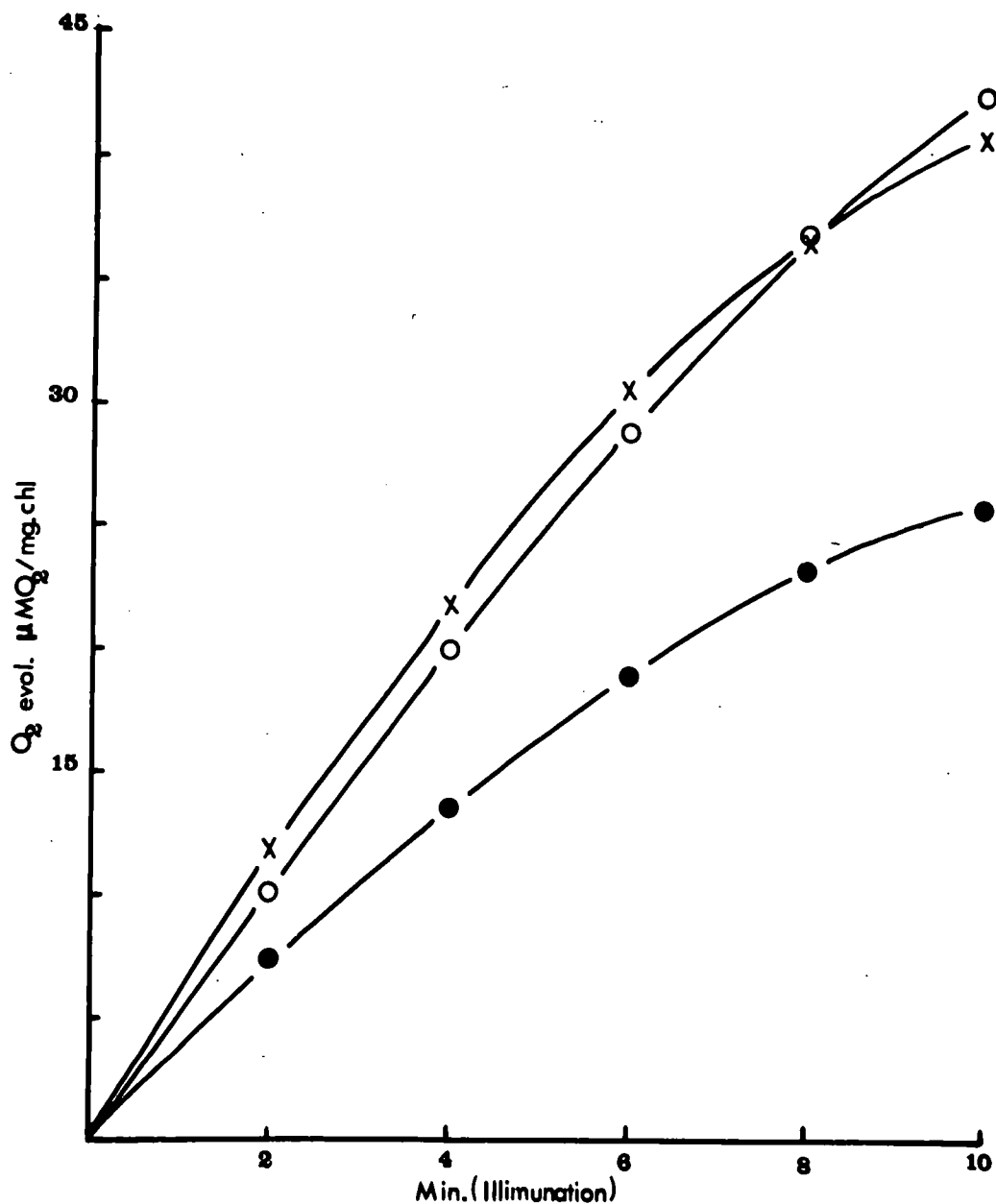


Fig.9

Time course of oxygen evolution by Chloroplasts isolated from healthy (○ ○), *U. fabae* (X X) and *B. fabae* infected leaves (● ●) (6 days after infection). Values vary with  $\pm 0.5 \mu\text{M O}_2$  and are average of 3 determination using a combined chloroplasts preparation from three plants.

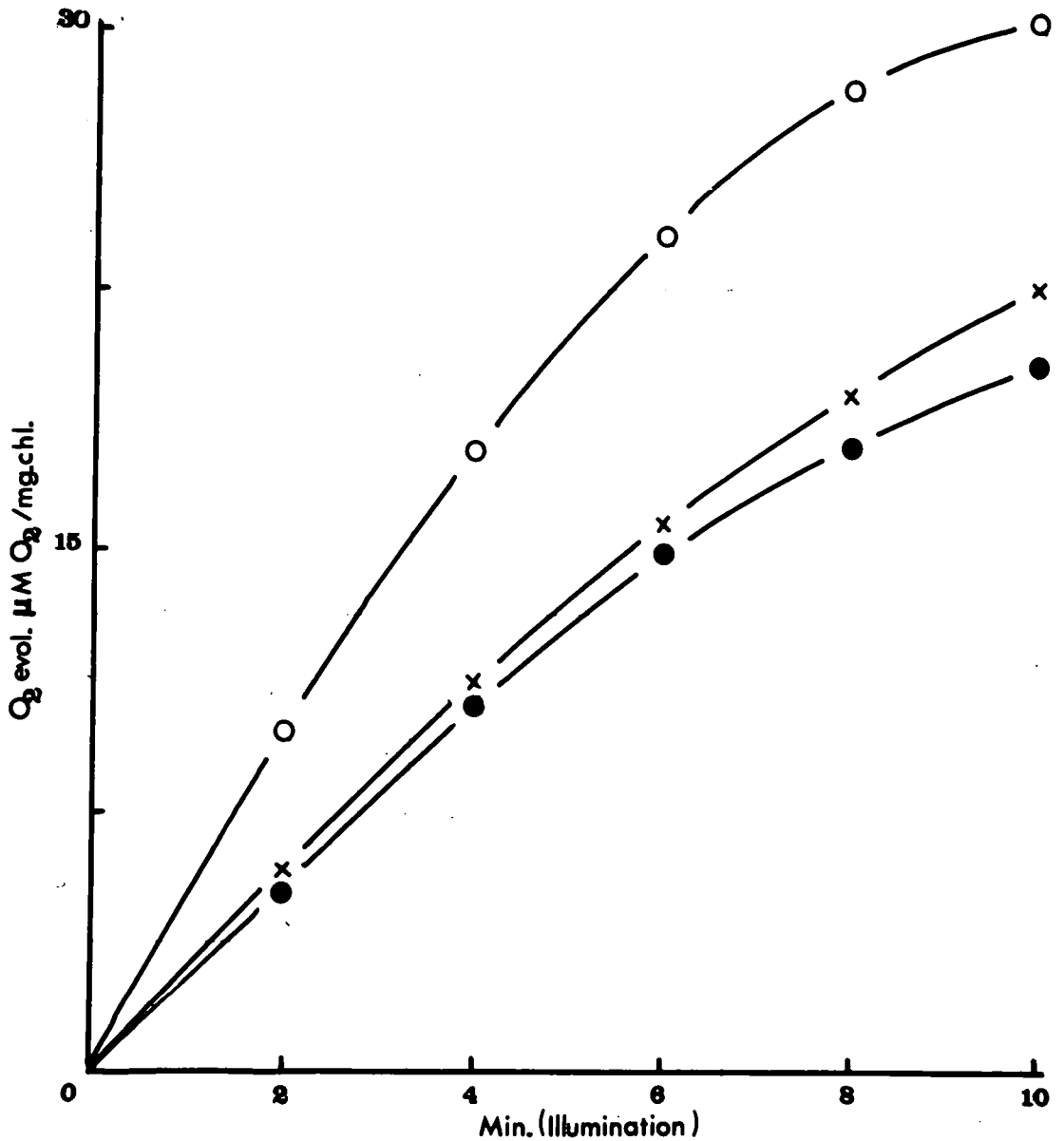


Fig. 10

Time course for oxygen evolution by chloroplasts from healthy (○ ○), *U. fabae* (X X), and *B. fabae* (● ●), infected leaves (16 days after infection). Values vary with  $\pm .05 \mu\text{M O}_2$  and are average of three determination using a combined chloroplasts preparation from three plants.



the oxygen output by the two types of chloroplast diminished gradually during the course of the experiments until the total amount of oxygen produced by both were almost equal. On the other hand, the amount of oxygen produced by chloroplasts from leaves infected by U. fabae (16 days after inoculation) (i.e. stage of decreased activity) (Fig.10) is considerably lower than that produced by chloroplasts from healthy leaves. This difference in Hill activity decreased during the course of the experiment.

#### B. Effect of infection with B. fabae

In contrast to the infection of V. faba by U. fabae, the infection of bean by B. fabae appeared to be much faster, and it was manifested by the appearance of 'chocolate' spots as early as 12 hours after inoculation. Likewise, the effect of this infection on the Hill reaction activities of chloroplasts isolated from such leaves can also be seen as early as 24 hours after infection. As can be seen from Table 9, the rate of Hill reaction of chloroplasts isolated from Botrytis infected leaves dropped considerably below that of chloroplasts isolated from healthy leaves. This decrease in Hill reaction was first noticed in assays done on material 2 days after inoculation, and it continued to fall to the twentieth day, after which the experiment was usually terminated. By following the oxygen output by chloroplasts from both healthy and infected leaves over a period of 10 minutes after illumination, it

was clear that the Hill activity of chloroplasts from Botrytis infected leaves are very much inhibited at all stages of disease development (6-16 days). Time course experiments showed that the differences between the activities of the two types of chloroplasts increased considerably through the sixth to the tenth minute of illumination (Figures 9 and 10).

C. Effect of mixing chloroplasts from healthy leaves with those from infected plants on the rate of Hill reaction.

As shown previously, depending on the stage of infection, the Hill reaction activities of chloroplasts from rust infected leaves were either less or greater than those isolated from uninfected leaves. On the other hand, chloroplasts from Botrytis infected leaves were always found to be less active than those from healthy leaves. The present experiments were conducted to find out whether or not chloroplasts from infected plants can influence the activities of those from healthy leaves. The results of these experiments can be found in Table 9. Mixing chloroplasts of healthy leaves with those of Botrytis infected leaves resulted in a reduction in the predicted Hill reaction activity. The same results were also obtained upon mixing chloroplasts of the healthy leaves with those from rusted leaves (14 days after infection). The percentage of inhibition of Hill activities using chloroplasts from Botrytis infected leaves was greater than with chloroplasts from Uromyces infected leaves.

TABLE 9

Effect of the infection of *V. faba* leaves by *B. fabae*  
on the Hill reaction activities of isolated chloroplasts

Days after inoculation	Rate of O <sub>2</sub> evolution ( $\mu\text{M}\text{O}_2/\text{mg.ch/h}$ )		% inhibition
	chloroplast from healthy leaves	chloroplast from Botrytis infected leaves	
2	176.0	160.0	9.1
4	168.0	142.0	15.5
6	100.0	75.0	25.0
8	80.0	80.0	00.0
10	70.0	61.0	12.8
12	79.0	58.0	26.0
14	64.0	50.0	21.8
16	96.0	68.0	29.2
18	72.0	62.2	13.8
20	54.0	24.0	55.5

(Value vary with  $\pm 5\mu\text{M}\text{O}_2$  and are average of three determination  
using a combined chl. prep. from three plants.)

TABLE 10

Effect of chloroplasts isolated from infected plants on the Hill reaction activity of chloroplasts isolated from healthy leaves

healthy leaves conc.(mg./ml)	Source of chloroplasts in reaction		final chlorophyll conc (mg./ml)	Rate Hill reaction*	
	U. fabae infected leaves conc.(mg/ml)	B. fabae infected leaves conc.(mg./ml)		$\mu\text{M O}_2/\text{mg/ml}$ Predicted	Exp. Cond.
0.11	0	0	0.11	-	97.5
0	0.11	0	0.11	-	72.5
0	0	0.11	0.11	-	15.0
0.055	0.055	0	0.11	84.9	75.0
0.055	0	0.055	0.11	56.2	47.5

\* Values vary with  $\pm 3 \mu\text{MO}_2$  and are average of three determinations using a combined chloroplast preparation from three plants.

No difference was obtained when chloroplasts from Uromyces infected leaves (4-8 old days after infection) were mixed with equal proportions of chloroplasts from healthy leaves. Moreover, supernatant from any of the infected chloroplast preparations resulted in little or no inhibition of the activity of healthy chloroplasts.

VII. Influence of infection on the rate of coupled phosphorylation by isolated chloroplasts

The results of experiments conducted using chloroplasts isolated from healthy V. faba leaves indicated once again, that such chloroplasts do not reduce NADP and consequently neither oxygen evolution nor ATP synthesis was detected. However, both reactions were found to be stimulated when Potassium ferricyanide was added to the reaction mixture at low concentration (0.26 mM). When higher concentrations of this oxidant was used, little or no phosphorylation could be detected due probably to the interference by the coloured ferricyanide ion in the colorimetric method for the assay of phosphate esterification. The pH of the reaction mixture was also of importance; pH values less than 7.0-7.5 were shown to be inhibitory to the phosphorylation reaction, but had little or no affect on oxygen evolution. The ratio of  $ATP:2e^-$  under the specified condition (Table 11) was found to vary throughout the 20 days (assayed at 2 day intervals) in which assays were made. The average  $ATP:2e^-$  ratio during this period of time (20-40 days after planting) was 0.36, (Table 11).

TABLE 11

Ratio of ATP:2e<sup>-</sup> as influenced by U.fabae and B. fabae infection

Days after inoculation	Source of chloroplasts		
	Healthy leaves ATP/2e <sup>-</sup>	<u>U. fabae</u> infected ATP/2e <sup>-</sup>	<u>B. fabae</u> infected ATP/2e <sup>-</sup>
2	0.3	0.13	0.07
4	0.3	0.14	0.06
6	0.2	0.11	0.11
8	0.38	0.11	0.32
10	0.7	0.15	0.4
12	0.5	0.2	0.3
14	0.58	0.11	0.26
16	0.35	0.27	0.5
18	0.11	0.29	0.4
20	0.29	0.29	-

All values are average of three determination (<sup>±</sup>0.1) using combined chloroplast preparation obtained from three plants.

A. Effect of *U. fabae* infection on the coupled phosphorylation

The results in Table 12 show that the rate of both reduction (oxygen evolution) and ATP synthesis (coupled phosphorylation) were affected by infection. As far as the reduction reaction is concerned, chloroplasts isolated from rust infected leaves (2-20 days after infection) showed a similar type of response to that previously discussed elsewhere in this study (uncoupled oxygen evolution) i.e. small initial reduction followed by a stimulation shortly before the flecking stage (6-10 days), then a decrease during the period of fungal sporulation until the experiment was terminated (10-20 days after infection). In contrast to this, the rate of coupled phosphorylation was found to be reduced throughout the entire period of assay (Table 12). Accordingly, the observed stimulation in oxygen evolution (6-10 days after infection) was not accompanied by any stimulation in ATP synthesis, suggesting that such stimulation in oxygen evolution was probably due to partial uncoupling of noncyclic phosphorylation caused by the infection. The data in Table 11 represents the calculated ratio of  $\text{ATP}:2e^-$  during the twenty day period after infection. As can be seen from this data in the table, rust infection caused a reduction in the  $\text{ATP}:2e^-$  ratio during the period 2-16 days after infection. Although the rate of both reactions (oxygen evolution and coupled phosphorylation) were also reduced in the later stages of disease development (18-20 days), the ratio of  $\text{ATP}:2e^-$  during this period was approximately equal

TABLE 12

Effect of U. fabae and B. fabae infection on  $O_2$  evolution and coupled phosphorylation isolated chloroplasts

Days after inoculation	Source of chloroplasts in reaction					
	Healthy leaves		<u>U. fabae</u> infected		<u>B. fabae</u> infected	
	Hill reaction $\mu M O_2$ /mg.chl/h	Coupled phosp. $\mu M/ml.PO_4$ ester.	Hill reaction $\mu M O_2$ /mg.chl/h	Coupled phos. $\mu MPO_4$ ester mg.chl/h.	Hill reaction $\mu M O_2$ /mg/chl/h	Coupled phos. $\mu M PO_4$ ester mg.chl/h.
2	180.0	58.9	155.0	20.9	140	10.6
4	171.4	52.4	170.0	25.4	150	10.6
6	174.5	39.3	188.2	20.2	169.0	20.4
8	150.9	58.9	116.4	12.4	112.7	36.8
10	108.0	80.0	135.9	20.0	108.8	36.8
12	110.7	55.6	130.9	27.8	89.9	36.8
14	117.2	69.5	95.5	10.66	68.5	18.0
16	122.6	43.4	68.9	18.7	40.9	20.6
18	98.2	10.6	54.5	14.9	30	12.2
20	93.6	27.8	54.5	16.0	-	-

All values are average of three determination using a combined extract from three plants  
Reaction time 5 minutes.



to that of healthy chloroplasts.

B. Effect of *B. fabae* infection on coupled phosphorylation

The results in Table 12 also illustrate the effect of Botrytis infection on coupled phosphorylation and accompanying reduction (oxygen evolution) by isolated chloroplasts. As can be seen from the table, the rate of oxygen evolution was inhibited from the first to the twentieth day after infection. This is in agreement with results given previously for oxygen evolution, suggesting that Botrytis infection has an inhibitory effect on the coupled and uncoupled oxygen evolution during all stages of disease development. As far as the coupled phosphorylation is concerned, the data also indicated that such infection resulted in a considerable inhibition especially in the early stages of infection (2-6 days after infection). Such inhibition in the rate of coupled phosphorylation continued during all stages of disease. However, the difference between the rate of healthy and infected was found to decrease gradually. Similarly, the calculated ratio of  $ATP:2e^-$  was also found to be reduced by the infection, indicating that such infection not only reduced the rate of the two reactions but also altered the ratio of  $ATP:2e^-$ . (Table 11)

VIII. Influence of infection on the rate of cyclic phosphorylation by isolated chloroplasts

A. Chloroplasts from healthy leaves

All chloroplast preparation used in this assay were usually washed once with tris buffer to remove any inorganic phosphate in the isolation medium. The results of these experiments using N-methylphenazonium methosulphate (PMS) as a cofactor are found in figure 11, which represents the rate of cyclic phosphorylation catalysed by healthy chloroplasts and by chloroplasts isolated from either Uromyces or Botrytis infected leaves at various intervals after infection. As shown in the graph, the rate of cyclic phosphorylation seemed to be greatly influenced by the age of plants from which chloroplasts were isolated. Chloroplasts isolated from 22 day old plants attained a rate of  $31.6 \mu\text{M Po}_4 \text{ est./mg./h.}$  (Average of three determinations with preparations consisting of chloroplasts from three identically treated plants). The rate was found to drop considerably throughout the eighth day (28 day old plants), to the twentieth day (40 day old plants) after infection, when it reached an average rate of about  $15 \mu\text{M Po}_4/\text{mg.chl./h.}$  (Average of three replicates).

Table 13 shows some day to day variation in the rate of cyclic phosphorylation of chloroplasts isolated during the period of assay (20 days at 2 day intervals). Experimental and preparative procedures seem to contribute very little to this variation. For example, three replicate preparations from healthy plants grown under similar conditions and harvested at the same time, gave activities of 33.5, 34.5 and 32.4 units. The major variation in the reaction

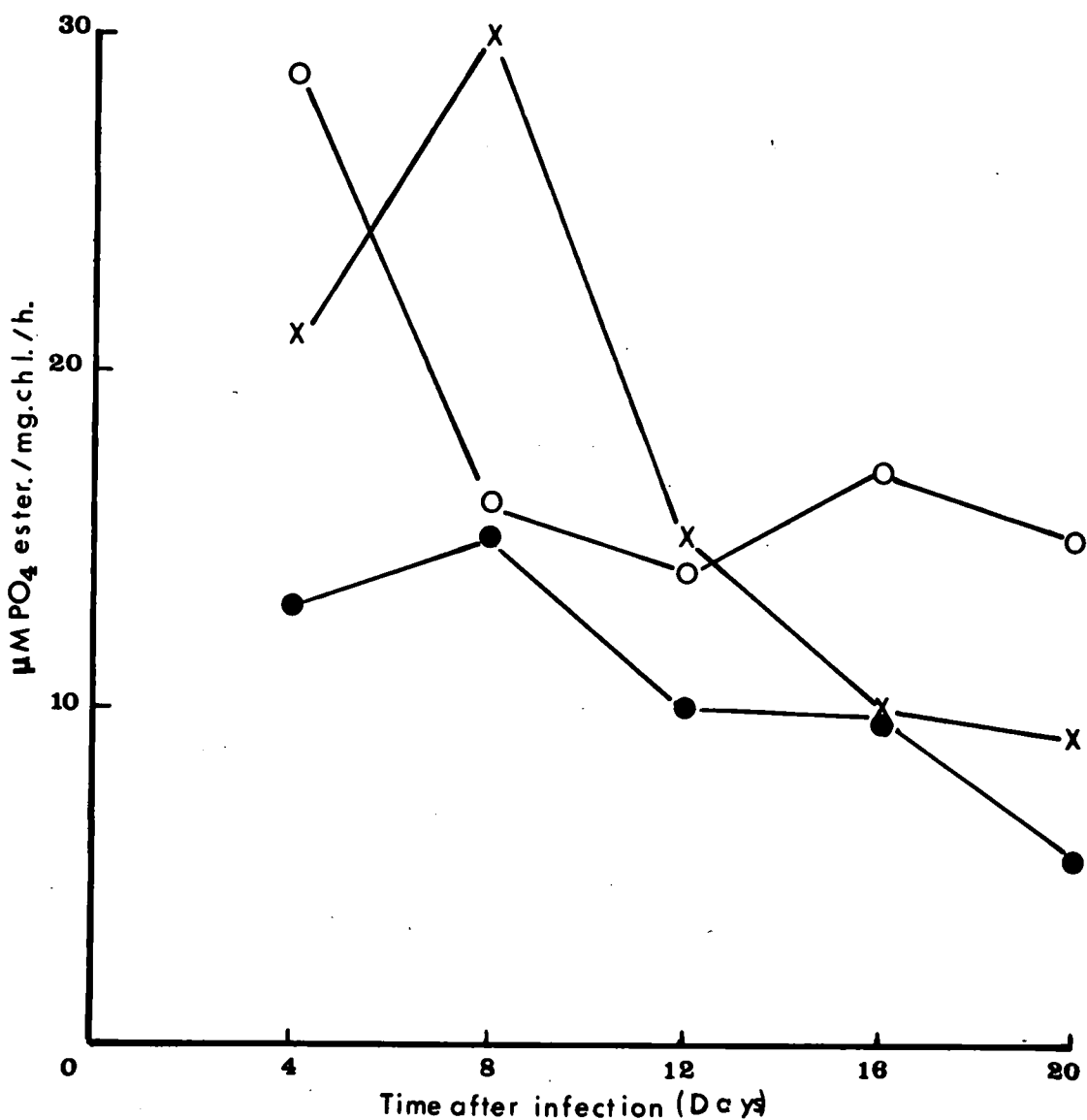


Fig. 11

Comparison between the rate of cyclic phosphorylation of chloroplasts from healthy (○ ○), with those from U. fabae infected (X X) and B. fabae (● ●) infected leaves. (Values are average of three determination by combined chloroplasts preparation obtained from three plants.)

TABLE 13

Influence of infection of V. faba by U. fabae and B. fabae, on the cyclic phosphorylation of isolated chloroplasts

Days after infection	Healthy			Rate of cyclic phosphorylation of chloroplasts from ( $\mu\text{MPO}_4/\text{mg.chl/h}$ )*					
	Exp.1	Exp.2	Exp.3	U. fabae infected		B. fabae infected			
	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
4	56	18	16	50	18	13	28	8	3
8	26	9	10.0	50	18	17.5	10	8	8
12	15	14	10	20	16	12	10	8	5
14	21	16	12	12	11	7	25	10	5
16	18	14	12.5	12	10	5	10	5	5.5

\* Each value is an average of 3 determination. Preparations consisted of the combined components from three plants.

is probably due to various sources, some of which are, (i) environmental variation, (ii) plant growth variation, (iii) diurnal variation (Spencer and Possingham, 1960), and (iv) seasonal variation (Zaitlin and Jagendorf, 1960). The calculated rate for this reaction was computed from the results obtained after 20 mins. of illuminated incubation. However, when phosphate esterification was assayed in the light at various intervals a drop in the amount of inorganic phosphate esterification was frequently observed after 40-60 minutes (Figure 12). This phenomena was general and occurred with all types of infected material. The reason for the drop is not clear and it probably is due to hydrolysis of ATP or phosphate ester.

#### B. Chloroplasts from U. fabae infected leaves

The results concerning cyclic phosphorylation activity as affected by U. fabae infection can be seen in figure 11. The rate of the reaction of chloroplasts from infected plants in the first assay (4 days after infection) decreased, in comparison with the rate of the reaction of chloroplasts from healthy leaves. However, the activity of these chloroplasts were found to be higher than healthy chloroplasts at about the eighth to the twelfth day after infection. This effect is reproducible and was noted from three different experiments carried out at various times (Table 13). The results of all these

experiments also indicated that this increase in the activity is limited to a short period of time (8-12 days after infection) which is the period of sporulation of the pathogen. After this period of increased activity, the rate of reaction was found to decline through the sixteenth day to the twentieth day after infection, when the experiments were usually terminated. Accordingly, the effect of Uromyces infection on cyclic phosphorylation follows the same general pattern observed and discussed elsewhere in this study for other parameters (i.e. oxygen evolution).

C. Chloroplasts from B. fabae infected leaves

Following the rate of cyclic phosphorylation of chloroplasts isolated from B. fabae infected leaves at various intervals after inoculation, indicated that the rate was drastically reduced as early as 2 days after inoculation. This reduction in rate continued at all stages (Fig. 11). These results are in agreement with other photochemical reactions tested (namely Hill reaction and coupled phosphorylation).

VII. Amino acid incorporation into peptidyl material by isolated chloroplasts

Results discussed elsewhere in this study, indicated that chloroplast activity is affected by the age of the plants from which they were isolated. For this reason, preliminary experiments were conducted to investigate the effect of plant age on the ability of

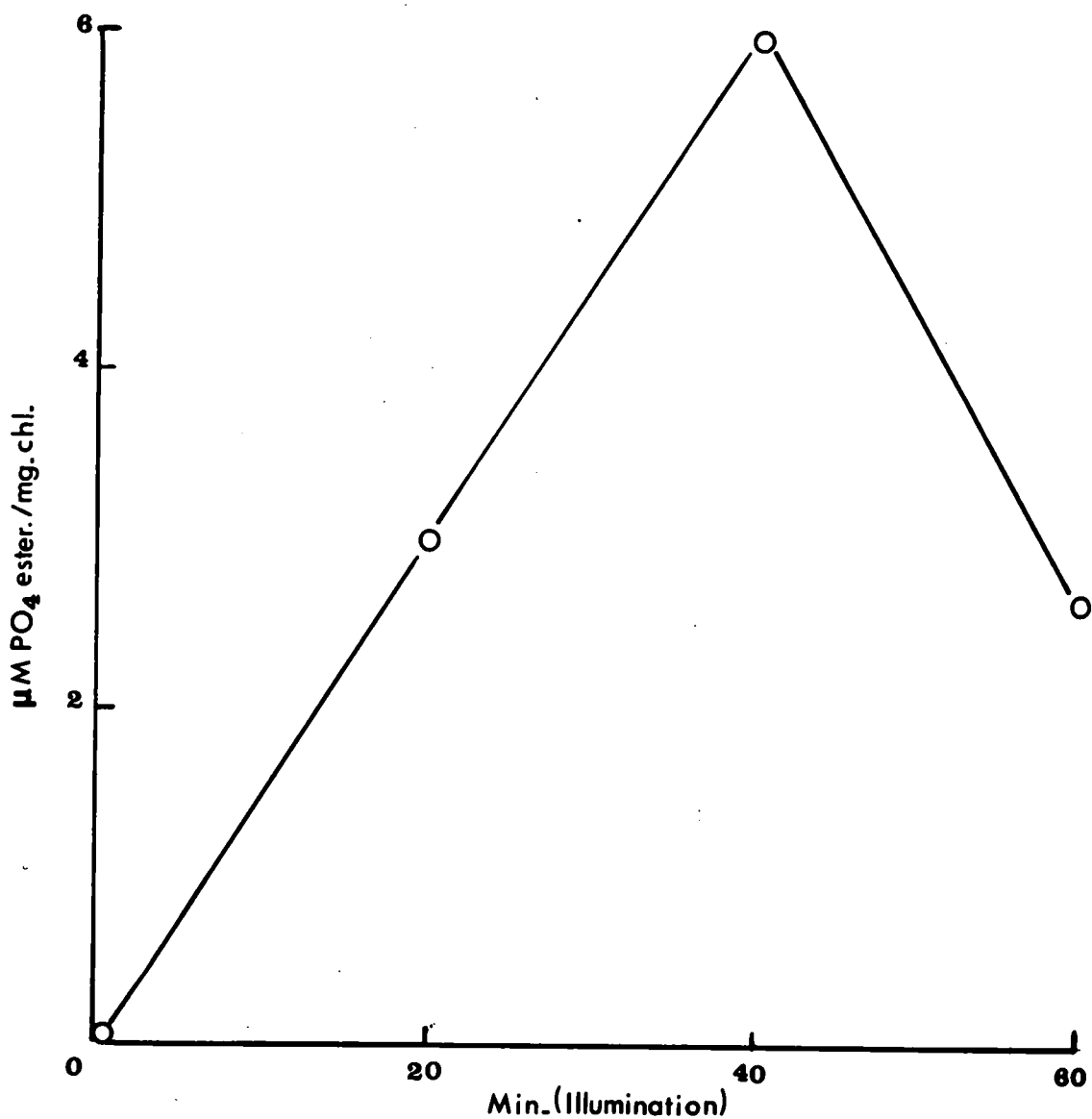


Fig. 12

Time Course of cyclic phosphorylation by isolated chloroplasts from healthy leaves (Values are average of three determination using a combined chloroplasts preparation obtained from three plants.

isolated chloroplasts to incorporate radioactive amino acids into protein. Accordingly, chloroplasts were prepared from plants in the early stages of development (15 days after planting) and from plants in the relatively later stages (26 days). This choice of plant age is of particular importance, since the ages of plants used in all other aspects of this study were between 20-25 days old; infection by both fungi was found to be uniform and reproducible during this period of plant growth.

The results of the radioactive incorporation studies (Table 14) indicated that chloroplasts isolated from 26 day old plants do not incorporate amino acids. On the other hand, chloroplasts prepared from 15 day old plants are relatively more active. The results in the table also show that the incorporation observed is almost exclusively into a triton soluble fraction. This fraction is assumed to be chloroplastic in origin since triton X100 is known to readily solubilise chloroplast membrane (Bamji and Jagendorf, 1966; Spencer, 1965). Possible contaminants of the original chloroplasts preparation (e.g. nuclei, bacteria or whole cells), which might be expected to contribute to the observed incorporation, are not susceptible to the triton X100 treatment, and form the triton insoluble fraction. This fraction is shown to contain negligible radioactivity, indicating that all the observed incorporation was into the chloroplastic material.



TABLE 14

Incorporation of C<sup>14</sup> leucine by chloroplasts isolated from  
plants at different stages of growth

	Incorporation by chloroplast isolated from	
	15 day old	25 day old
	c.p.m./mg/chl.	
1. TCA insoluble (pellet)	85	0
2. Triton insoluble	$\pm$ 1	0
2. Triton soluble	437	0

1. TCA added at end of incubation - Counted

2. Triton added at end of incubation.

The quantitative discrepancy between the amount of incorporation detected using the two assay procedures, was reproducible and whilst the reason for this has not been investigated it would seem that triton treatment of the incubation is a necessary step in the assay procedure.

Due to the apparent inability of chloroplasts isolated from older plants (20-40 days old) to incorporate radioactive amino acids, these experiments were discontinued.

IX. Free amino acid content of leaves of *V. faba* infected with *U. fabae* or *B. fabae* as compared with healthy leaves

The results of preliminary experiments using both Yemm and Cocking analysis and thin-layer chromatography indicated that the method of extraction used gave approximately the same value for the amount of amino acids e.g. values for the total free amino acids present in three similar leaves were 4.4, 4.5 and  $4.5 \times 10^{-3}$   $\mu$ M amino acid per g. of leaf material respectively. Figure 13 is a diagram of a typical thin-layer chromatographic separation and gives the pattern of amino acid obtained. This pattern was reproducible with extracts of leaf samples of similar origin.

The results in figure 14 represent the total amount of amino acid nitrogen found in 1 g. of leaves sampled at various intervals after infection. The amount of free amino acids in healthy leaves

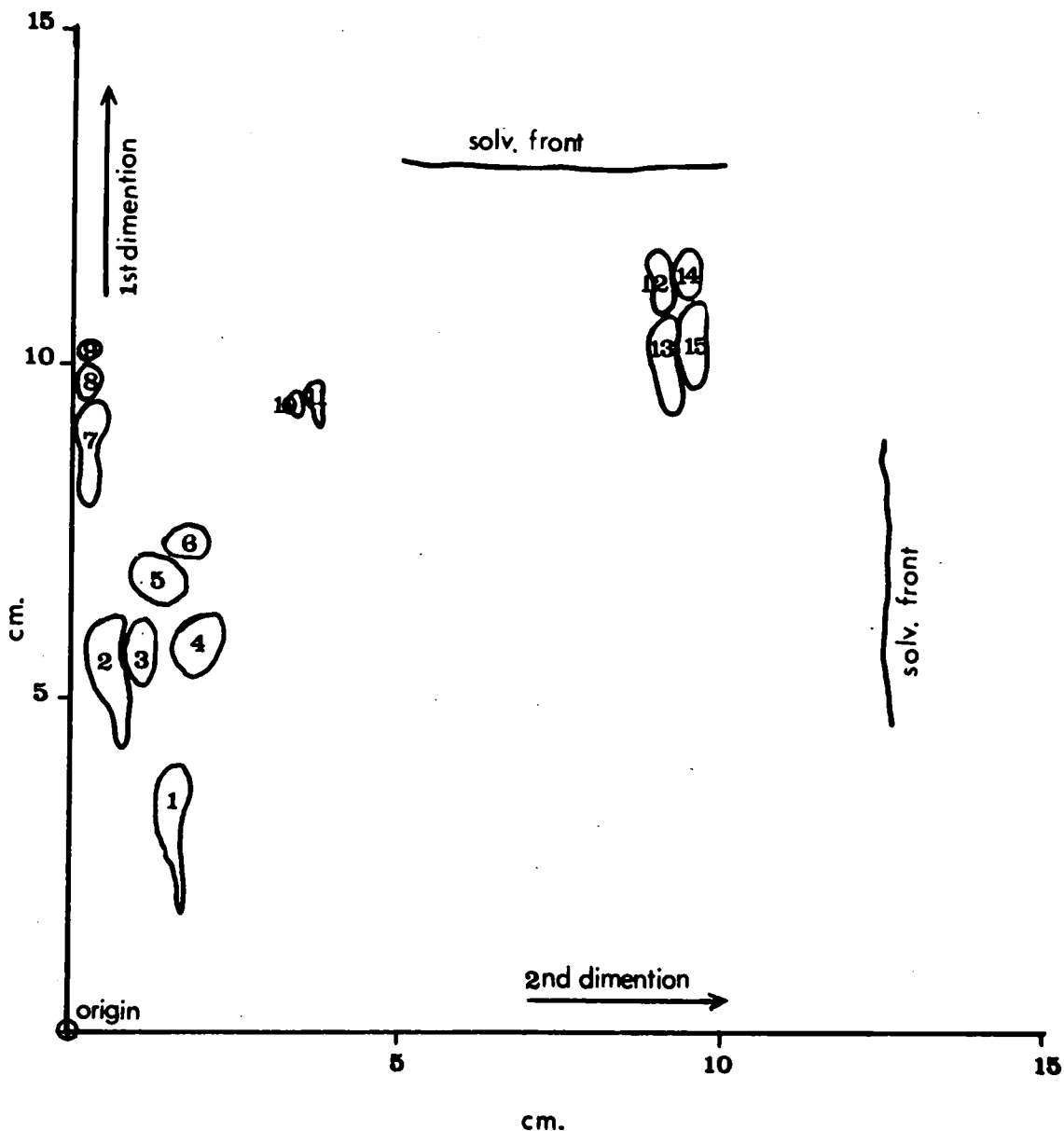


Fig. 13

Schematic representation of two dimensional thin layer chromatograms showing a typical pattern of amino acids separation on thin layers of cellulose powder (1st. dimension propan-2 ol-Formic acid-water, 40:2:10 v/v) 2nd. dimension test-butanol, methyl ethyl ketone,  $\text{NH}_3$ , water 50:30:10:10: v/v)

(1) Arg; (2) Glu; (3) Gly; (4) Lys; (5) Ser; (6) Ala; (7) ASP; (8) Prol; (9) (?); (10) Hist; (11) Meth.Sulphate (?); (12) Phen; (13) Tyr; (14) Leu; (15) Val.

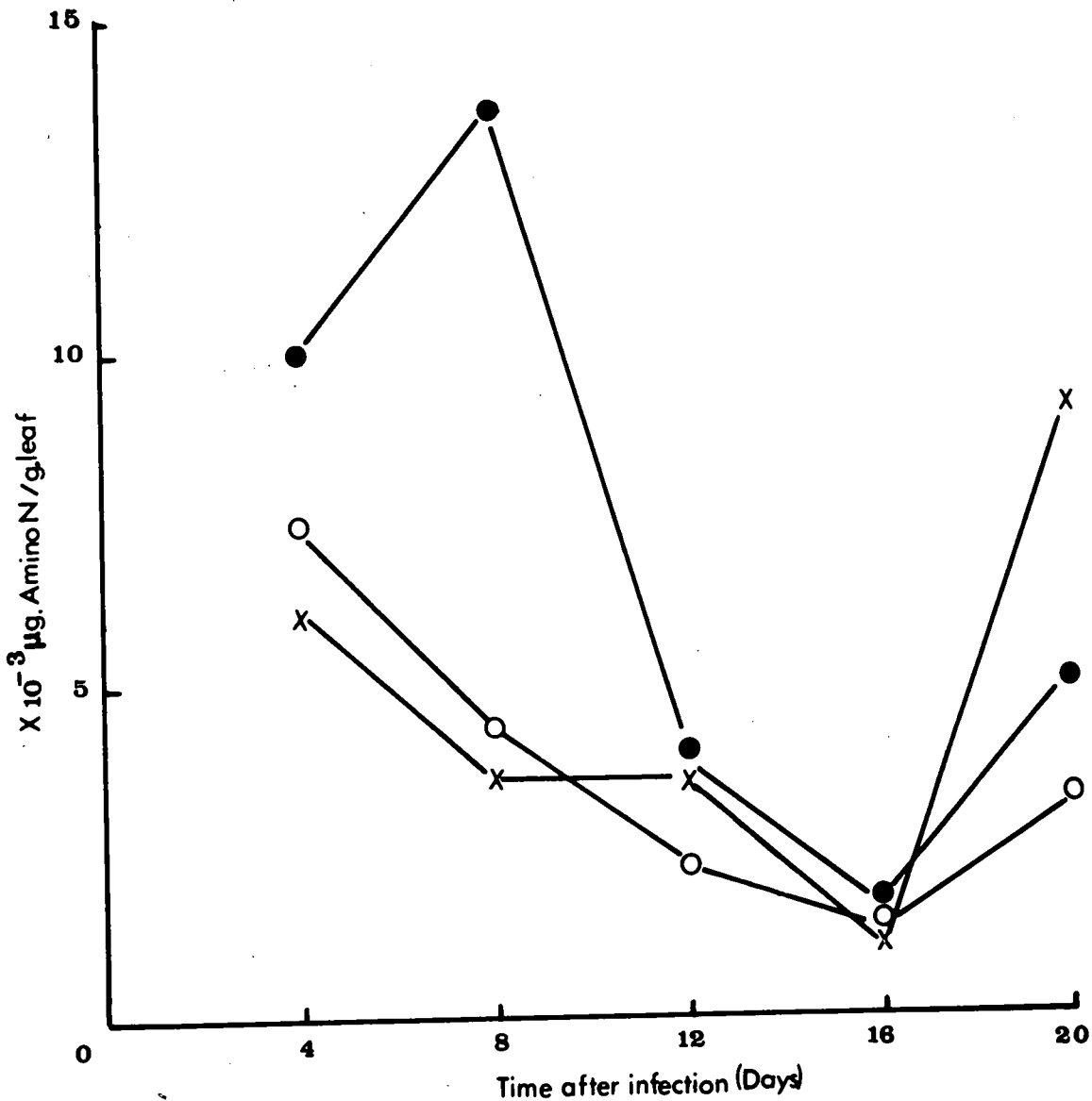


Fig. 14

The effect of infection of *V. faba* (○ ○), by *U. fabae* (x x) and *B. fabae* (● ●) on the total free amino acids (values are average of combined extract prepared from three plants, total amino N determined by Yemm and Cocking method, 1955)

Extra.

declined from the eighth (28 day old plants) to the sixteenth day (36 day old plants). By the twentieth day (40 day old plants) the amount of total free amino acids was found to have increased considerably.

In comparison, the total amount of free amino acids in plants infected with U. fabae sampled at the fourth and eighth day after infection, was found to be less. By the twelfth day, samples from leaves infected with U. fabae were found to contain higher amounts of amino acids in relation to those of a comparable age from healthy plants. As the infection progressed (16-20 days after inoculation) the amount of free amino acid in rusted leaves increases considerably, reaching amounts of three times that of the healthy leaves.

With regard to the samples obtained from Botrytis infected leaves, the results show that the infection induced the accumulation of a considerable amount of free amino acids, particularly during the period of the fourth to the eighth day after infection. After this period of time the amount of free amino acids started to decline rapidly in a pattern similar to that of the healthy leaves. Although the amount of free amino acids in Botrytis infected leaves was found to be higher than those of the healthy leaves, the differences between both were found to decrease gradually with ageing of leaves.

'Auto Analyser' Analysis

The data in figure 15 shows a typical record of a Technicon, 'Auto Analyser' analysis using 1 ml of an extract (2.8  $\mu$ M of amino N) from healthy V. faba (24 days old). As shown in this record, the free amino pool is formed of the following amino acids which have been identified with reference to a standard amino acid mixture. These amino acids are (in sequence), ASp, Sera, Glu, Gly, Ala, Val, Tyr, Phen, Unknown 1, Ornith, Lys, His and Arg. The results in Table 14 show the differences in the concentration of these amino acids in healthy leaves, leaves infected with U. fabae, and leaves infected with B. fabae. As can be seen from the table, free amino acids in B. fabae infected leaves (4 days after infection) has accumulated in relatively high quantities as compared to the control. The data indicate the presence in U. fabae infected leaves (4 days after infection) of the same amino acids as those found in both extracts from healthy leaves and leaves infected with B. fabae. However, the concentration of each amino acid is less than those of the control. As both infections progress (16 days after infection) the quantity of free amino acids from leaves infected with U. fabae increased and a new amino acid (canavanine) was observed. This amino acid has not been found in extract from either healthy or B. fabae infected leaves. The free amino acids from B. fabae infected leaves (16 days after infection) continued to be maintained at a higher level than those of the control.

TABLE 14

Comparison between the amino acids contents and concentration  
in extracts from healthy leaves, leaves infected with U.  
fabae and infected leaves with B. fabae.

Amino acids	Healthy leaves	U. fabae infected leaves		B. fabae infected leaves	
		4 days after infection	16 days after infection	4 days after infection	16 days after infection
Aspartic acid	6.7	3.8	12.0	12.9	13.5
Serine	52.0	28.5	21.0	29.6	21.5
Glutamine	6.5	1.9	9.0	8.7	6.6
Glycine	1.3	0.95	2.5	2.5	1.6
Alanine	1.3	1.14	2.0	4.4	1.95
Valine	0.52	0.38	2.5	0.37	0.75
Tyrosine	0.5	0.28	1.5	0.74	0.52
Phenylalanine	28.6	21.4	15.0	24.0	25.2
Ornithine	0.52	0.38	13.0	1.1	1.3
Unknown	0.52	0.38	6.0	1.1	1.82
NH <sub>3</sub> (in Buffer)	6.5	0.76	12.0	12.4	6.6
NH <sub>3</sub>	1.8	0.57	5.0	2.8	2.65
Lysine	1.3	1.33	2.0	2.66	6.5
Histidine	3.9	1.33	4.0	4.66	3.9
Canavanine	-	-	5.0	-	-
Arginine	3.5	-	6.0	6.0	-



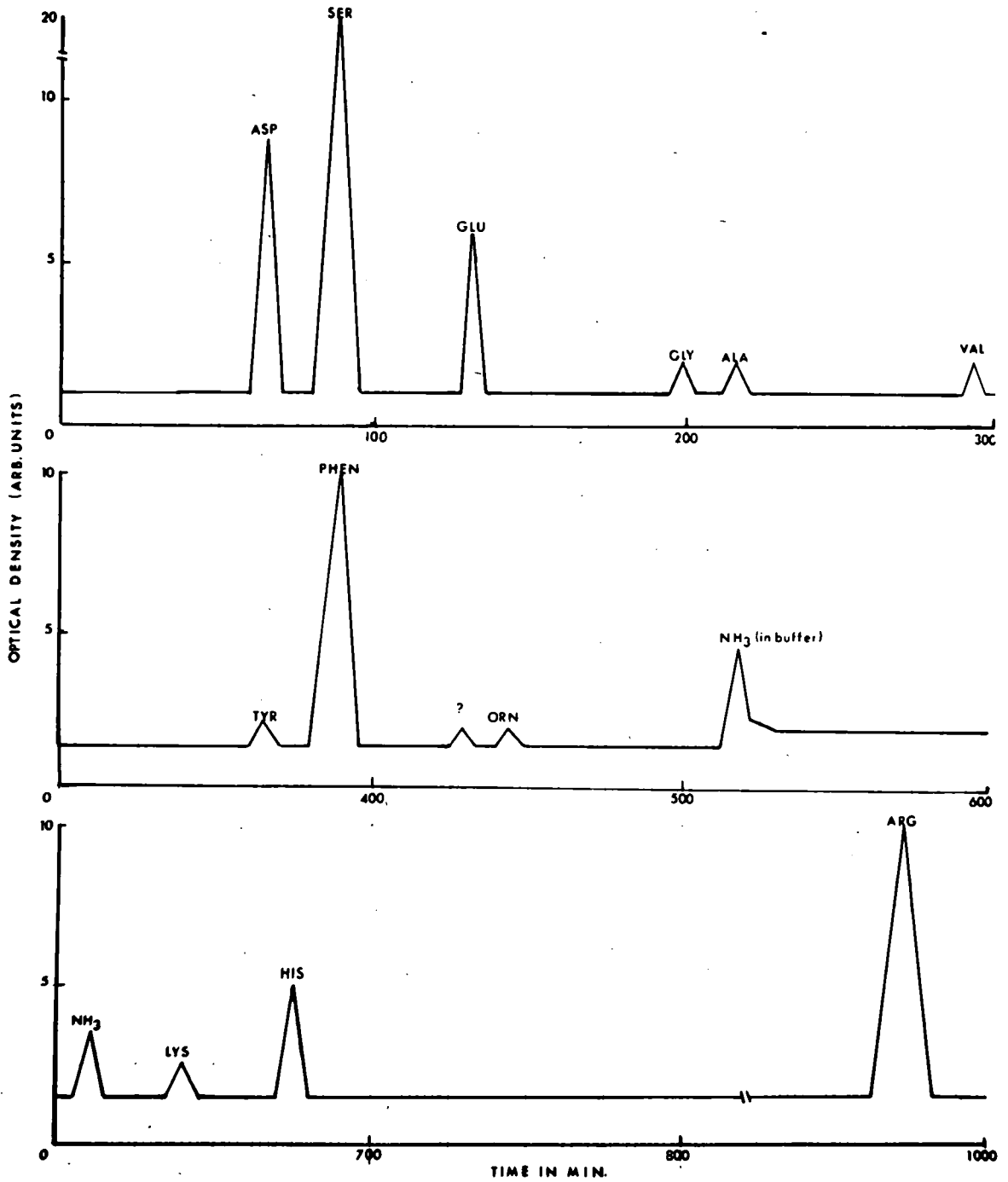


Fig. 15

Typical record of a Technicon 'Auto Analyser' analysis showing the different amino acids found in extract from healthy Vicia faba leaves.



## Discussion

Since the development of the electron microscope and its consequent use in biological science, a great deal of work has been published using various host parasite combinations. However, most of these studies are concerned with the interaction of the host plant with obligate parasites, and ultrastructural information is scarce with respect to the relationship of non-obligate parasites to the host cell. The reason for this situation is partly due to the fact that infection with obligate parasites are less destructive especially in the earlier stages, and infection usually lasts longer and undergoes various stages; the perseverance of the host cells fine structure and the duration of the obligate parasite infection, facilitates the investigation. Nevertheless, Bracker (1967a) suggests the need for more fine structural studies involving non-obligate parasites. Such studies not only reveal the nature of the fine structure of the interaction of these pathogen with their host, but also aid in a better understanding of the differences between this group and obligate parasitism. Moreover, it is also desirable that the descriptive ultrastructural studies, be accompanied with biochemical studies orientated towards resolving the function and significance of ultrastructural patterns in the pathogen and their respective hosts. Despite the importance of such studies, very few reports are available.

In the present investigation, attempts were made to compare and correlate the infection of broad bean (Vicia faba) with Uromyces and Botrytis fabae from both structural and functional angles. Also to examine and compare the fine structure of both fungi. As mentioned previously, the fine structure of the cytoplasm mesophyll cells of healthy V. faba does not differ much from that of other plants studied (Esau et al., 1966; Weintraub and Regetli, 1966; Ehrlich and Ehrlich, 1968). However, it is worth mentioning that some unidentified bodies were frequently observed in the cytoplasm of healthy leaves. The first of these bodies, which are the size of the mitochondria but without any crista, have been previously reported by Weintraub and Regetli (1966). These authors have also reported the presence of another type of inclusion in the cytoplasm and the vacuoles of healthy mesophyll cells of V. faba. According to them, these inclusions are spherical or hemi-spherical, 400-1000  $m\mu$  in diameter. In the present study similar inclusions were also found in healthy mesophyll cells. However, the inclusions found in the present investigations are 16-36  $\mu$  in diameter and were always found to be in the vacuole attached to the tonoplast. The exact nature and significance of these bodies is not clear, but according to their general appearance, they are probably the <sup>n</sup>tanin globules which could be identified in the light microscope

by staining with ferric chloride (Weintraub and Regetli, 1966).

Other features of the fine structure of the healthy mesophyll cells of broad bean are the presence of 'lomasome'-type bodies in pockets made of invaginated plasma membrane (Plates 9, 10). Similar structures have been reported to be found in the tracheary elements of various plants (Esau et al., 1966). While these structures are often called 'Boundary formation' they resemble and occupy the same position as those designated as lomasomes, and it is this term which is used here. In the field of host parasite relations, 'lomasomes' have recently been involved as a factor in disease resistance by Manocha and Shaw (1964), who worked with two varieties of wheat, Khapli (resistant to Puccinia graminis-tritici) and 'Little Club' (susceptible to the same parasite). According to these authors lomasomes are a type of resistant mechanism which is found naturally in the resistant variety (Khapli) not found in the susceptible variety (Little Club), and it is implied that the reason for resistance in Khapli is due to the presence of lomasomes. However, Ehrlich et al (1968) indicated that, while such structures appear in response to the infecting fungi, they were found in both resistant and susceptible varieties, which indicates that they are not fully associated with either resistance or susceptibility. Unfortunately, there was no

available variety resistant to either U. fabae or B. fabae, and the variety (triple white) that was used throughout the present investigation, is completely susceptible to both pathogens and so no comparisons were possible.

As stated previously, lomasomes are found in uninfected plants of broad bean, but the frequency of their appearance seemed to be enhanced after host infection, especially in the case of Botrytis infection. Accordingly, these structures probably play a role here similar to that reported by Ehrlich et al., (1968). In the case of rust infection, some induced lomasomes appear to contain electron dense material and resemble in appearance those bodies shown by Luke et al., (1966) (dark masses) in vectorin treated oat root. These dark staining lomasomes, designated in this study as dark vesicles (DV), are frequently found in pockets of the plasma membrane immediately underneath a developing haustorium. Their conspicuous occurrence around dead 'necrotic haustorium' strengthens the idea of a defensive role. Since dark vesicles were not found in either healthy or Botrytis infected mesophyll cells, it seems reasonable to suggest that they are probably specifically induced as a result of the rust infection.

As to their origin, Luke et al., (1966) think that the dark staining masses have originated from the cell wall. On the other

hand Ehrlich et al., (1968) believe that the lomasomes in wheat, infected with the rust fungi, probably have their primary origin in the host protoplast.

The evidence in this investigation, indicated that lomasomes of healthy cells and those found in Botrytis infected cells probably originated from the endoplasmic reticulum (Plate 10). The exact origin is not clear of lomasomes containing electron dense vesicles which were found only in U. fabae infected cells. Their position and staining properties suggest that they could have originated from the cell wall.

Lomasome structures are by no means restricted to plant cells, and similar structures have been reported in other organisms. Glauert and Hopwood (1959) described membranous structures in the vicinity of growing cross-walls of bacteria. Crawley (1965) and Barton (1965) have also reported independently that such organelles were found adjacent to the cell wall in the algae Chara and Nitella. Their presence in fungi was observed in the basidiomycete, Polystictus versicolor (Girbardt, 1958). The term lomasomes meaning 'Border Bodies' was in fact coined by Moore and McAlear in 1961, who reported these structures in several fungi. Since then, similar structures have been described in Botrytis cinerea (Hawker and Hendy, 1963), Albugo candida (Berlin and Bowen, 1964) Pernospora manshurica (Peyton and Bowen, 1963), and

various other fungi (see Hawker, 1965; Moore, 1965; Bracker, 1967).

In this investigation lomasome-like bodies were also observed in both B. fabae and U. fabae growing on the host tissues (Plate 27,35,39-42). As indicated in the literature the actual role of these structures is not entirely clear, and various functions have been assigned to them including involvement in wall synthesis (Wilsenach and Kessel, 1965), enzymatic hydrolysis (Calonge, et al., 1969a), absorption by haustoria (Peyton and Bowen, 1963). The evidence obtained in this study suggests that lomasomes found in B. fabae mycelium probably have a secretory role, since such structures are found abundantly when the fungus is grown on the host tissues or on medium containing complex substances (i.e. host extract, pectin, cellulose or starch). No such structures were found when the fungus was grown on media containing simple carbon source (i.e. glucose and medium X). These results agree with those of Pitt (1968), who demonstrated by histochemical methods the location of several acid and neutral hydrolytic enzymes in these particles, as did Calonge et al., (1969a) working on Sclerotinia fructigena. As far as U. fabae is concerned lomasome-like structures found in the intercellular mycelium probably have a similar secretory role, thus facilitating cell wall penetration. The fact that such structures have never been found in the haustoria of Uromyces and their prevalence in the

intercellular mycelium may suggest their secretory function (i.e. by containing enzymes which hydrolyse the cell wall and thus aid penetration). Apart from the lomasome structures, both tubular and vesicular bodies have been observed for the first time in the vacuoles of the haustorium and occasionally in the vacuoles of the intercellular mycelium of U. fabae (Plates 22,32,34). Such structures are probably a characteristic feature of the fine structure of this fungus, since no similar structures have been reported in comparable studies with other fungal/host interactions (Bracker, 1967; Ehrlich and Ehrlich, 1963; Berlin and Bowen, 1964; Calonge, 1969). The exact role of these bodies is not clear, but the fact that they are always found in the vacuoles might suggest their secretory nature. Whether these suggestions are true or not must await further experimental verification. As can be seen from the foregoing discussion lomasome structures are found in different organisms (plants and fungi) under different conditions, and Moore (1965) questions whether all structures that appear lomasome-like are in fact homologous. Such questions can only be solved by studies directed towards the significance and function of these structures and up till now, very little has been done on this line and most of the suggestions for their role are speculative (Moore, 1965; Bracker, 1967a).

As a consequence of infection, the walls of the host cell are penetrated. In the case of U. fabae, host wall penetration takes place between the second and sixth day after infection and the mechanism of penetration follows more or less the same general pattern outlined for various obligate parasites (Ehrlich and Ehrlich, 1963a; Bra cker, 1967; Calonge, 1969). However, the present studies could not confirm the observation made by Ehrlich and Ehrlich (1963a), that the initial contact between the nascent haustorium of Puccinia graminis and the host cell protoplast is between a naked protoplast without a fungal cell wall. As illustrated, the penetration of U. fabae is accomplished by the invagination of host cell wall by the haustorial mother cell, whose wall was found to be continuous at all stages with that of the haustoria. Penetration by this fungus seems to be accomplished both chemically (hence the dissolution of host cell wall), and mechanically, by virtue of the invagination of the host cell wall under the mechanical pressure of the developing haustorium. This agrees partly with both Ehrlich and Ehrlich (1963a) and Calonge et al., (1969) working with two species of Puccinia. Both authors think that penetration is primarily a chemical rather than a mechanical phenomena.

In bean cells infected with Uromyces, although the host wall breaks away to allow a passage of the haustorium, the host plasma



membrane was found to remain continuous around the developing haustorium. This result is consistent with the work of Bracker (1967) and Littlefield and Bracker (1970). In contrast, cell invasion by B. fabae is faster than with Uromyces and seems to be purely by a chemical mechanism as suggested in Sclerotinia fructigena (Calonge, 1969). B. fabae does not form haustoria, and host cell penetration is accomplished by the growing mycelium. This mycelium apparently secretes an enzyme (Deverall and Wood, 1961) which dissolves part of the cell wall, so facilitating fungal invasion. A proposed mechanism for this is illustrated and discussed elsewhere (see Plate 44).

Although the effect of B. fabae infection is very drastic the development of the fungal mycelium itself seems to be considerably limited. This limitation in the fungal growth is probably due to the reported inhibitor production (Purkaystha and Deverall, 1965a,b), resulting from the interaction of the parasite with the host tissues. In addition to the limited growth of the fungus its cytoplasm was found to contain many large vacuoles and very few organelles. On the other hand, the development of the fungus on artificial media was found to be significantly greater. The cytoplasm of the fungal cell grown on artificial media (including bean leaf extract) is denser, both as far as the cytoplasmic matrix and the number of cytoplasmic organelles. The fact that

fungus growth and features of fine structure are preserved even when grown on extract of the host leaves, suggests that the observed inhibition is probably due to the production of a substance which is produced as a result of the interaction between the living host and the parasite, i.e. a production of a Phytoalexin-like substance. In contrast to this idea however, Deverall (1966), in his studies comparing the biochemical changes taking place in the infection droplets containing spores of both B. cinerea and B. fabae indicated that a phytoalexin like substance is produced in high yield in the case of Botrytis cinerea, where, in the case of B. fabae comparatively small amounts of this inhibitor is produced. It should be pointed out however, that the criterion for the limited growth of B. fabae in this study, is based in relation to the growth of U. fabae. The growth and cellular development of U. fabae in the host tissues is considerably greater than that of Botrytis, as can be seen from Plate 11. Generally the fine structure of U. fabae cytoplasm does not differ from other obligate parasites (see review by Hawker, 1965; Moore, 1965; Bracker, 1967), and other than by the production of haustoria the general fine structure of the cells of this fungus does not differ much from those of normally developing B. fabae cells (grown on culture). The difference in fine structure of the cells of U. fabae from those of B. fabae growing on the host tissues, is in the reduction in the density and size of the cytoplasm and the cytoplasmic organelles of the latter,

and this effect can probably be explained by the production of inhibitor against B. fabae. Some support for this view can be derived from the results of the preliminary experiments conducted to study the effect of water extract from Botrytis infected leaves on the fine structure of cell of Botrytis mycelium incubated for varying times (24-43 hours). As it has been shown in Plate 67, most of the cell components of mycelium incubated in water extract from infected leaves, have disintegrated. On the other hand, mycelium incubated in healthy extracts showed very little disintegration of the cytoplasm.

From the foregoing discussion it seems reasonable to suggest that the unidentified structures in the extracellular spaces of Botrytis infected leaves (Plate 53-55) are probably remnants of fungal mycelium acted upon by the defensive mechanism of the host. However, further investigations on the nature of these structures should prove interesting. In contrast to B. fabae the fine structure of U. fabae was found to be preserved and the only similar effect to that described above was the occasional formation of necrotic haustoria (Plate 18a,b). As explained previously, (see Results) these types of haustoria and their mother cells were found to be blackened and apparently dead. Layers similar to, and continuous with, the host cell wall were occasionally found excluding these haustoria from the host cytoplasm. Such layers were

found to contain many electron dense vesicles (Plate 18a,b). It is not clear whether the apparent death and necrosis of such haustoria are due to the content of such vesicles (lomasomes) or due to the isolation of the fungus from the host cytoplasm, thus stopping the passage of nutrients from the cytoplasm of the host to these haustoria. Similar observations of necrotic haustoria were made by Ehrlich et al., (1968). In the case of normally developing haustoria, nutrients pass from the host to the fungus by a mechanism probably similar to that outlined by Ehrlich and Ehrlich (1963a) for Puccinia graminis i.e. by the invagination of the host plasma membrane adjacent to the haustoria, and the consequent formation of vesicles which discharge their contents into the incapsulation (sheath) surrounding the haustoria. Although invaginations similar to that observed by Ehrlich and Ehrlich (1963) were also observed in the host plasma membrane adjacent to the haustoria, this study does not confirm the presence of channel-like areas in the haustorial wall which projected into the incapsulation as reported by Ehrlich and Ehrlich (1963). As to the origin of the incapsulation (sheath) around U. fabae haustoria, it would seem reasonable to suggest that it might have originated from the fungus, since there is a clear continuity between such regions and fungus cell wall (Plate 31). This region is separated from the host plasma membrane by an electron transparent area (Plate 31). Although this conclusion is in

agreement with some reports, others think that this region has probably originated from both the host cell and the parasite.

From the fine structural observations, host cells infected by the rust fungus can be divided into two classes (1) cells which exhibit a reaction marked by an increase in the volume of the cytoplasm, an increase in the size of the ribosome rich host cell organelles, and an increase in the size of mitochondria; such changes indicate an increased capacity for synthesis. This reaction is characteristic of infected cells from 4-12 days after infection. (2) As infection progresses, host cells are characterised by the production of many vesicles and the disintegration of cytoplasmic organelles and membranes. This kind of reaction showed some similarities with the kind of degeneration described by Shaw and Manocha (1965a).

Cells infected with B. fabae can also be classified to two types, (1) in the immediate vicinity of the necrotic lesion and characterised by the complete breakdown and the blackening of the cell components; such cells can be seen as early as 24 hours after infection. As the distance increased from the centre of the necrotic lesion so less and less blackening and disintegration was observed. Interestingly, despite the disintegration and the blackening of the cytoplasm and the other host cell organelles, chloroplasts were found to show

relatively more resistance to these reactions (Plate 45).

(2) Cells at the periphery of the necrotic lesion (about 0.2-0.5 cm. from the centre of the lesion) exhibit an entirely different reaction. This reaction is characterised by the increase in the volume of the cytoplasm and in the numbers of cytoplasmic organelles. The response is characteristic of cells undergoing increased capacity for synthesis and probably these are the cells responsible for the production of factors limiting the growth and affecting the cell contents of the fungus. Only occasionally has a fungal mycelium been seen in these cells. Although this particular type of cell in Botrytis infection showed some resemblance with those in the early stages of U. fabae infection, they differ from them in some details, i.e. although the nuclei in both infections were found to be increased in size, the U. fabae of infected cells showed the following characteristic response to the infection. This response is characterised by the increased electron density of the interchromatic region which could represent a decreased DNA and RNA and an increased protein content (Littau et al., 1964). Such changes are consistent also with the results of Bhattacharya et al., (1965), who used cytochemical techniques to indicate an increase in RNA and protein, in nuclei of cells at the relatively early stages of infection (6-12 days after infection). After this period the response starts to diminish gradually marking the senescence

of the cells (see also Manocha and Shaw, 1966).

As stated previously, one of the common consequences of infection to both the peripheral cells of Botrytis necrotic lesions and the cells in the early stages of U. fabae infection, is the enlargement of both the nuclei and nucleoli. In the case of obligate parasites it is now generally accepted that this change is accompanied with an increase in the synthetic capacity of the cells (Bracker, 1967b; Peyton and Bowen, 1963; Bhattacharya, et al., 1965). Moreover, cells adjacent to wounds also respond by an increase in size of nuclei and nucleoli (Ziegler, 1965). Whether the enlargement of nuclei in the periphery cells of Botrytis necrotic lesions, is due merely to wounding or specifically to the infection, is not certain. The second possibility cannot be ruled out, in view of the reported association of the increase in the size of this organelle with specific facultative parasite infection (Braun, 1962; Owens, 1964).

The increase in the size of mitochondria observed in this study in both types of infection might be related to an increase in respiration; a basic principle of plant pathology, as well as animal pathology, is that most if not all infections increase respiration (Yarwood, 1966); increases of up to ten times that of healthy tissue have been reported (Bushnell and Allen, 1962).

Some of the observed increase is probably due to the activity of the invading parasite itself, and to correct for this factor, various methods have been used. Some of these are (a) selective killing of the parasite in situ by means of chemicals or heat (Pratt, 1938, Yarwood, 1953); (b) removing the parasite mechanically (Allen, 1966); (c) measuring the respiration in regions beyond those occupied by the parasite (Akazawa, 1956). Recent developments which allow the isolation of intact plant organelles may provide a useful tool in studying the effect of infection on host mitochondria without the interference of the parasite in the determinations. Aksenova et al., (1968) studying the properties of isolated mitochondria and ribosomes of cabbage infected with B. cinerea, noted increased ATPase activity of the former and an increase in ribosome content with infected tissues.

In contrast to respiratory responses, reduced photosynthesis is a usual consequence of disease development (Yarwood, 1966; Wood, 1967). The reduction in photosynthesis has been assumed to be proportional to the photosynthetic area invaded by the parasite (Chester, 1950). Such an assumption might not be very accurate, however, since different parasites vary greatly in the injury they cause in the same amount of tissue (Johnson and Miller, 1940). Moreover, some parasites exert effects distant from the invaded areas



(Yarwood, 1965). In the present studies, the two parasites differ in the amount of visible injury they caused to the leaves of V. faba. The time at which such injury occurs is also different in both organisms. Fine structural observations on chloroplasts of cells infected with the rust fungus (2-4 days after infection) did not reveal any anatomical difference from the healthy leaves. However, as the 'flecking' stage took place (6 days after infection), chloroplasts appeared to be reduced in size and contained many vesicles which seemed to be continuous with inner chloroplastic membrane. These vesicles were not found in chloroplasts of either healthy or Botrytis infected leaves. Similar vesicles are said to be common in the development of stages of tomato plant fruit plastids (Shalla, 1964; Rosso, 1967). Although Esau and Cronshaw (1967) observed similar vesicles in Nicotina tabacum in both control and TMV infected plants, Arnott et al., (1969) indicated that they are characteristically more prevalent in TMV infected tomato plants. In the present studies, these vesicles were only found in the inner membrane of chloroplasts from 6-20 days old rust-infected leaves. With Botrytis infection the results indicate that, although most of the cytoplasm and cell components were completely blackened, some chloroplasts were relatively preserved. The chloroplasts found in the centre of the necrotic lesion however, either lost their stroma or became very blackened probably indicating that they had lost some or all of their photosynthetic activities.

In order to test this possibility and also to study the significance of the changes which occurred in chloroplasts in both types of infection, biochemical studies were made on isolated chloroplasts from infected leaves. The idea behind choosing to study the photosynthetic reaction on isolated chloroplasts without the presence of any other parts of the cell is (a) to simplify and limit the scope of the experiment to the effect of infection on photosynthesis, without the interference of the respiratory activities of both the host and the parasite; (b) to investigate whether or not the reported effect of infection (be it increase or decrease) is limited to the in vivo experiments; (c) to exclude the interference of the parasite metabolism.

Accordingly, it was hoped that the isolation and separation of chloroplasts from healthy and infected plants would elucidate the biochemical function of any changes which take place after infection. Also whether or not such changes have any direct effect on the photochemical reactions namely carbon dioxide fixation, Hill reaction, photophosphorylation and protein synthesis by isolated chloroplasts.

#### Healthy Tissues

Chloroplasts from V. faba were only active in protein synthesis when they were isolated from very young seedlings (10-14 days after planting). Little or no in vitro protein synthesis was detected when

chloroplasts were prepared from older plants. Due to the inability of chloroplasts from older plants (older than 20 days after planting) to incorporate radioactive amino acids, experiments on protein synthesis were discontinued, since the ages of plants used throughout this study owing to the infection cycle was 20-40 days.

Unfortunately chloroplasts prepared from V. faba using conventional methods of isolation, were found not to fix carbon dioxide. The reason for this is not clear. However, according to Baldry et al., (1970) it could be explained in terms of specific inhibition of the carboxylation reaction of the PCR cycle by quinones produced from endogenous phenolic compounds. This was based on the established findings that the enzymes associated with this step are particularly sensitive to incubation with SH compounds (Losada et al., 1960; Robin and Trown, 1964). Moreover, Pierpoint (1966) has suggested that the inhibition of plant enzymes may be due to the products of enzymic oxidation of chlorogenic acid complexing SH groups in protein.

Chloroplasts isolated from V. faba leaves, regardless of the age of plants from which they were prepared, were unable to carry out Hill reaction activities in the absence of added electron acceptor. Of the three electron acceptors used,  $(K_3Fe(CN)_6)$ , NADP, DPIP) the

former was the only acceptor to support oxygen evolution. Although this problem was not investigated in detail, it seems possible that a situation might exist, similar to that described by Baldry et al., (1970) for sugar cane chloroplasts, namely that the inhibition of oxygen evolution in the absence of exogenous electron acceptor was due to inhibition; thus o-diphenols and quinons extracted from sugar cane leaves were shown to inhibit oxygen evolution (in the absence of external electron acceptor) of Spinach chloroplasts. It was postulated that these compounds act either as electron donor or acceptors resulting in uncoupling of the normal pathway of photosynthetic electron flow or changing the redox state of intermediates of the electron transport chain (Baldry et al., 1970). Furthermore, Mehler (1951) found that manganese ion stimulated oxygen uptake by isolated chloroplasts in the presence of catalase and ethanol. The inability of V. faba chloroplasts to reduce NADP or DPIP however, remains unexplained. In view of this, studies analogous to that reported by Baldry et al (1970) on chloroplasts of V. faba should prove interesting.

#### Infected Tissues

Plant infection causes a reduction in the rate of photosynthesis (Kuprwiez, 1957; Scott and Smillie, 1963; Yarwood, 1966; Wood, 1967). This is true whether plants are infected with obligate or facultative

parasites. From the few reported cases of increased photosynthesis, all are agreed that increases are limited to the early stages of infection (Shaw, 1961; Livne, 1964; Harding et al., 1968). Reasonable amounts of information are available concerning the influence of physiological and viral diseases on the photosynthetic reactions by isolated chloroplasts (Spike, 1955; Zailten and Jagendorf, 1960; Spencer and Possingham, 1966). On the other hand, the only report concerning the influence of fungal infection on Hill reaction activities has been made on the rust fungi of Rye (Pentzkaya and Kurprovesh, 1968). No information is available concerning the influence of facultative parasites on any of the chloroplast enzymic reactions. In the present investigations the influence of U. fabae (obligate) and B. fabae (facultative) infections on the number of chloroplasts, chlorophyll content, Hill reaction and photosynthetic phosphorylation, was studied. Both infections resulted in the destruction of the photosynthetic area but with varying speeds and in varying proportions. Consequently, as would be expected, both infections resulted in a reduction in the number of chloroplasts per  $\text{cm}^2$  of leaf area. Furthermore, infection (10-20 days and 2-20 days after infection in the case of Uromyces and Botrytis respectively) by both organisms reduced the amount of chlorophyll content per chloroplast. The results with Hill reaction activities using potassium

ferricyanide as an external electron acceptor, indicate that there was an increase in activity of chloroplasts isolated from rust infected leaves (6-8 days after infection). Prior to this increase there was an occasional reduction in the rate which might be due to a mechanical injury resulting from the inoculation procedure. The increase reported in this study and also that by Pentzkaya and Kurprovesh (1968) (6-8 days after infection) are not due to an enhancement of chlorophyll synthesis, since the amount of chlorophyll used was standardised in both reactions with healthy and infected chloroplasts. Furthermore, results from the present studies suggest that the stimulation is probably not due to the formation of chemical compounds on infection, since no stimulation occurred with healthy chloroplasts when 'infected' supernatant was added to them or when equal proportions of chloroplasts from infected tissues were mixed with healthy. Electron microscope observations on chloroplasts isolated from infected leaves (6-8 days after infection) revealed no significant structural differences between them. However, electron microscope observation on cells containing the parasite haustoria showed the presence of many vesicles in chloroplasts and their formation coincide in time with the reported stimulation. Whether or not this stimulation is due to any structural changes must await investigations. As the rust infection progresses the rate of the

Hill reaction of chloroplasts isolated from infected leaves starts to decline in comparison with chloroplasts from healthy leaves. These results are in agreement with the reports of Pentzkaya and Kurprovesh (1968). Although these authors did not comment on the possible cause of the decrease, evidence in the present studies suggests that this was probably due to the formation of inhibitors which are produced as a result of the infection. This evidence is derived from the results of some experiments in which chloroplasts from infected plants were mixed with those from healthy plants resulting in a reduction in the rate of Hill reaction of the centres, although this reduction was not very large. The suggestions seem possible in view of Baldry et al., (1970) results concerning the inhibition of ferricyanide catalysed oxygen evolution by plant phenolic compounds and the formation of phenolic compounds as a result of infection has been clearly demonstrated (Farkas and Kiraly, 1962; Wood, 1967). In contrast to the idea of inhibitor formation, Zailtin and Jagendorf (1960) ~~pro~~posed that the reduction in the Hill reaction activities of chloroplasts from TMV infected tobacco, is due mainly to the depletion of or limitation of the intercellular nitrogen supply. Such a possibility cannot be ruled out as a factor in the reduction of Hill reaction activities of chloroplasts isolated from infected V. faba leaves especially as both pathogens

caused a senescing (in the case of Botrytis disintegration) of the leaf. This would inevitably result in the breaking down of protein bringing about a net export of nitrogen. Whether the early inhibition (24 hours after infection) of chloroplasts isolated from Botrytis infected leaves is due mainly to inhibitor production or to depletion of nitrogen supply or both, must await further investigation. Although the foregoing results were obtained from reactions containing no added phosphorylating reagent, the addition of ADP to the reaction mixture resulted in a similar type of response as in the case of Botrytis infection. However, with U. fabae infection, the enhanced rate of oxygen evolution (6-8 days after infection) was found consistently to last longer (6-12 days after infection). At this period of time, the rate of coupled phosphorylation of chloroplasts from both Botrytis and Uromyces infected leaves was found to be reduced in relation to those from healthy leaves. These results suggest that the observed enhanced rate of the phosphorylation accompanied oxygen evolution in the case of Uromyces infection, might be due to uncoupling of the electron flow. The results also indicate that both infections had an adverse effect on the coupled phosphorylation.

The effect of Botrytis infection on the cyclic phosphorylation was shown to be of a more destructive nature, and this effect can be



noticed as early as 48 hours. It follows the same general pattern of response as that of other photochemical reaction (i.e. uncoupled and coupled oxygen evolution; coupled phosphorylation). In contrast, the effect of U. fabae on the cyclic phosphorylation activities of isolated chloroplasts was found to be stimulative in the early stages (8-12 days after infection), followed by a gradual decline as the host started to senesce. These results, do not confirm the reports of Wynn (1965) working with chloroplasts isolated from rust infected oats, in which chloroplasts isolated from rust infected leaves carried out photosynthetic phosphorylation at essentially the same rate as chloroplasts from comparable healthy leaves. However, it should be pointed out that the results of Wynn did not include the period of the latter stages (10-20 days) of disease development on the photosynthetic activities of isolated chloroplasts. The present studies indicate a drop in the activity starting from 12-20 days after infection. This decline in activity is associated with the senescing of leaves and disorganisation of chloroplasts (Plate 25,26). Furthermore, the reported stimulation of Hill reaction and photosynthetic phosphorylation in the case of the early stages of rust infection (6-12 days after infection), is consistent with the argument of Cutter (1951) that obligate parasites require photosynthetic intermediates for their development. On the other hand, the decline in the activities of the Hill reaction

and photosynthetic phosphorylation of both Botrytis (2-20 days after infection) and Uromyces infected leaves (12-20 days after infection) are not due to the loss of total chlorophyll concentration, but rather to a loss of enzyme activity. If the results given in this study are calculated on the basis of the number of chloroplasts per reaction or on a leaf area basis, the rates of inhibition recorded would be greater than indicated above. Thus, in addition to the loss of enzyme activity per unit of chlorophyll, fungal infection (particularly in the case of Botrytis) also induced a loss of total chlorophyll and a reduction in the active leaf area which results in a reduction in the number of chloroplasts in infected tissues.

Despite the progress being made on the techniques and methodology of the in vitro study of the enzymic reactions of photosynthesis, very little has been done on the effect of fungal infection on such reactions. The results presented here suggest that this line of study is both interesting and promising.



Explanation of Figures

Capital letters refer to the Cellular constituents in the host,  
small letters refer to the fungus.

BC	Blackened Cell
BHC	Blackened host cytoplasm
C	Collar
CH	Chloroplast
CR	Chromatin regions
CW, cw	Cell wall
DI	Electron dense inclusion
DB	Electron dense band
Dr	Electron dense ring
Dv	Electron dense vesicles
Dw	Electron dense wall
EB	Electron dense bodies
EL	Electron dense bladder
en	Encapsulation
ER	Endoplasmic reticulum
GB	Golgi bodies
GR	grana
h	Hyphae
HC, hc	Host cytoplasm
hn	haustorial neck

ho	haustorial opening
ICR	Interchromatin region
Ih	Intercellular hyphae
Ich	Intercellular hyphae
LB	Lipid bodies
LG, lg	Lipid globules
LO, lo	lomasomes
LT	Intact chloroplasts
M, m	Mitochondria
ML	Chloroplast without the outer membranes
MVB, mvb	Multivesicular bodies
nh	necrotic haustorium
NE, ne	Nuclear envelope
NU, nu	Nucleus
NUS, nus	Nucleolus
PD	Plasmadesmata
PM, pm	Plasma membrane
S	Septum
SG	Starch grain
Sh	Sheath
Sp	Septum pore
ST	Stroma
TP, tp	Tonoplast

TS	Tubules
TH	Thickness
tw	electron transparent wall
V, v	Vacuole
Vs, vs	Vesicles
VI	Vesicular inclusions
XV	Xylem vessel

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PLATE 1

Photograph showing the pattern of growth and the size of plants used. Plants in this stage (20 day old) were normally used for artificial inoculations with the pathogens.







PLATE 2

Photograph illustrating the types of symptoms resulting from infection with U. fabae (centre) and B. fabae (right), healthy control (left) (10 days after infection).

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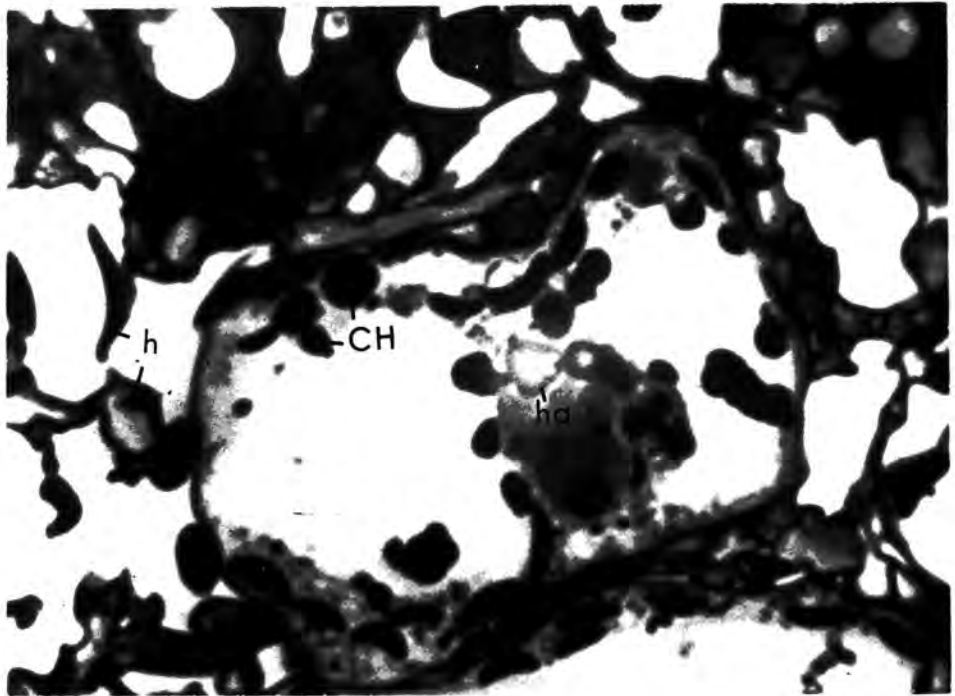
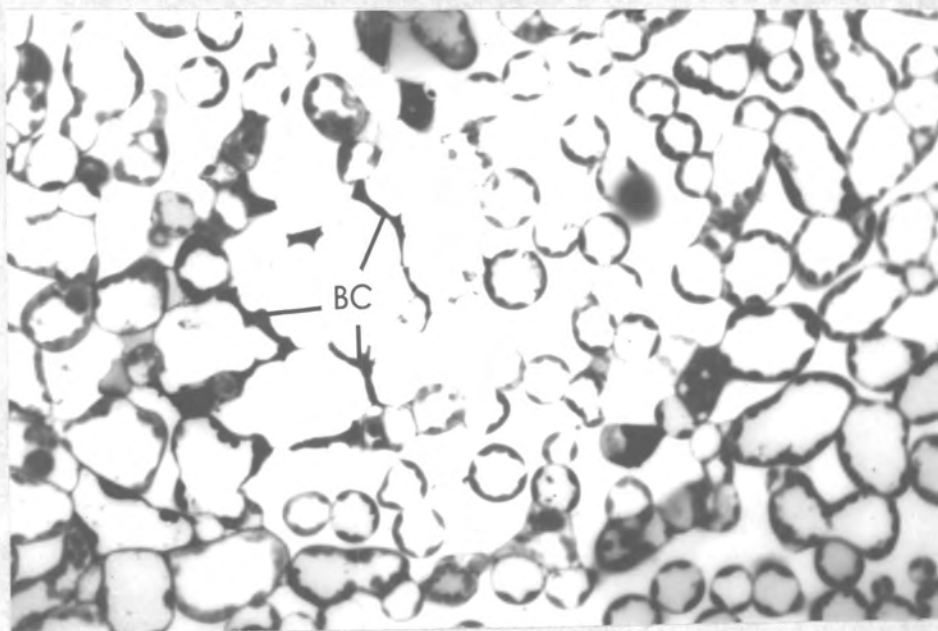


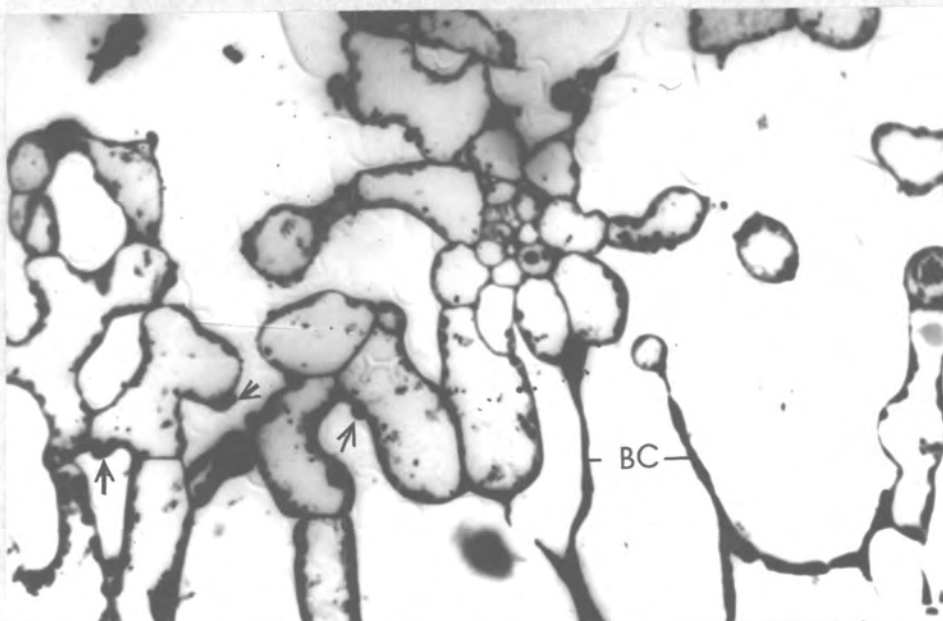
PLATE 3

A longitudinal section of an infected V. faba leaf showing the distribution of U. fabae mycelium (h) in the extracellular spaces of the mesophyll cells. Note the numerous haustoria (ha) inside the cell in the middle portion of the photograph (Mag.120X)





a



b

PLATE 4

(a) A longitudinal section of a *V. faba* leaf infected with *B. fabae*, showing both the blackened necrotic cells (BC) and the apparently healthy cells at the periphery of the necrotic lesion, (b) a cross section showing extracellular (electron dense bodies) Arros. Mag. 30X.

PLATE 5

Low magnification Electron micrograph showing  
the organisation and the general fine structure  
of healthy mesophyll cells of V. faba.

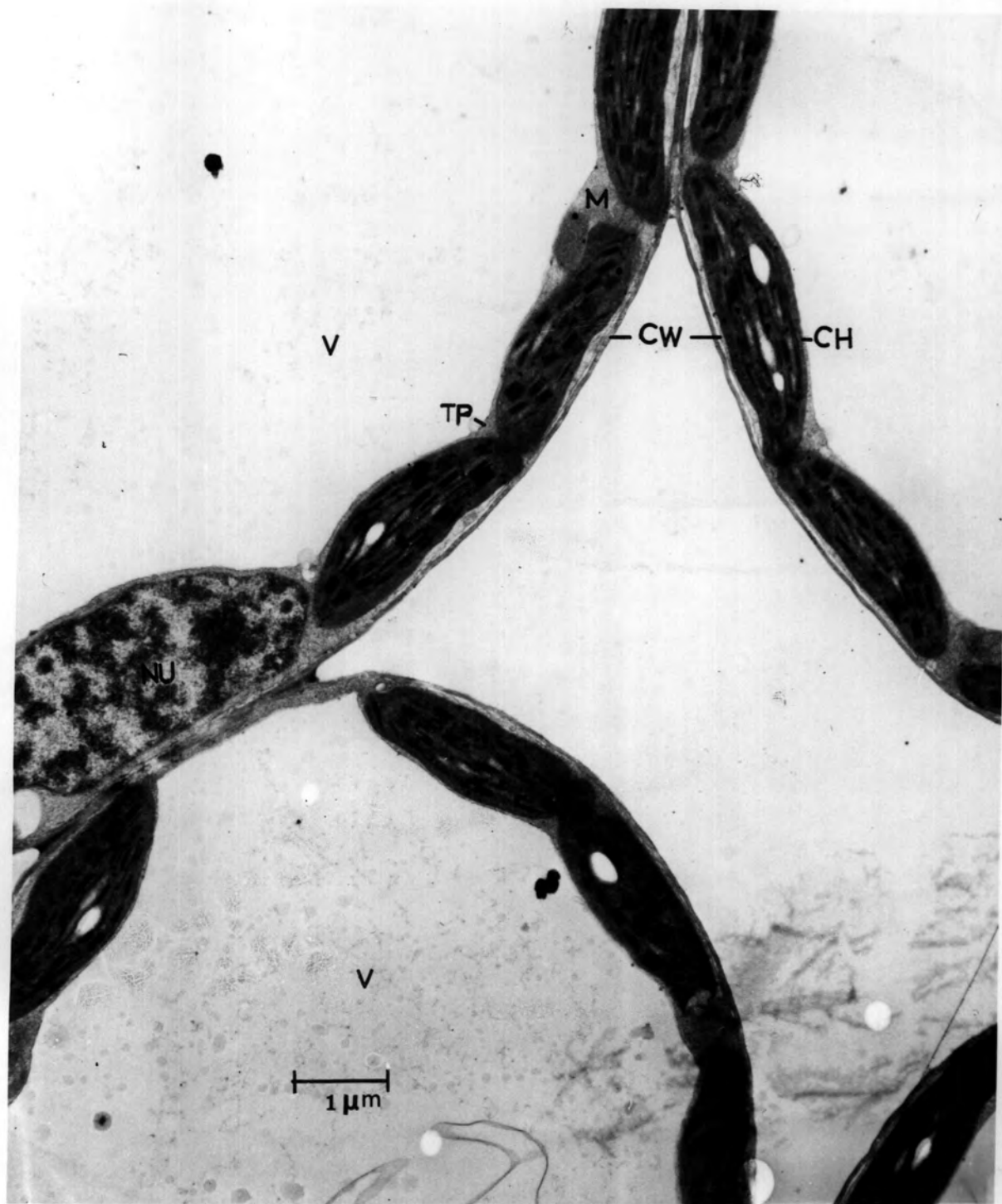
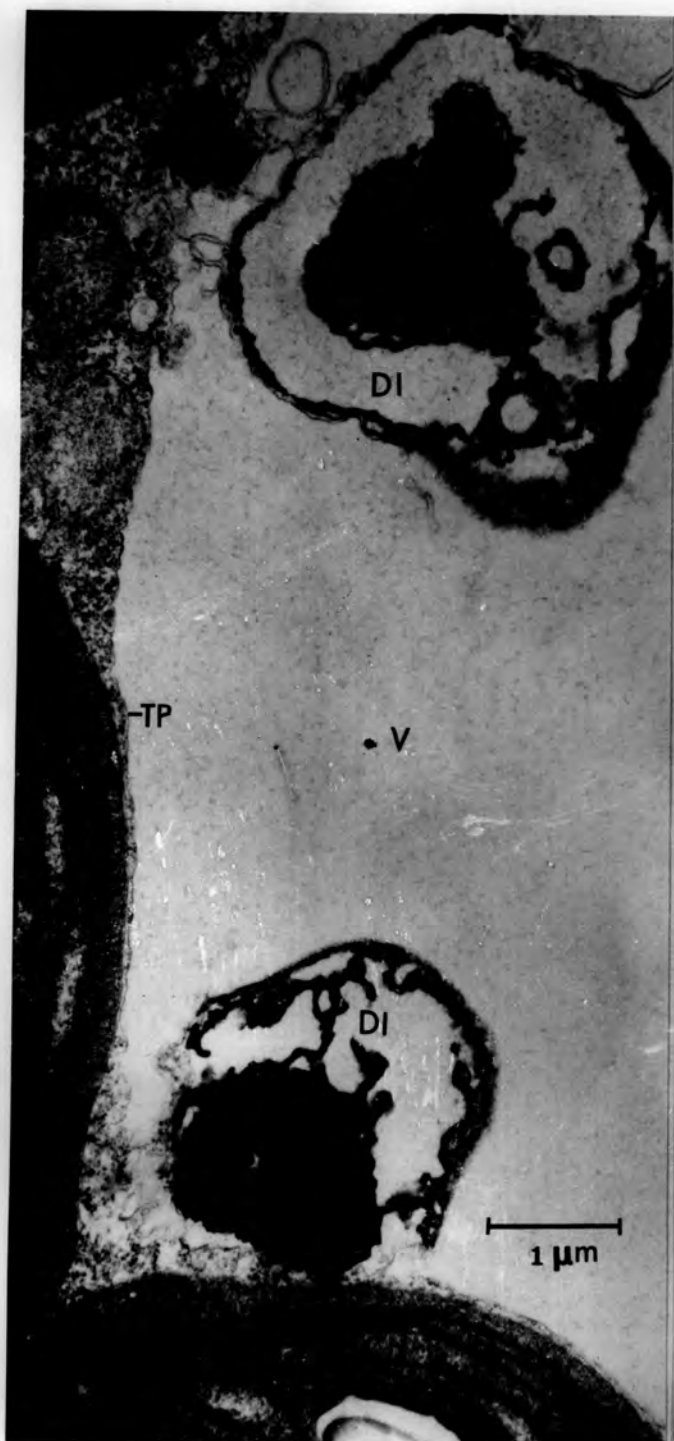


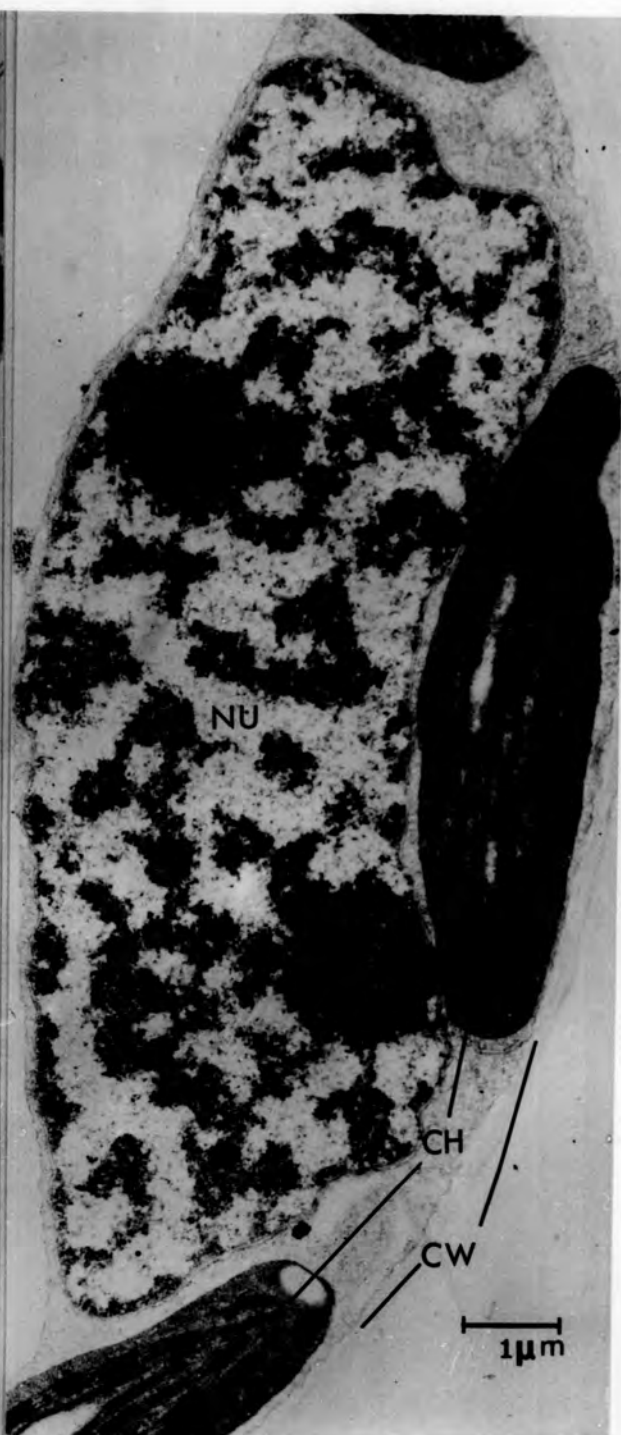
PLATE 6

(a) Electron micrograph of a portion of a healthy mesophyll cell showing two of the electron dense inclusions (DI) frequently found in the vacuole (V).

(b) Microphotograph showing the arrangement and the fine structure of the chloroplasts (CH) and the nucleus (NU)



a



b

PLATE 7

High power Electron micrograph showing the fine structural details of the chloroplasts in healthy mesophyll cells of V. faba.

(G) grana

(O) Osmophilic globules

(St) Stroma

(L) Lamella

(SG) Starch grain



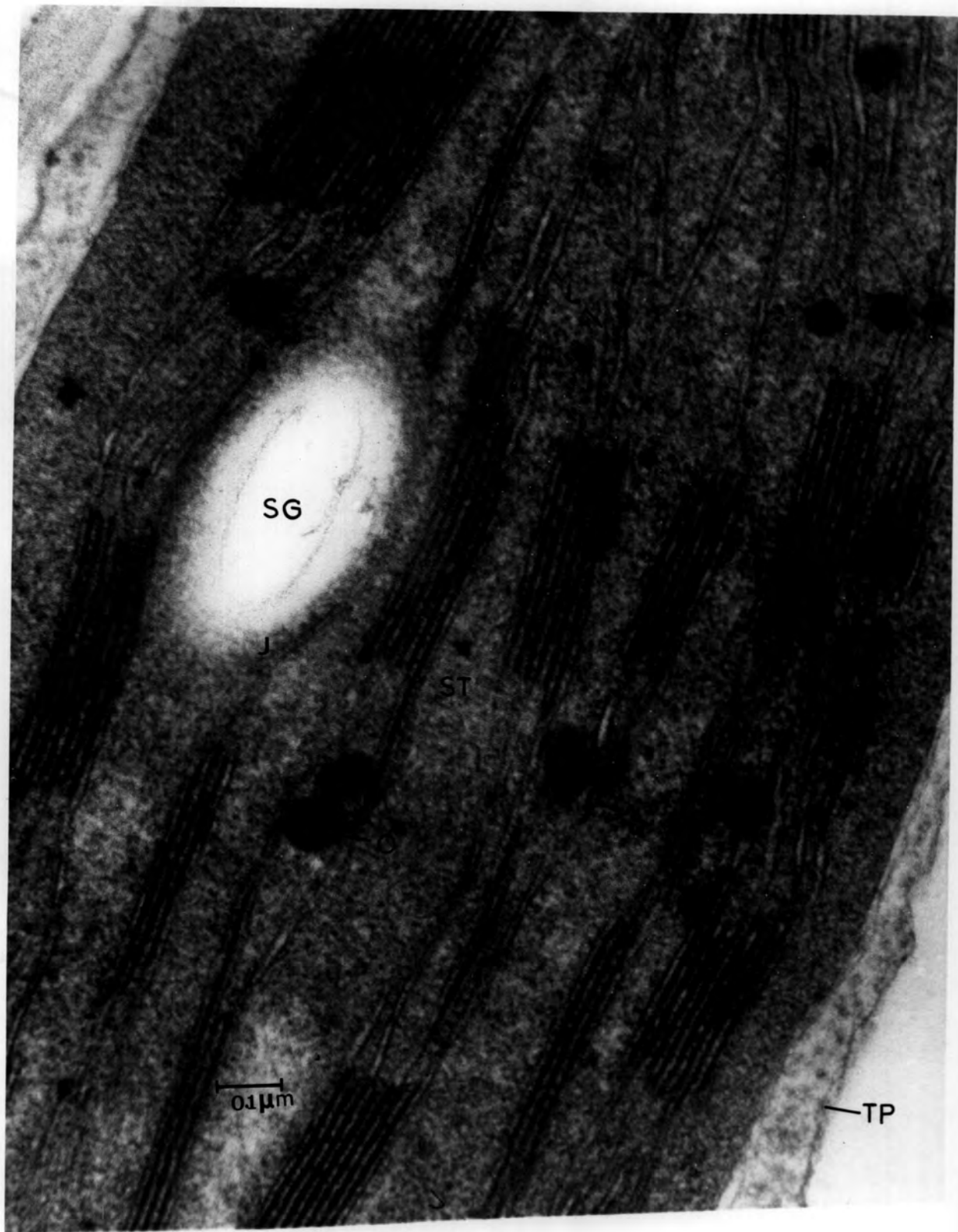


PLATE 8

High power Electron micrograph showing the distribution and the proportion of the chromatin (CR) and the interchromatin (ICR) regions in the nucleus of healthy mesophyll cells.

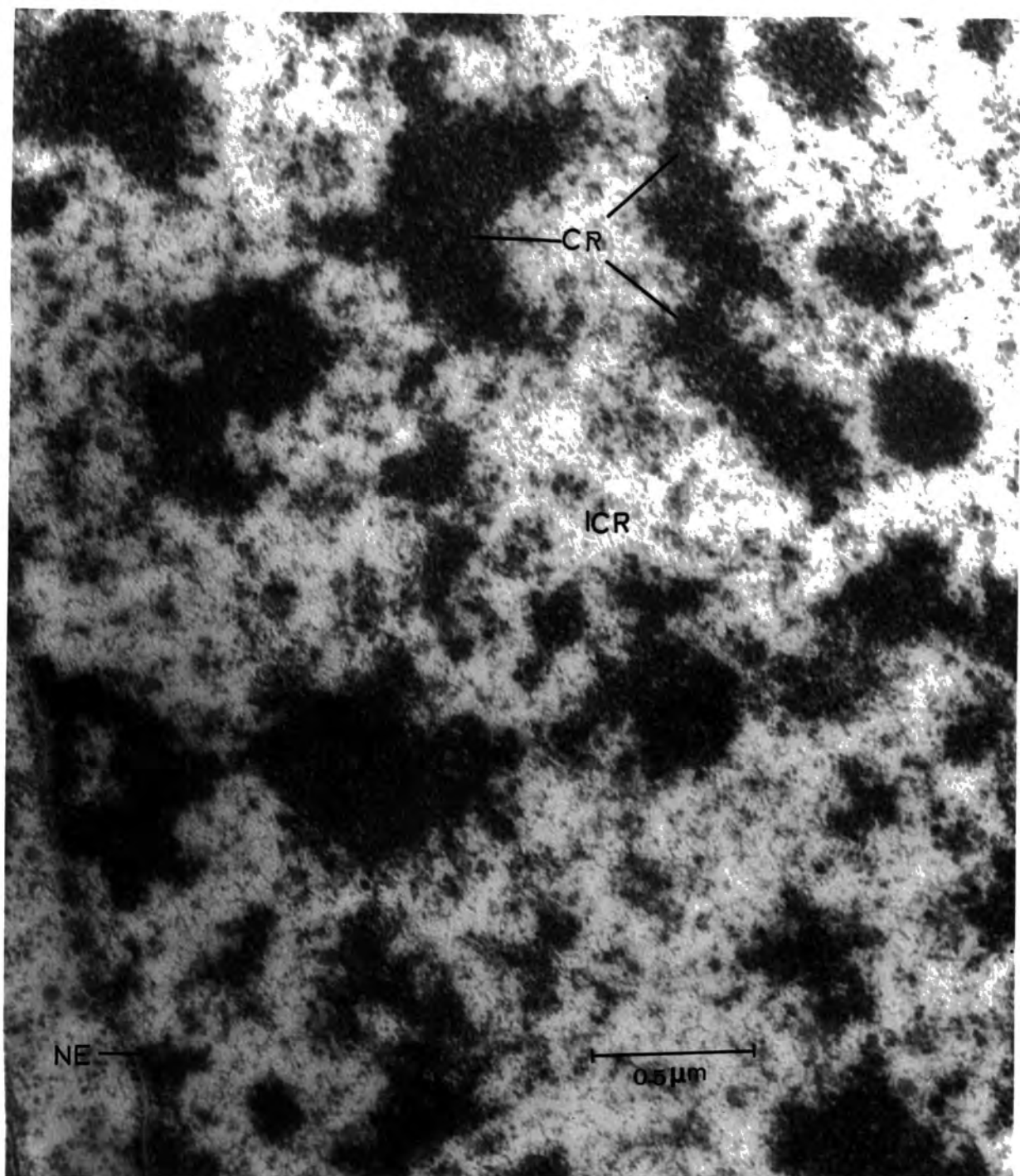


PLATE 9

Electron micrograph of parts of two mesophyll cells of healthy V. faba showing the location of lomasomes-like bodies (LO) surrounded by the plasmamembrane (PM). Also note the vesicles migrating from one cell to another through what looks like a plasmadesmata (Arrow).

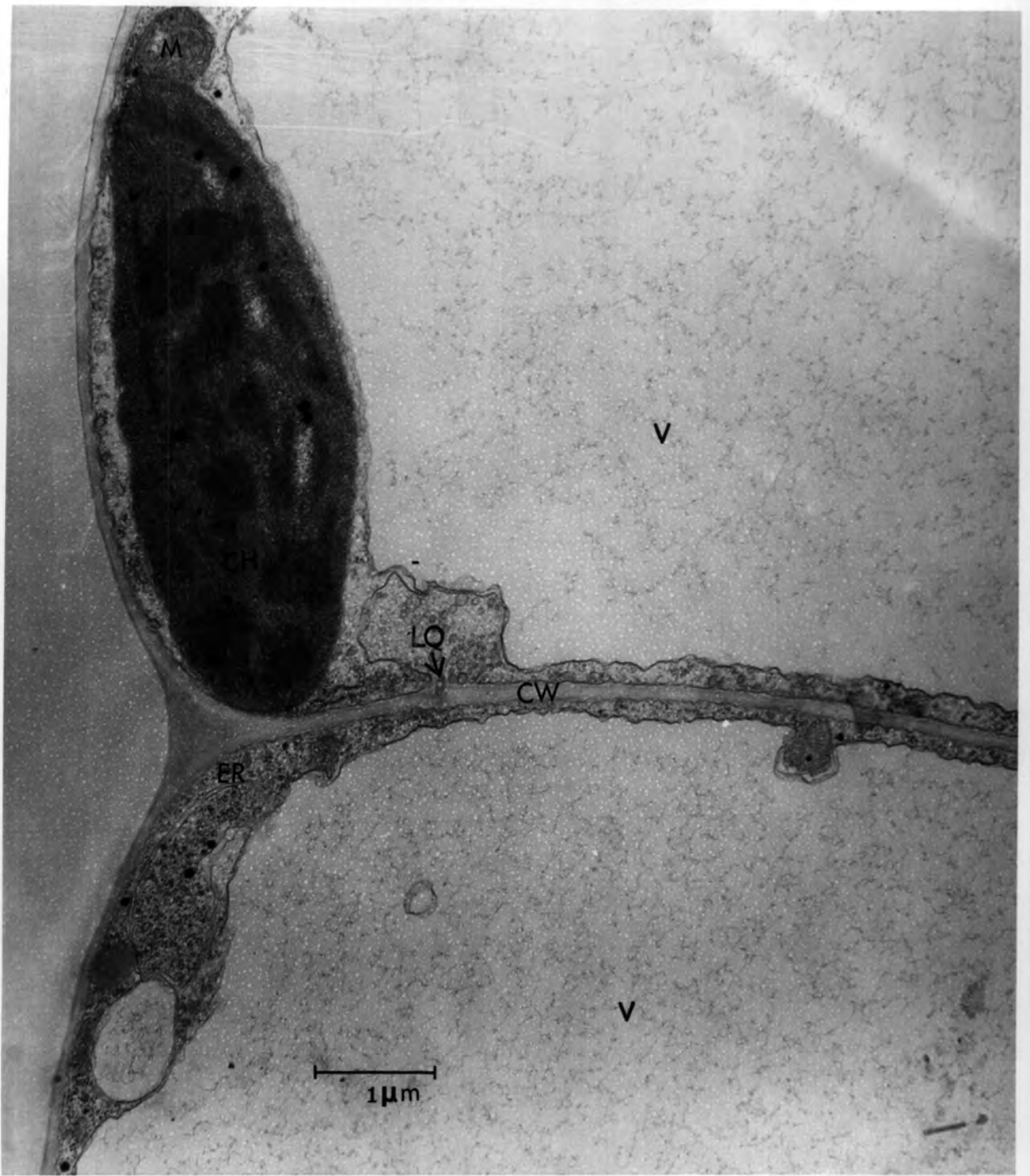


PLATE 10

High magnification Electron micrograph showing parts of two mesophyll cells of healthy V. faba. Note the association of vesicles (VS) with the endoplasmic reticulum (ER)

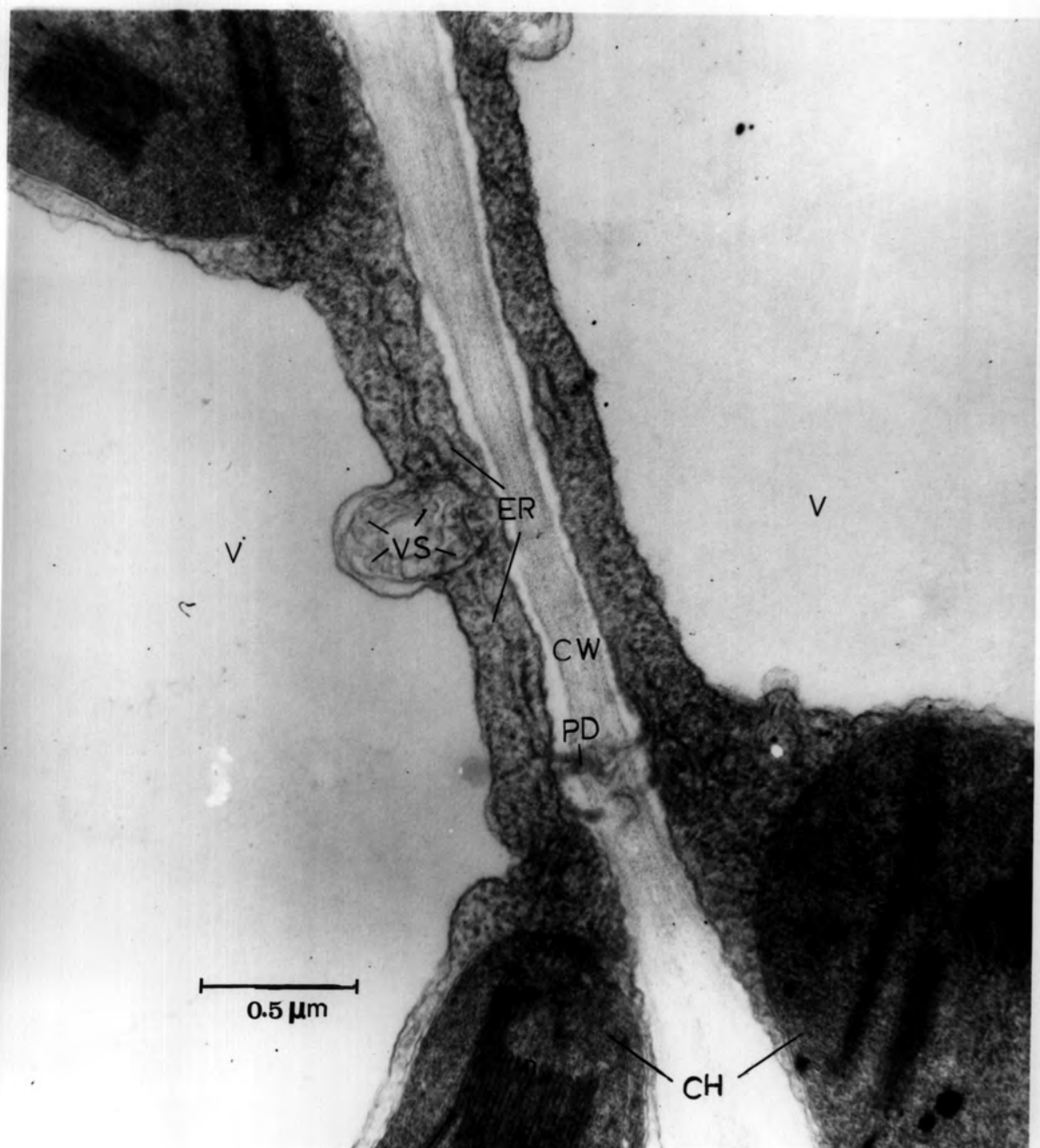


PLATE 11

Low power electron micrograph showing some of the mesophyll cells of V. faba infected with U. fabae (5 days after infection). Note the proliferation and the distribution of the intercellular hyphae (h) growing between the host cell wall (CW) and in the intercellular spaces (IS)





PLATE 12

(a) Electron micrograph showing some of the earlier stages of host cell penetration - note the invagination of the host plasma-membrane (PM) and the production of dense vesicles (DV).

(b) showing the invagination of the host cell wall (CW) as a result of fungal penetration.

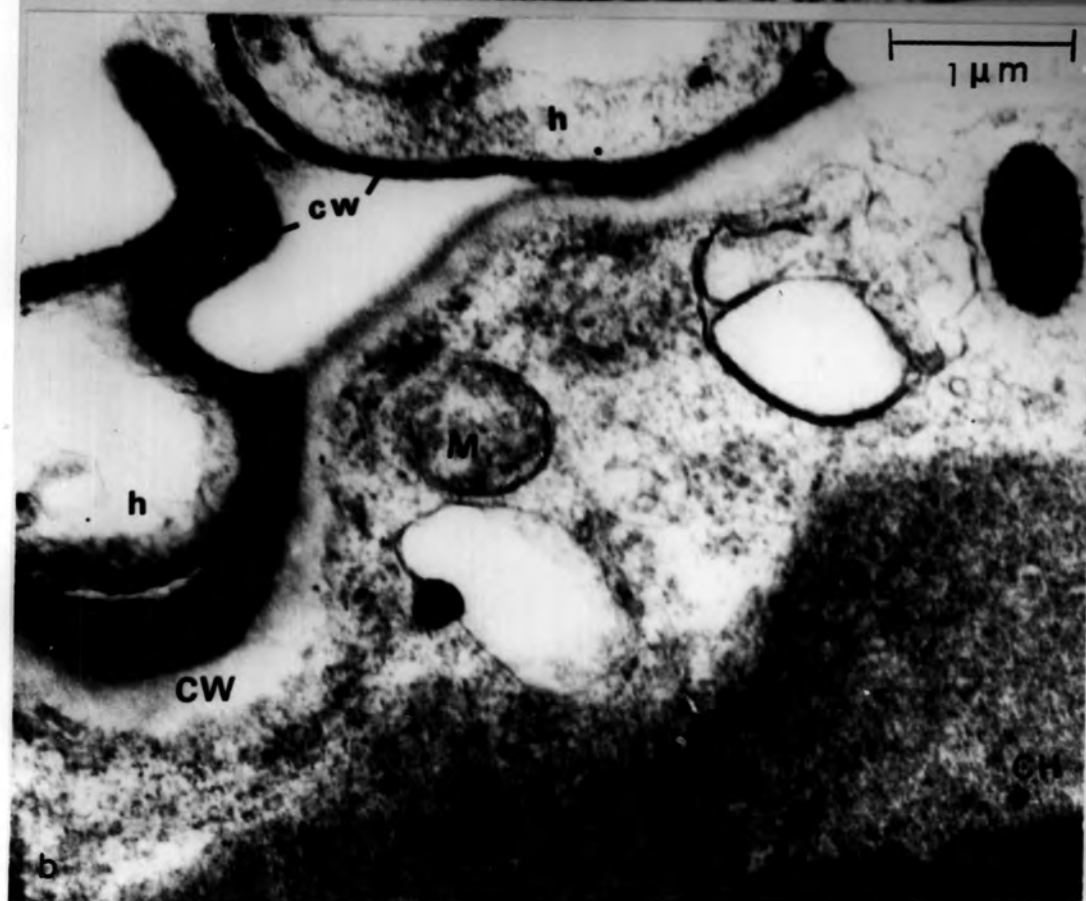
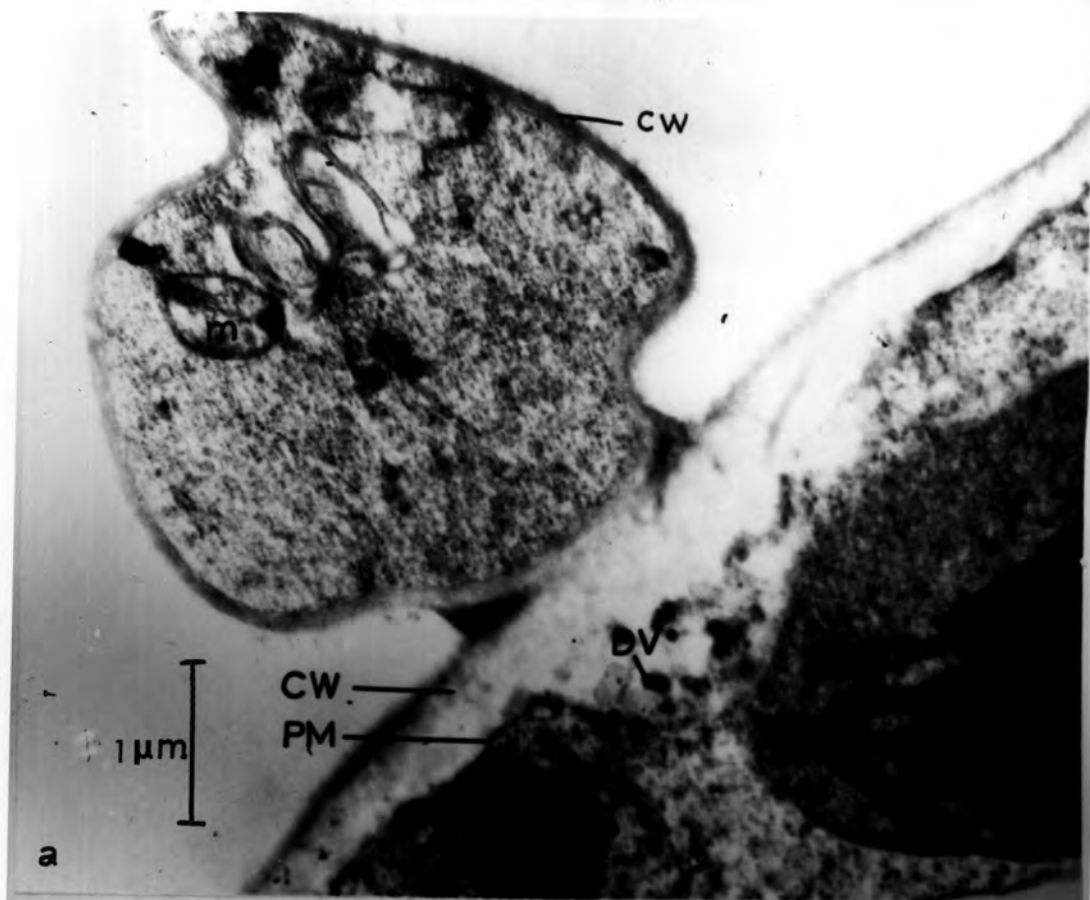


PLATE 13

Electron micrograph showing one of the earlier stages of the formation of the haustorium of U. fabae. Note that invaginated Host Cell Wall (CW) and plasmamebrane (PM) is still intact. Also observe the presence of dark vesicles (VS) in the host cell wall.

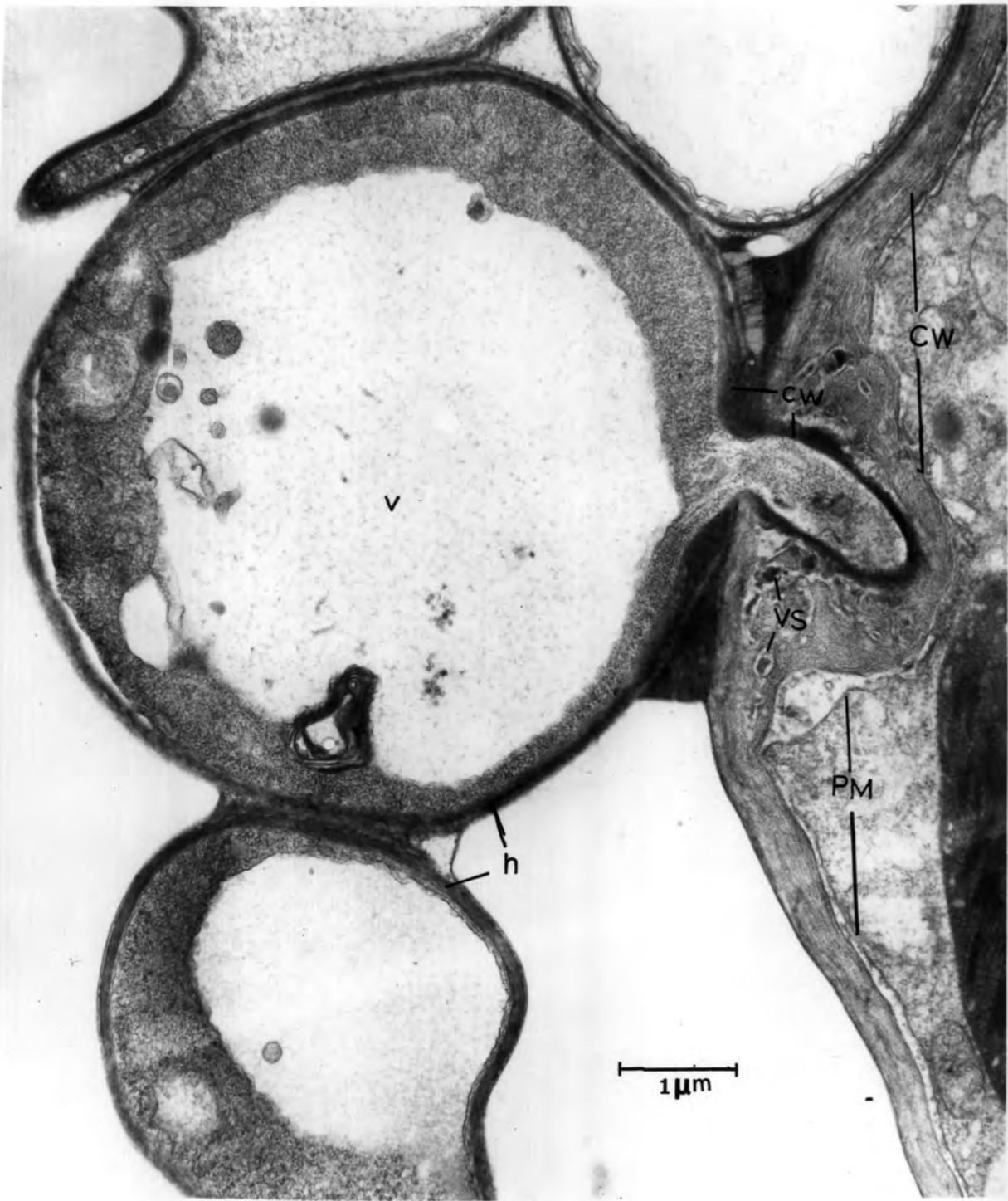


PLATE 14

Electron micrograph illustrating a relatively advanced stage of host cell penetration and the formation of haustorial neck (n). In this stage the host cell wall (CW) is pierced. The cytoplasm and cell wall of intercellular hyphae (h) is continuous with that of the haustorium neck. Note also the presence of dark vesicles (DV) in the area of penetration.

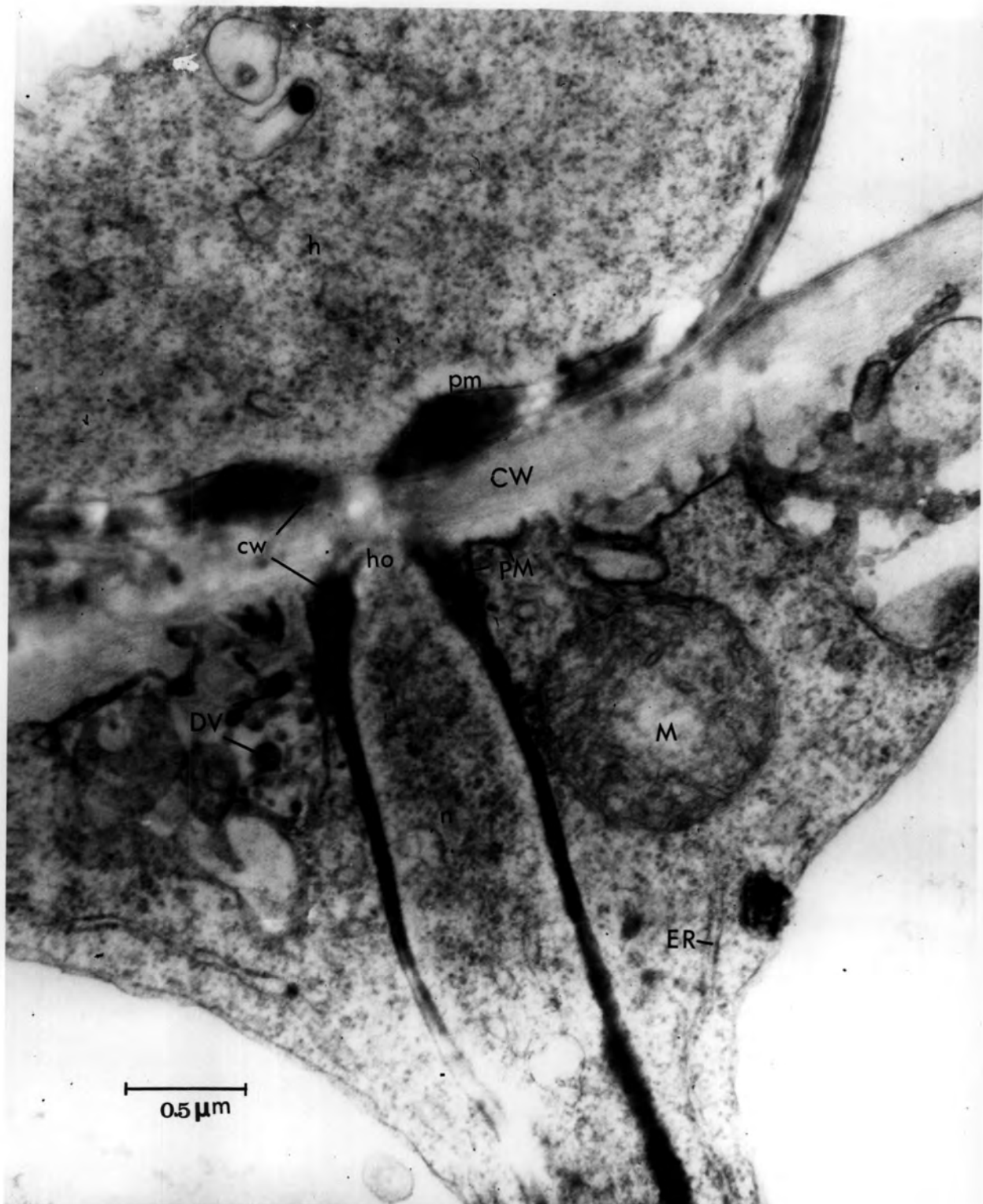


PLATE 15

Electron micrograph showing the last stages in the formation of U. fabae haustorium. A dense ring (dr) can be seen around the middle portion of the haustorium neck.



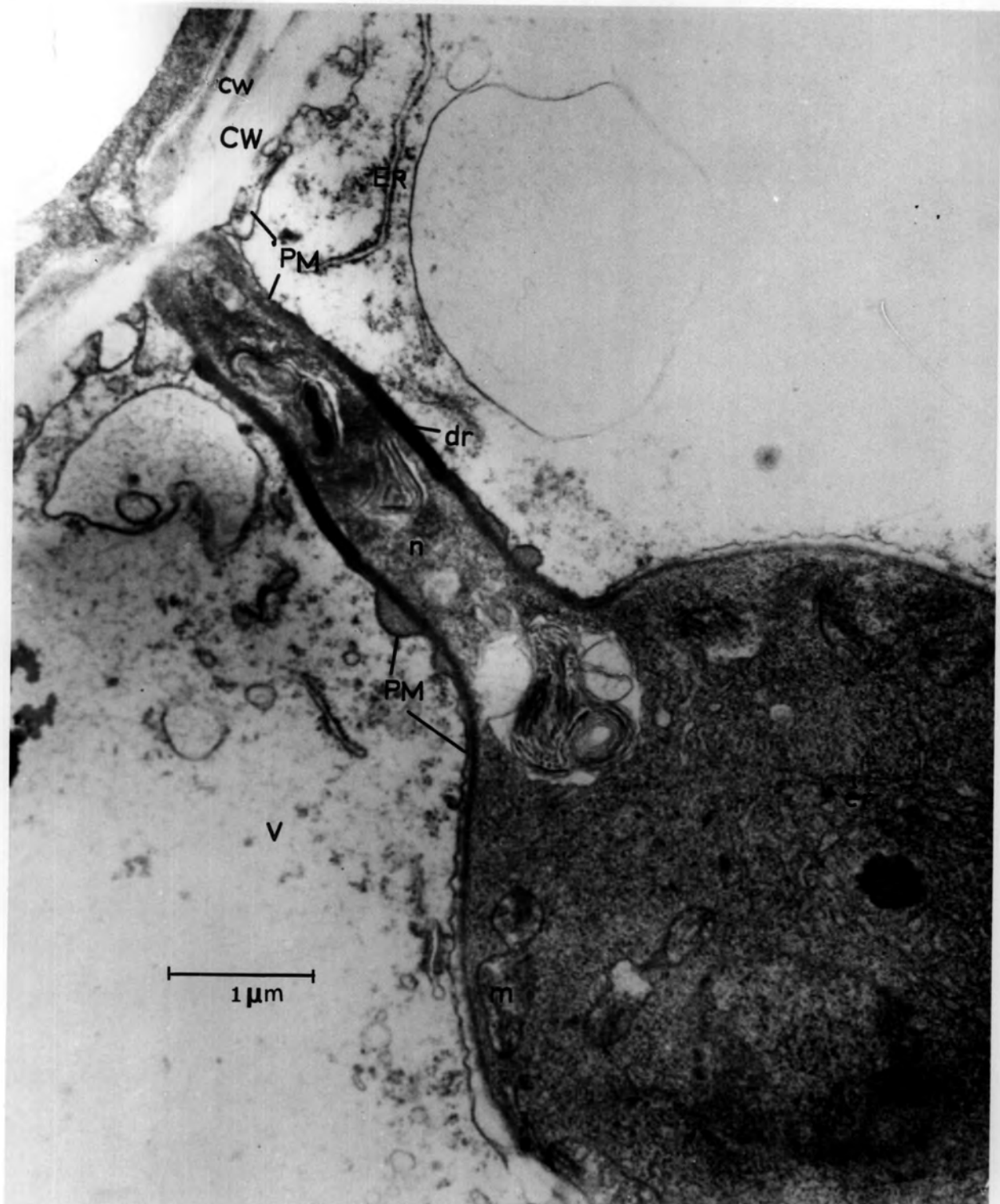


PLATE 16

Electron micrograph showing the physical relationship between host cell components with the 'finger shaped' haustorium of U. fabae.

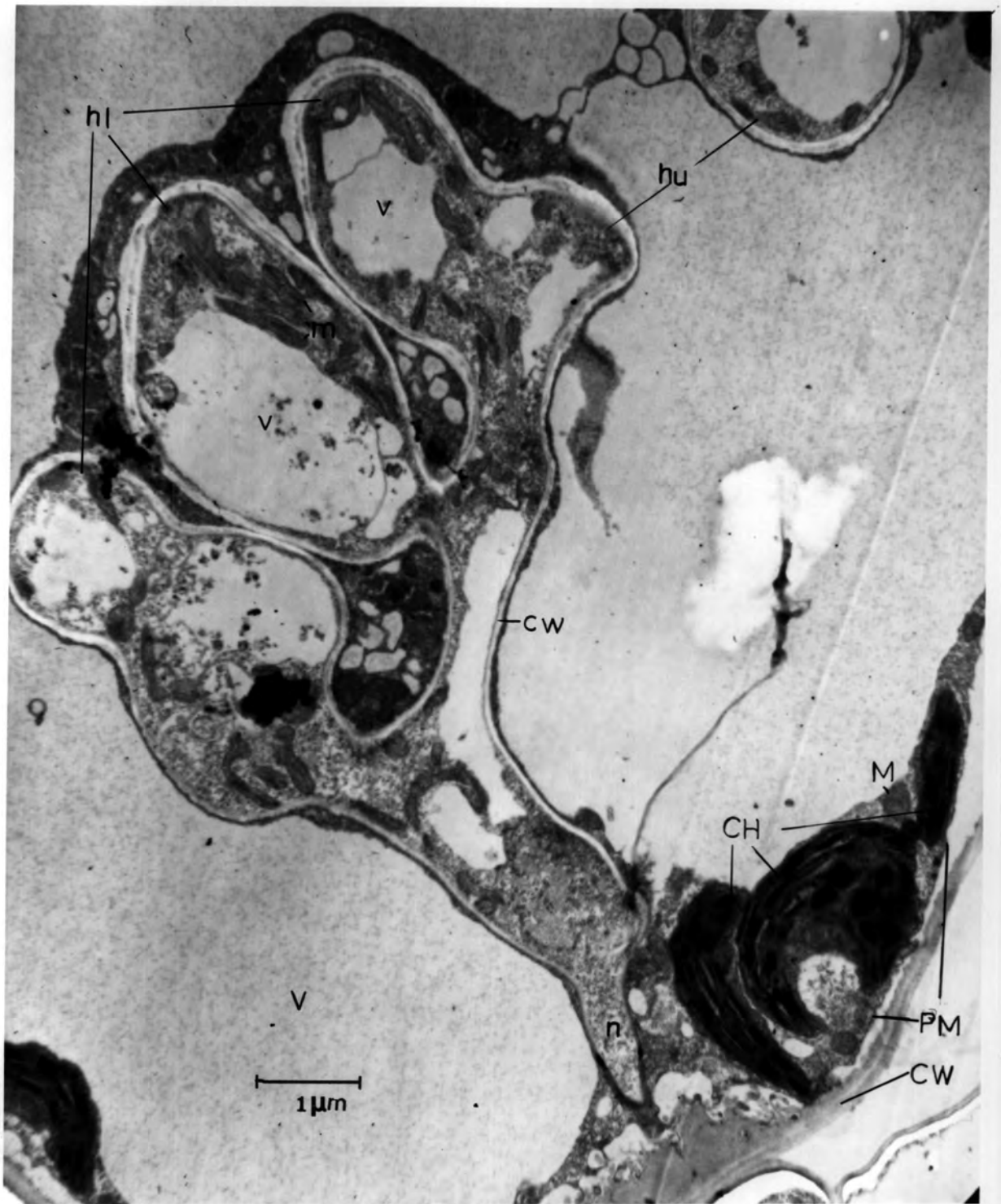


PLATE 17a,b,c

Diagrammatic representation of the early host reaction (production of dark vesicles)(DV) (a) and the method of penetration preceded by host cell wall invagination (b) Plate 17c represents also a diagrammatic interpretation of the relationship between the developed haustorium to the host cell component.

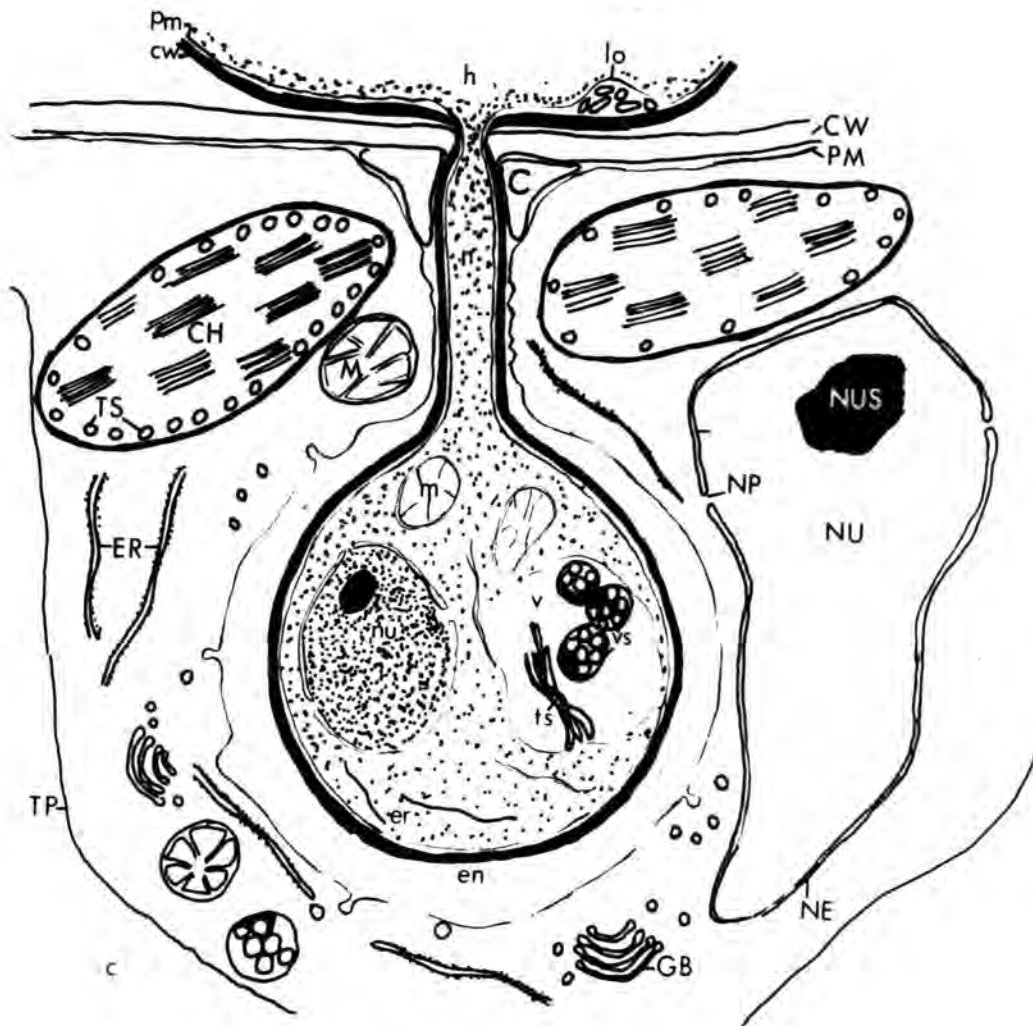
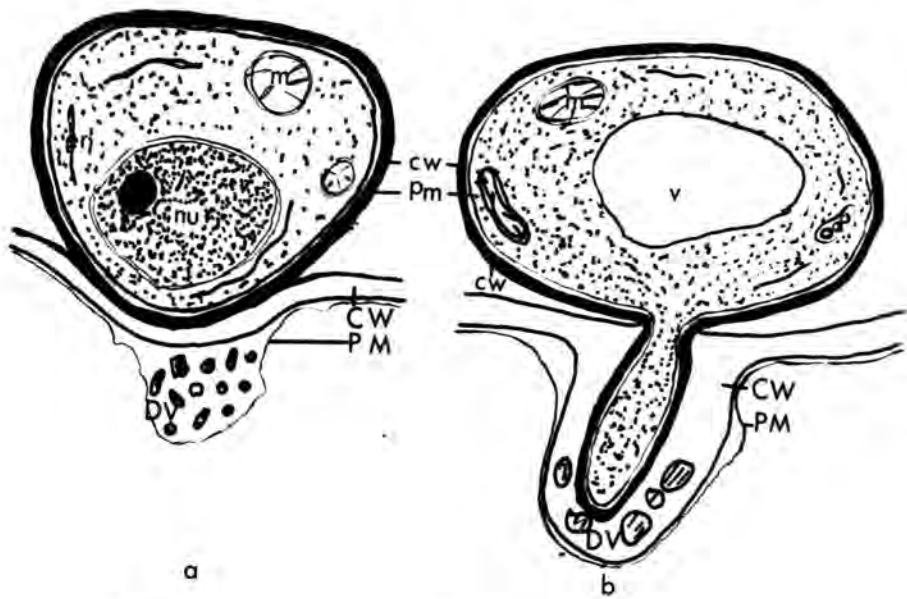
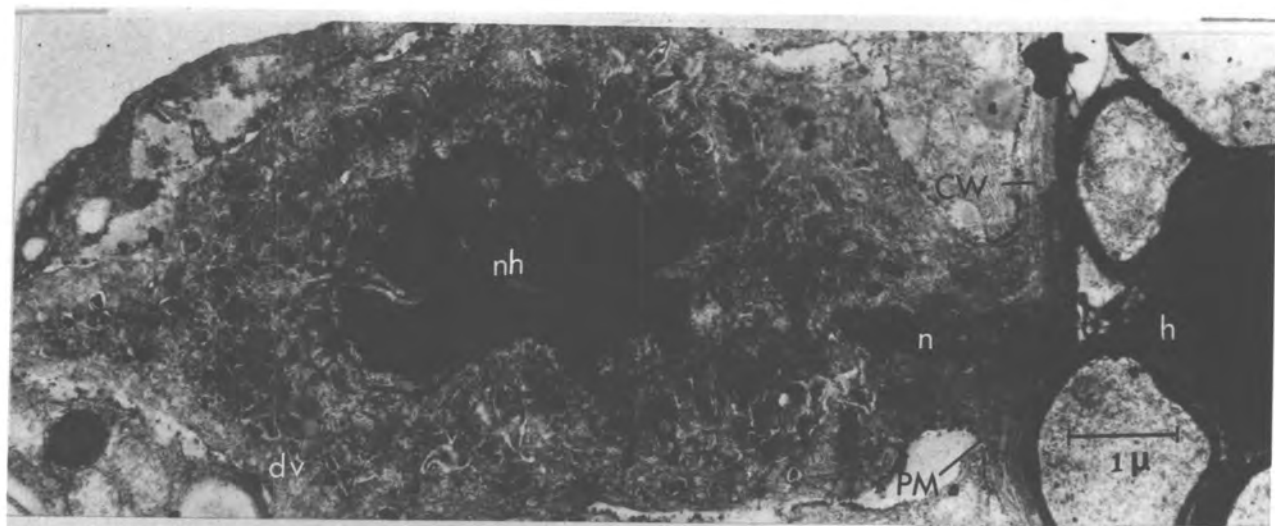
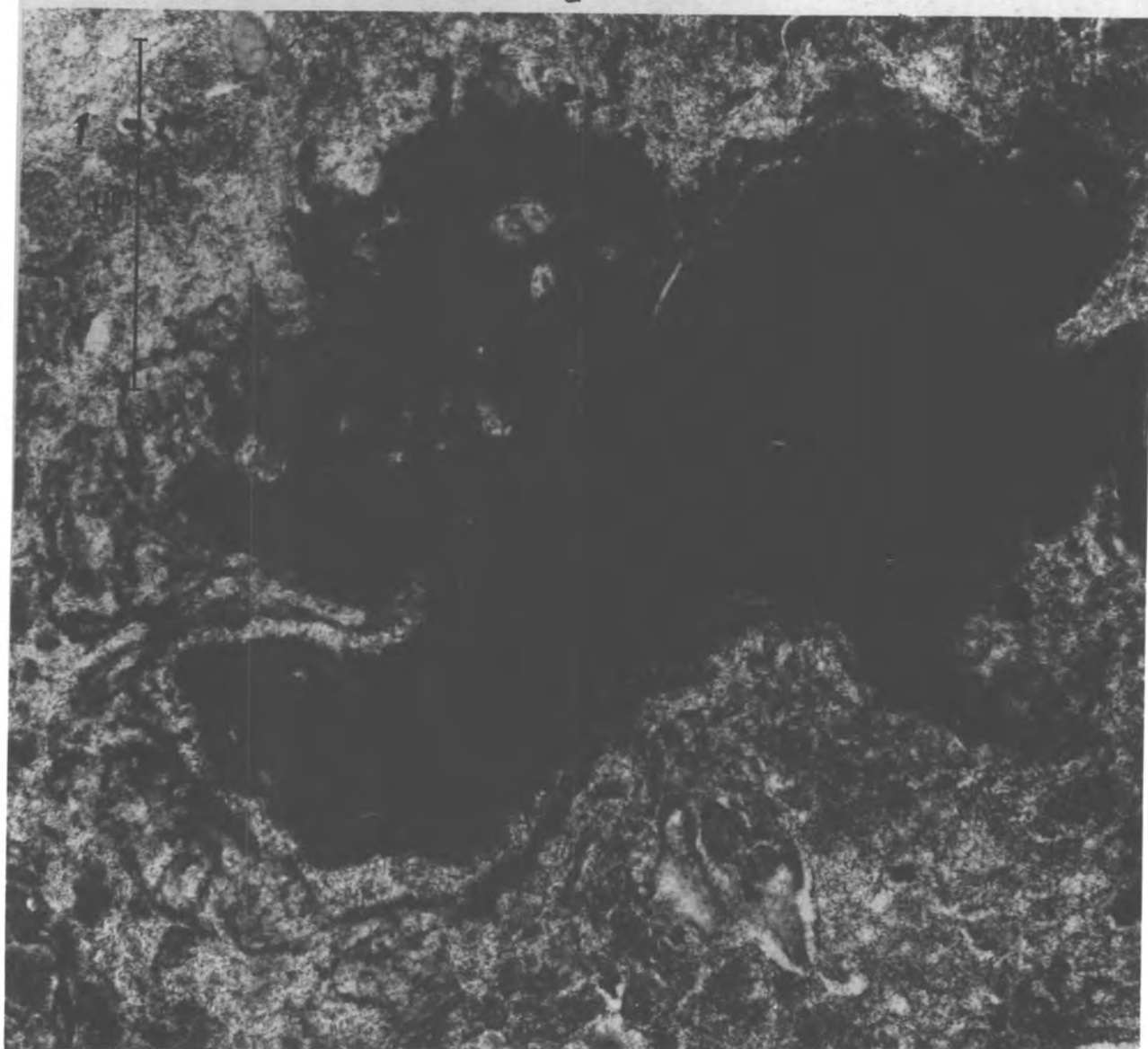


PLATE 18

- (a) Electron micrograph showing the relationship of the necrotic haustorium (nh) to the intercellular hyphae (h). Observe the 'walling off' material and dense vesicles (dv) which are continuous with the host cell wall (CW)
- (b) enlarged view showing the necrotic haustorium body.



a



b

PLATE 19

Electron micrograph of a portion of a mesophyll cell infected with U. fabae, showing the increase in the volume of the host cytoplasm. Also note the presence of three haustorial bodies in host cytoplasmic matrix.



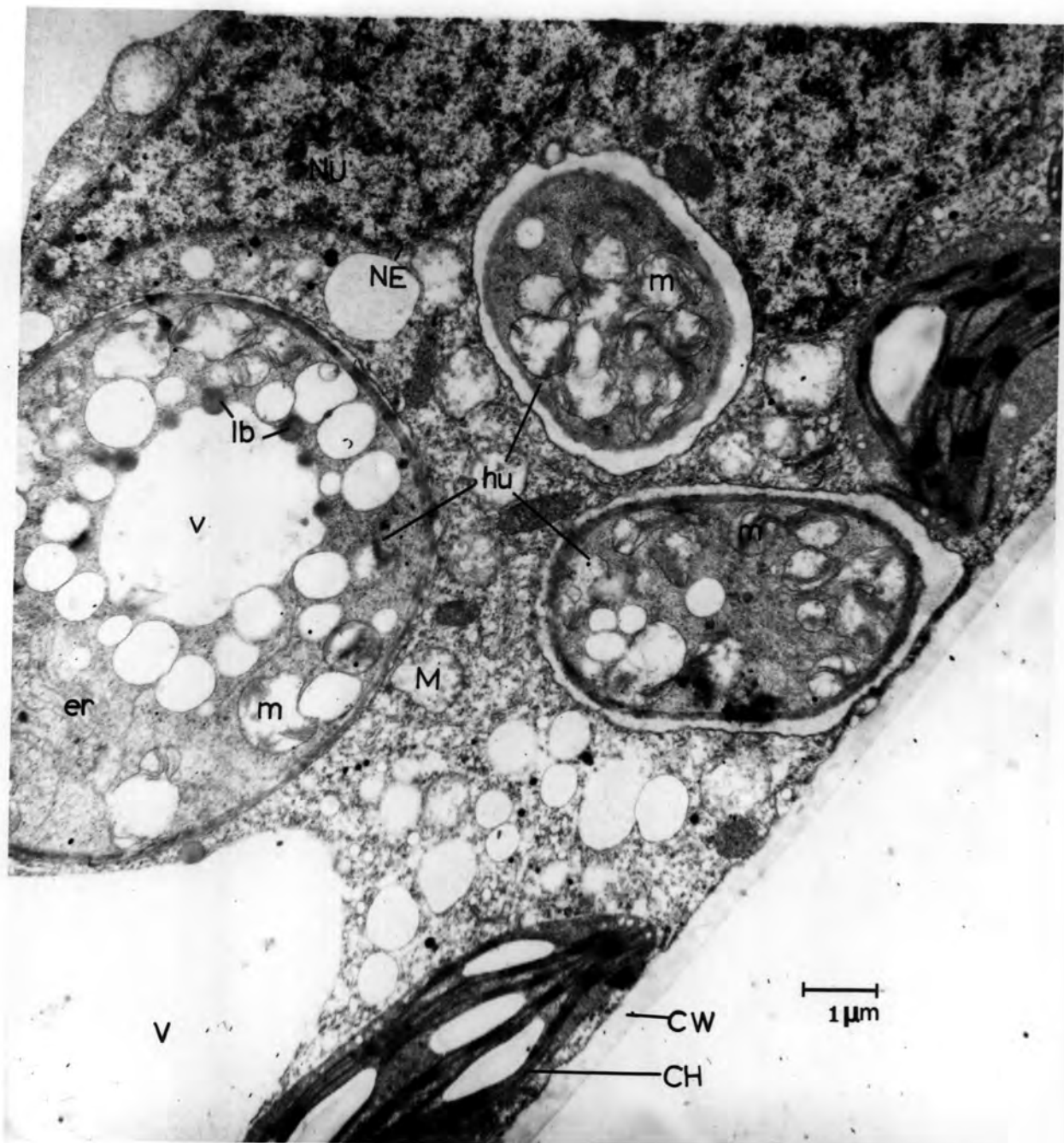
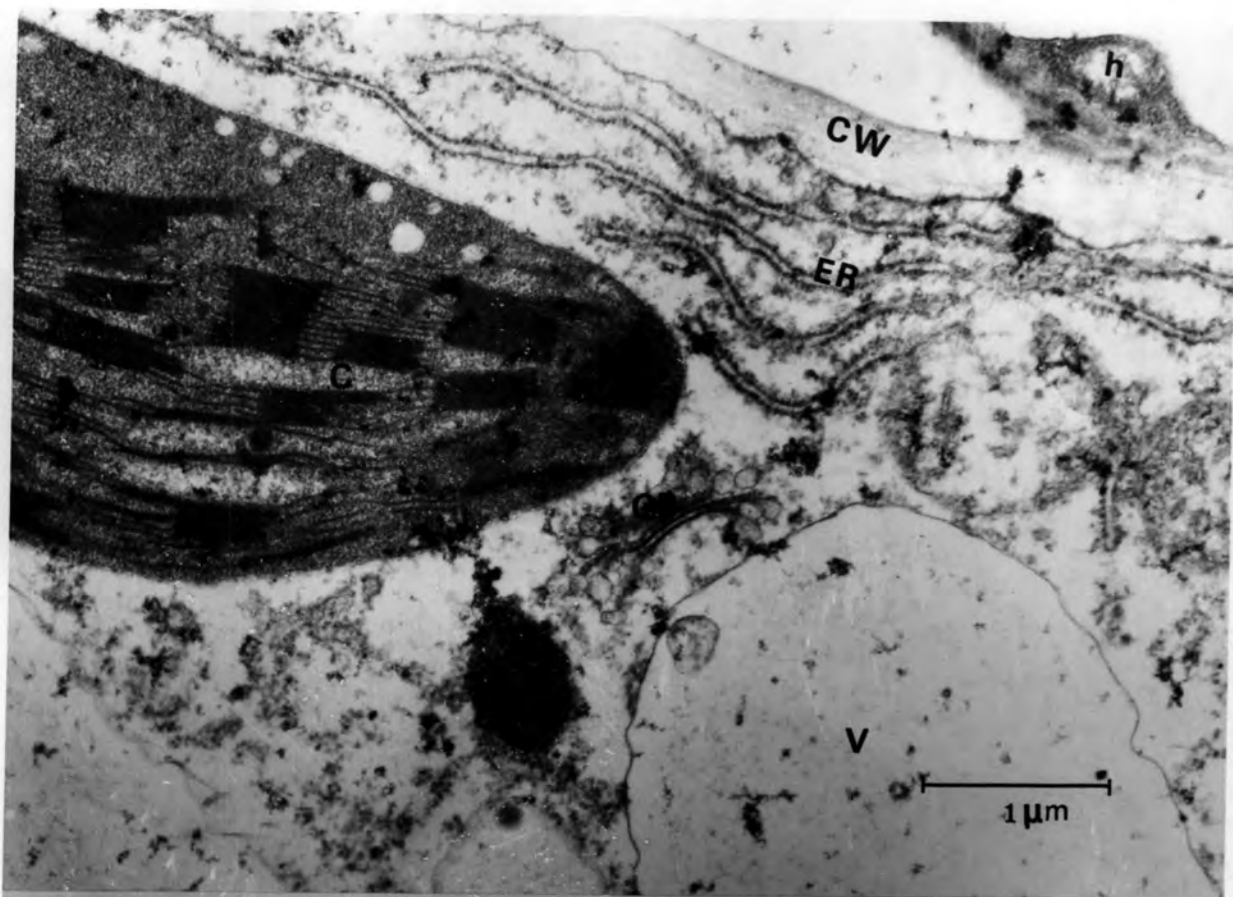


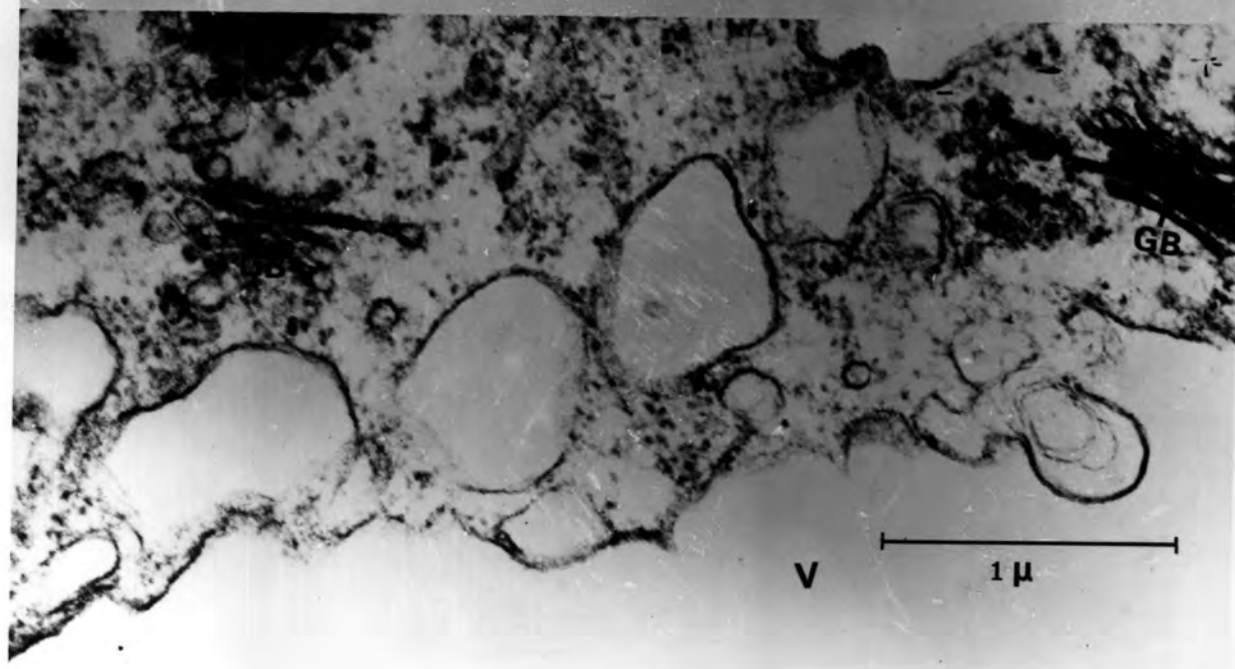
PLATE 20

(a) High power electron micrograph for a mesophyll cell of V. faba infected with U. fabae showing the increased proliferation of host rough endoplasmic reticulum (ER).

(b) shows the presence of host Golgi bodies in the cytoplasm of bean cell infected with U. fabae.



a



b

PLATE 21

Electron micrograph of a portion of a mesophyll cell infected with U. fabae showing both a cross section of the parasitic haustorium and the host response to the infection marked by the increase in the concentration of endoplasmic reticulum (ER). Also note the presence of vesicular structure in host cytoplasm in the vicinity of the haustorium and in the vacuole of the haustorium.

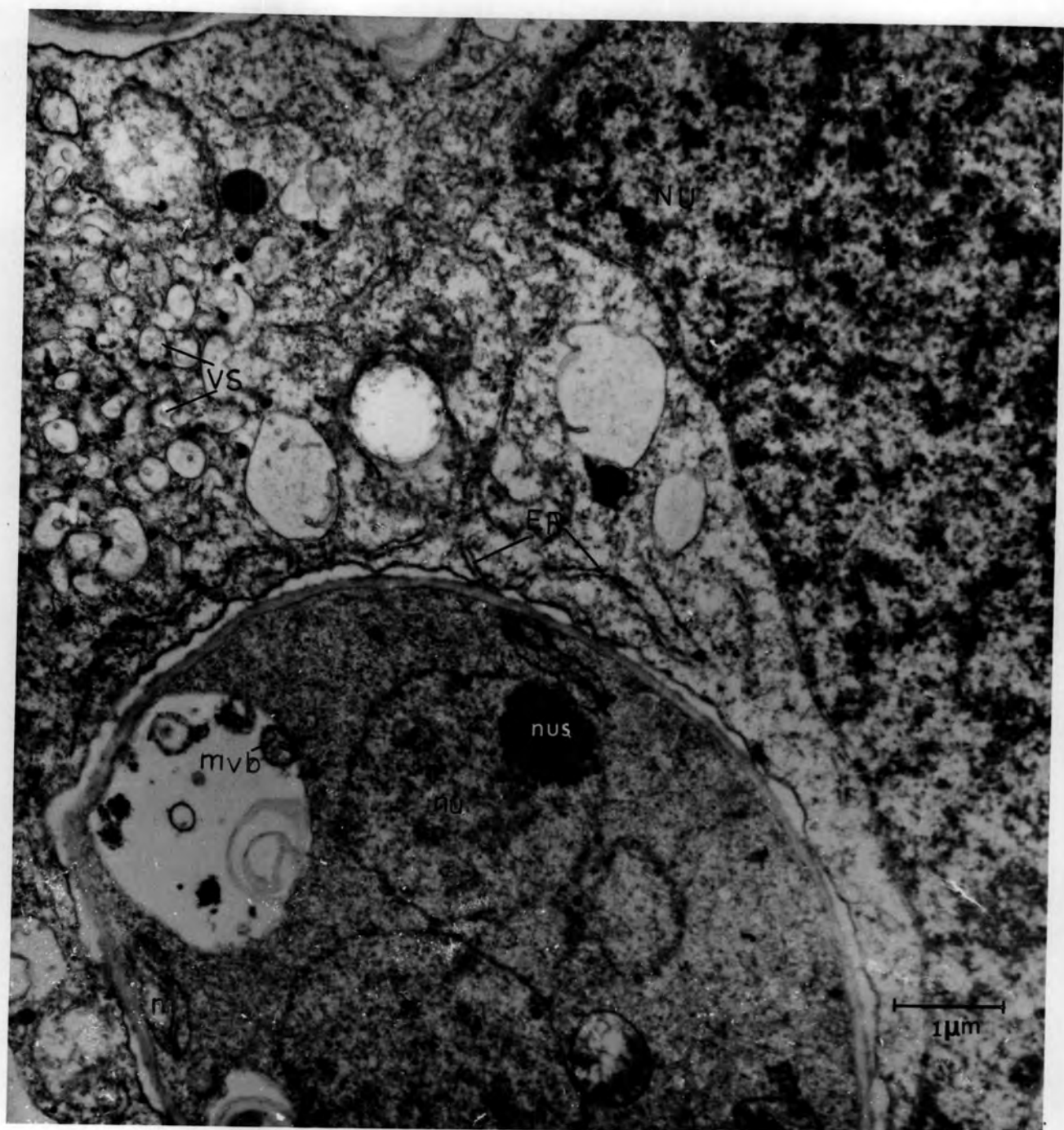


PLATE 22

Cross section of a U. fabae haustorium intruded into the host vacuole but still bound by the host tonoplast.

Observe vesicular structures with electron dense ornamentation in the vacuole of the haustorium. Similar vesicular and tubular structures occur in the host's cytoplasm around the haustorium.

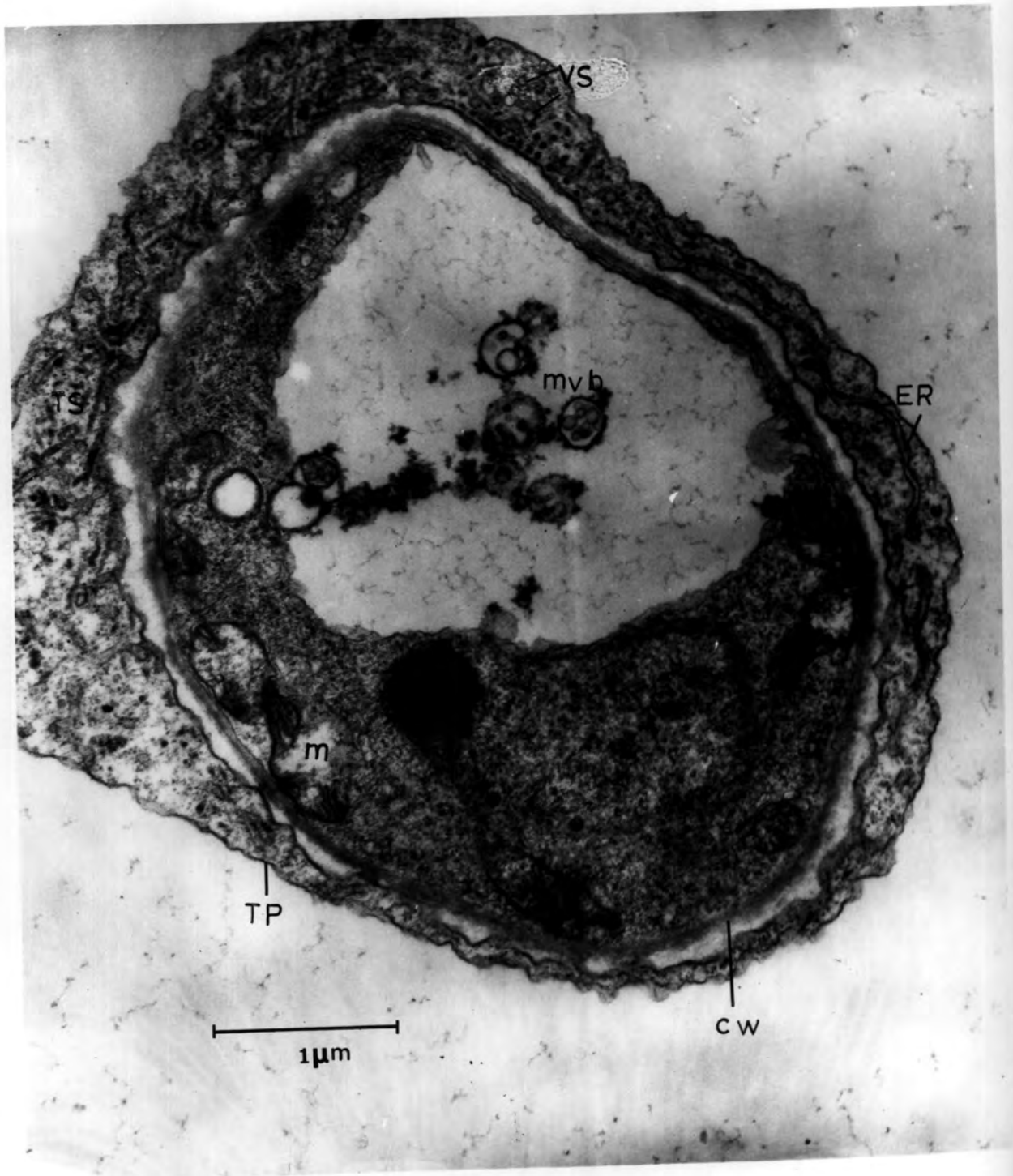


PLATE 23

High power microphotograph of a chloroplast from  
mesophyll cell infected with U. fabae.



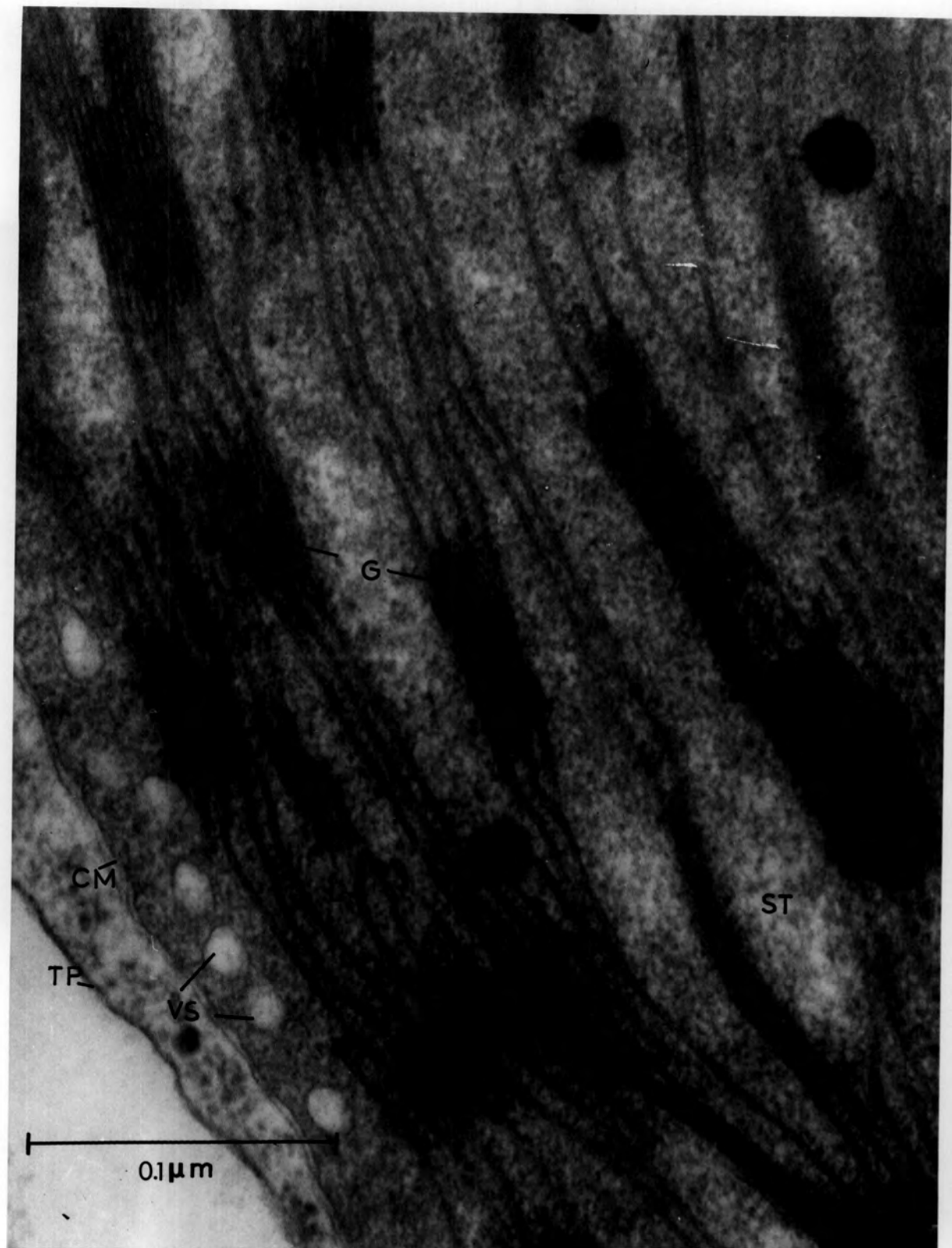


PLATE 24

Electron micrograph of a portion of infected mesophyll cell of V. faba showing some of the effect of U. fabae infection on the fine structure of chloroplasts (i.e. the production of vesicles from the inner chloroplastic membrane).

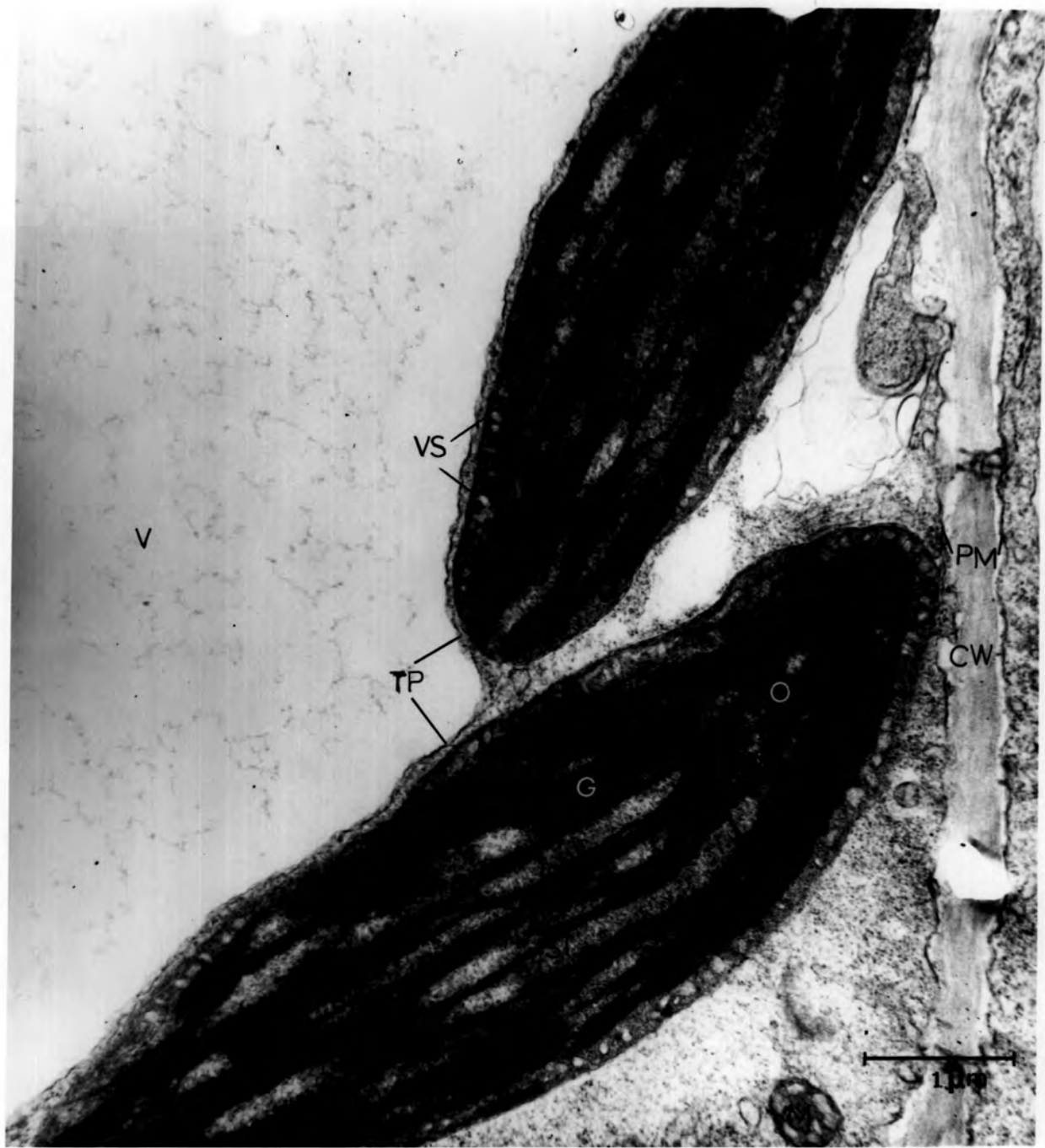


PLATE 25

Low power electron micrograph showing the general appearance and disorganisation of host cytoplasm (Arrows) as a result of U. fabae infection (20 days after infection). Also note the reduction in the size of chloroplasts. At this stage fungal senescence can also be observed.

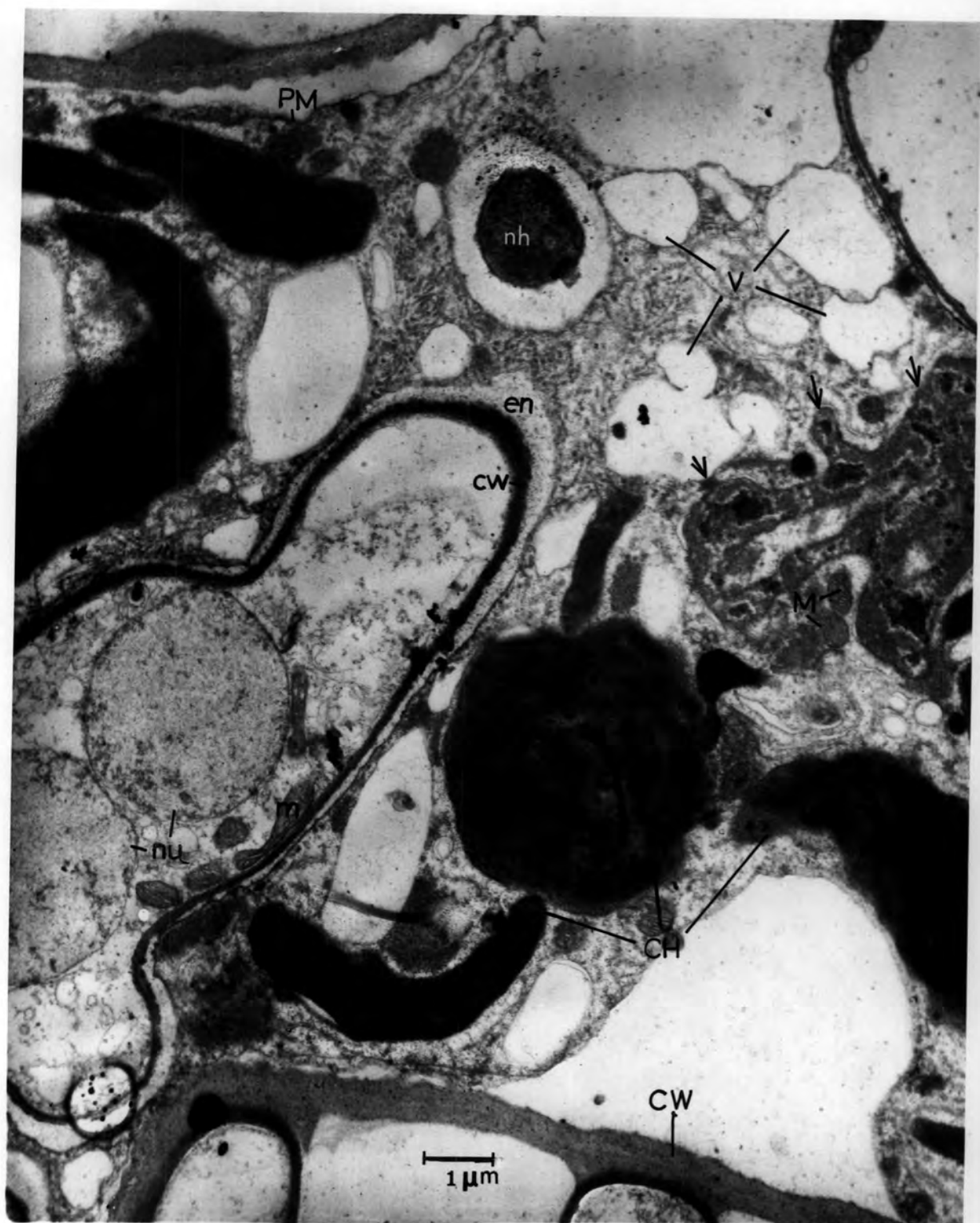


PLATE 26

High power Electron micrograph showing the fine structure of chloroplast from mesophyll cell infected with U. fabae (20 days after infection). Note the presence of vesicular aggregate in such chloroplasts.



PLATE 27

Portion of a mesophyll cell infected with U. fabae showing the formation of vesicles and curved extrusion from the chloroplasts. Also observe the production of lomasomes (lo) in the intercellular hyphae (h).





PLATE 28

Electron micrograph of a mesophyll cell infected with U. fabae showing some fine structural changes taking place in the host nucleus. Observe the size of the nucleus and the nucleolus.

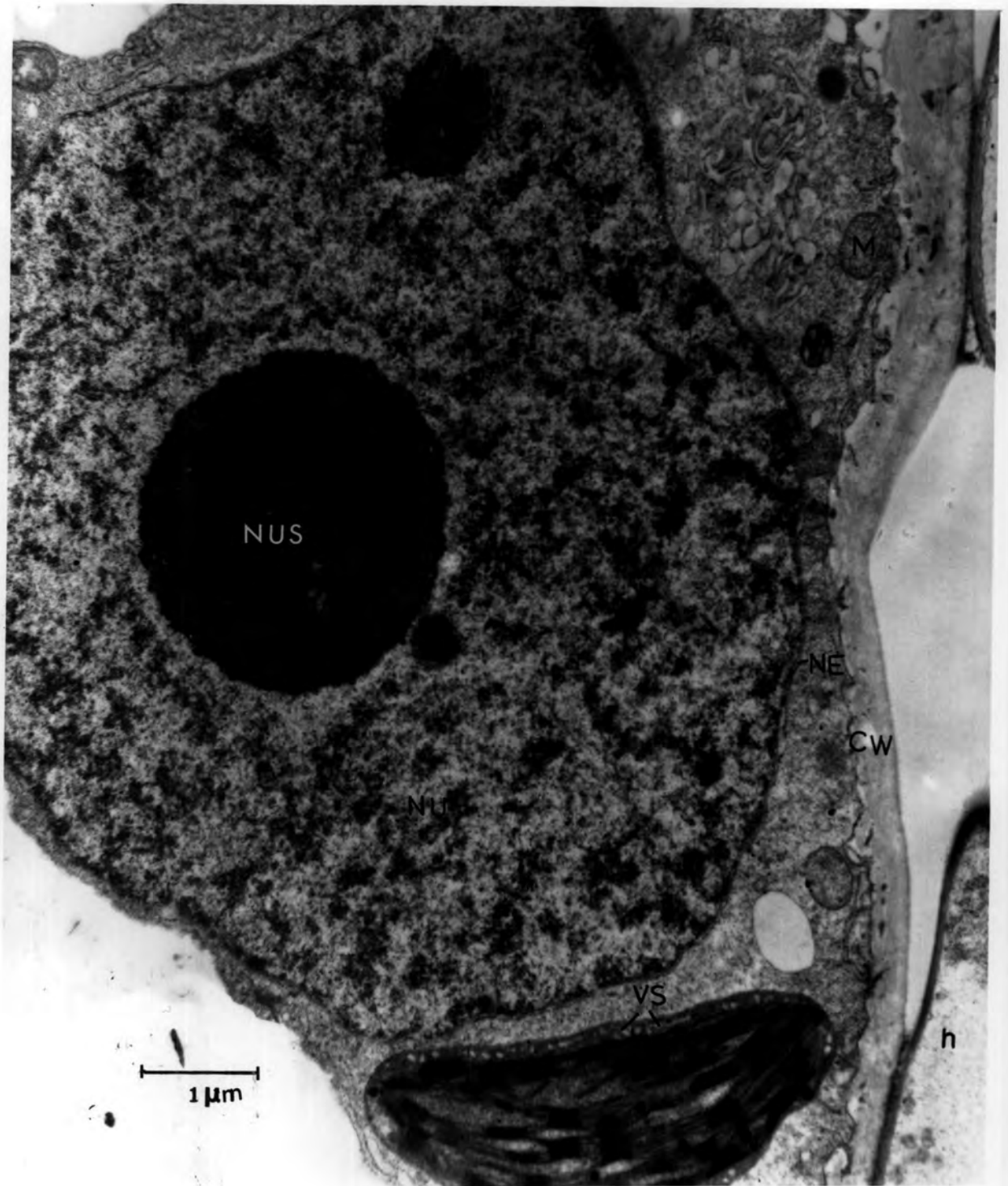


PLATE 29

A high power Electron micrograph of a nucleus from U. fabae infected mesophyll cell of bean showing the distribution and proportion of the chromatin (CR) and the interchromatin (ICR) regions.

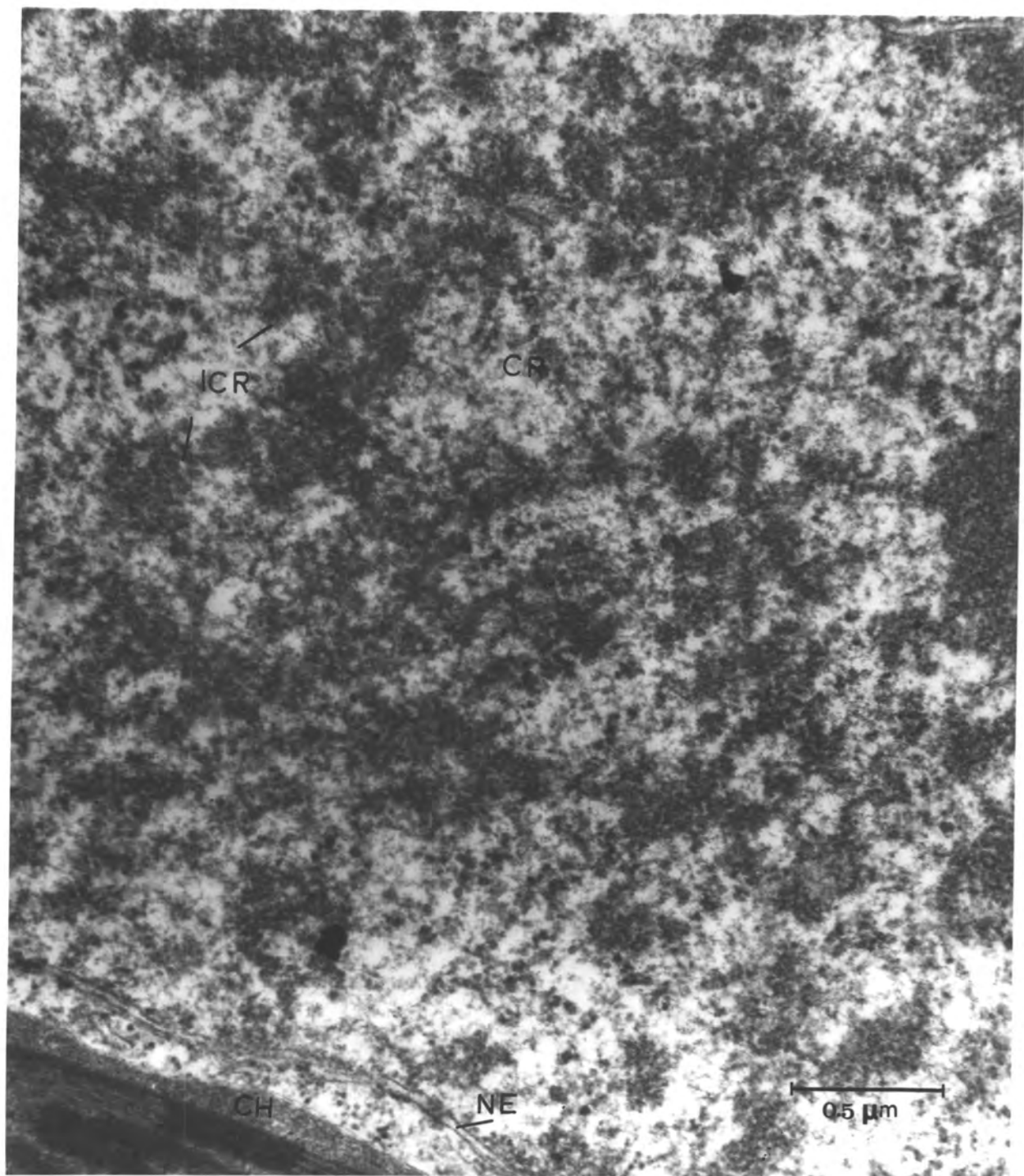


PLATE 30

Electron micrograph showing a necrotic haustorium (nh) which lacks the walling off material. Note that the host cytoplasm is unaffected. (Compare with haustorium in Plate 18a,b).

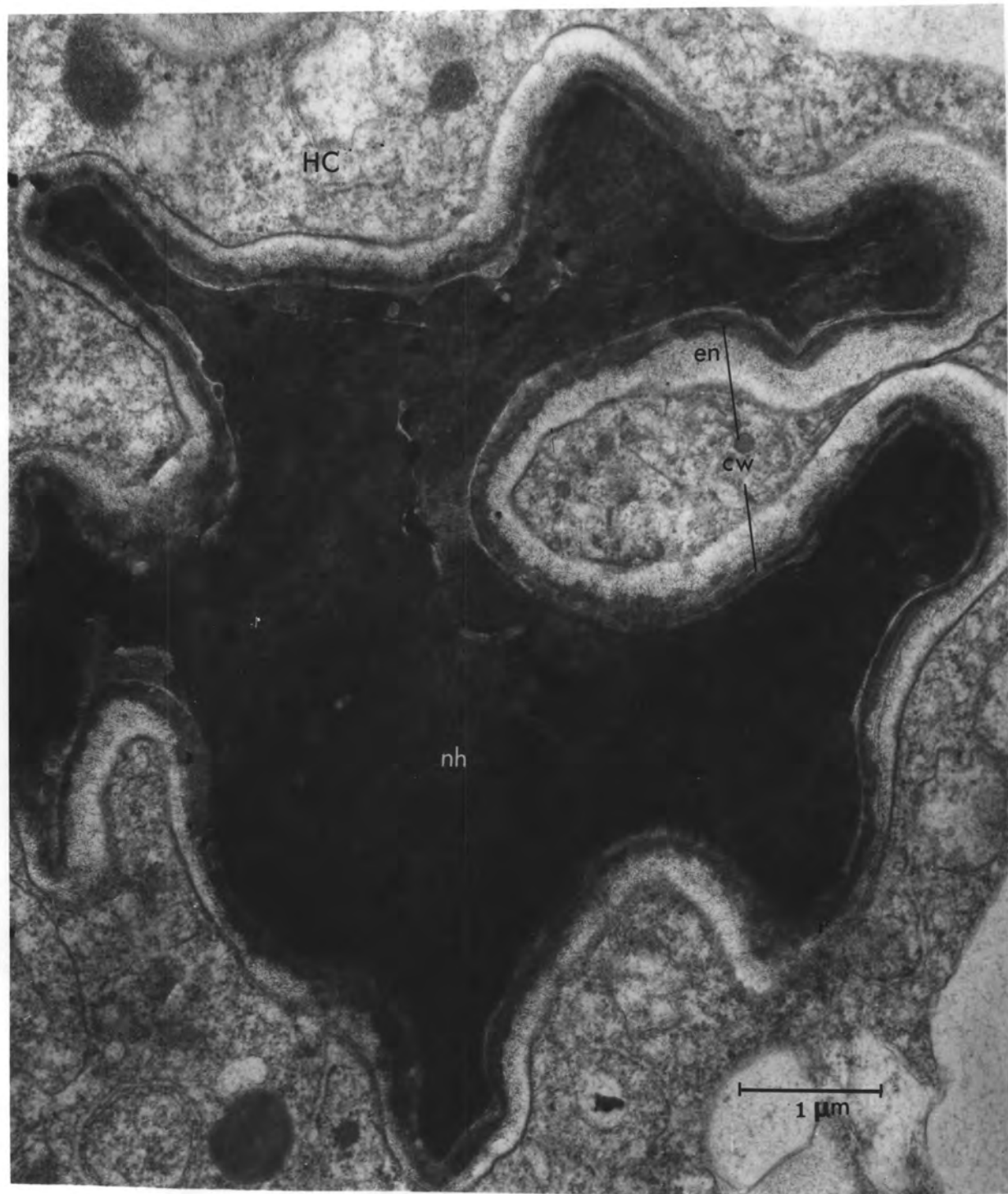


PLATE 31

Electron micrograph showing some of the configuration and the fine structure of the haustorium of U. fabae in relation to the host cytoplasm. Note the presence of thick layer of encapsulation (en) surrounding the haustorial bodies.





PLATE 32

Electron micrograph of a cylindrical shaped haustorium of U. fabae containing two nuclei. Observe the connection between the haustorium cytoplasm and electron dense vesicular structure in the vacuole.

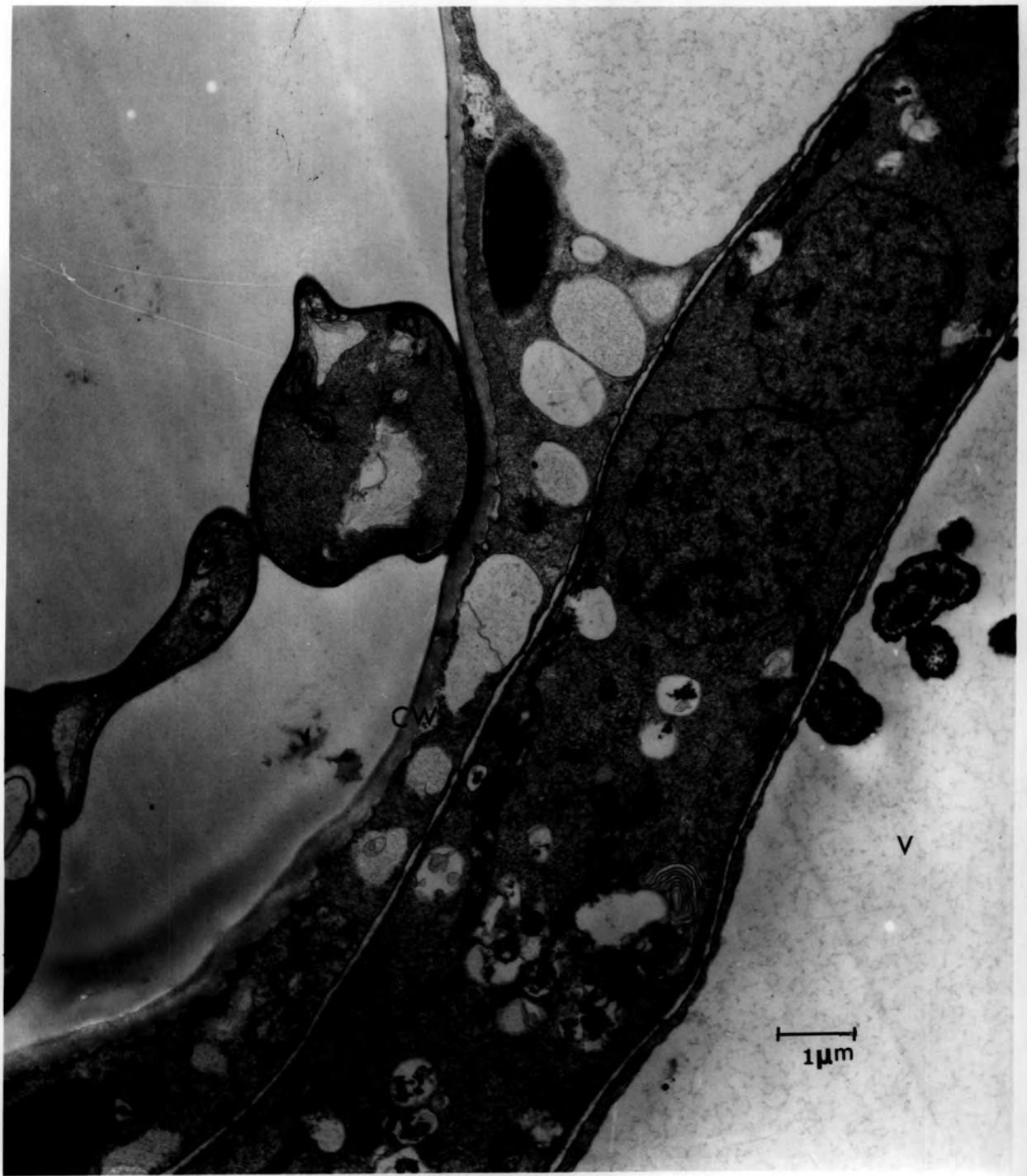
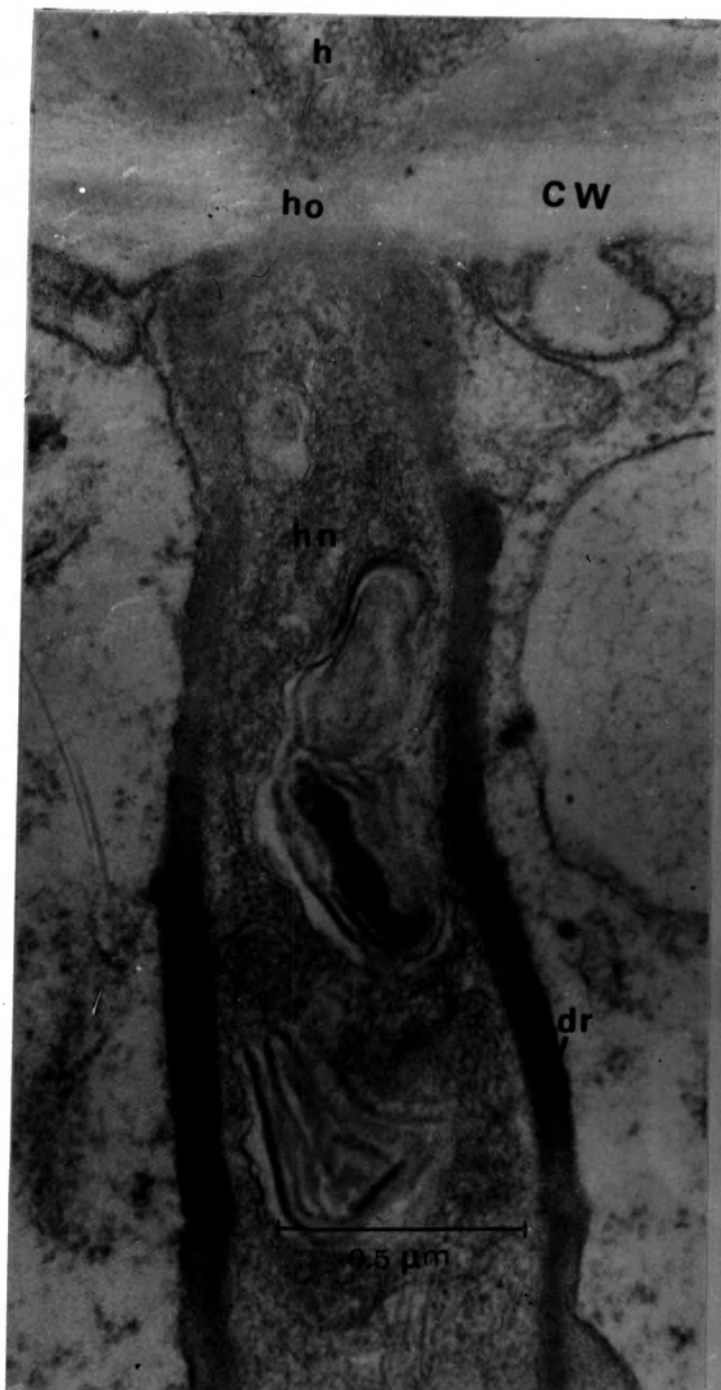


PLATE 33a,b

Electron micrographs of two haustorial necks (hn) of U. fabae haustoria. Note the presence of Electron dense ring (dr) in the middle part of the neck in Plate 33a and the absence of such structure in the neck in Plate 33b.



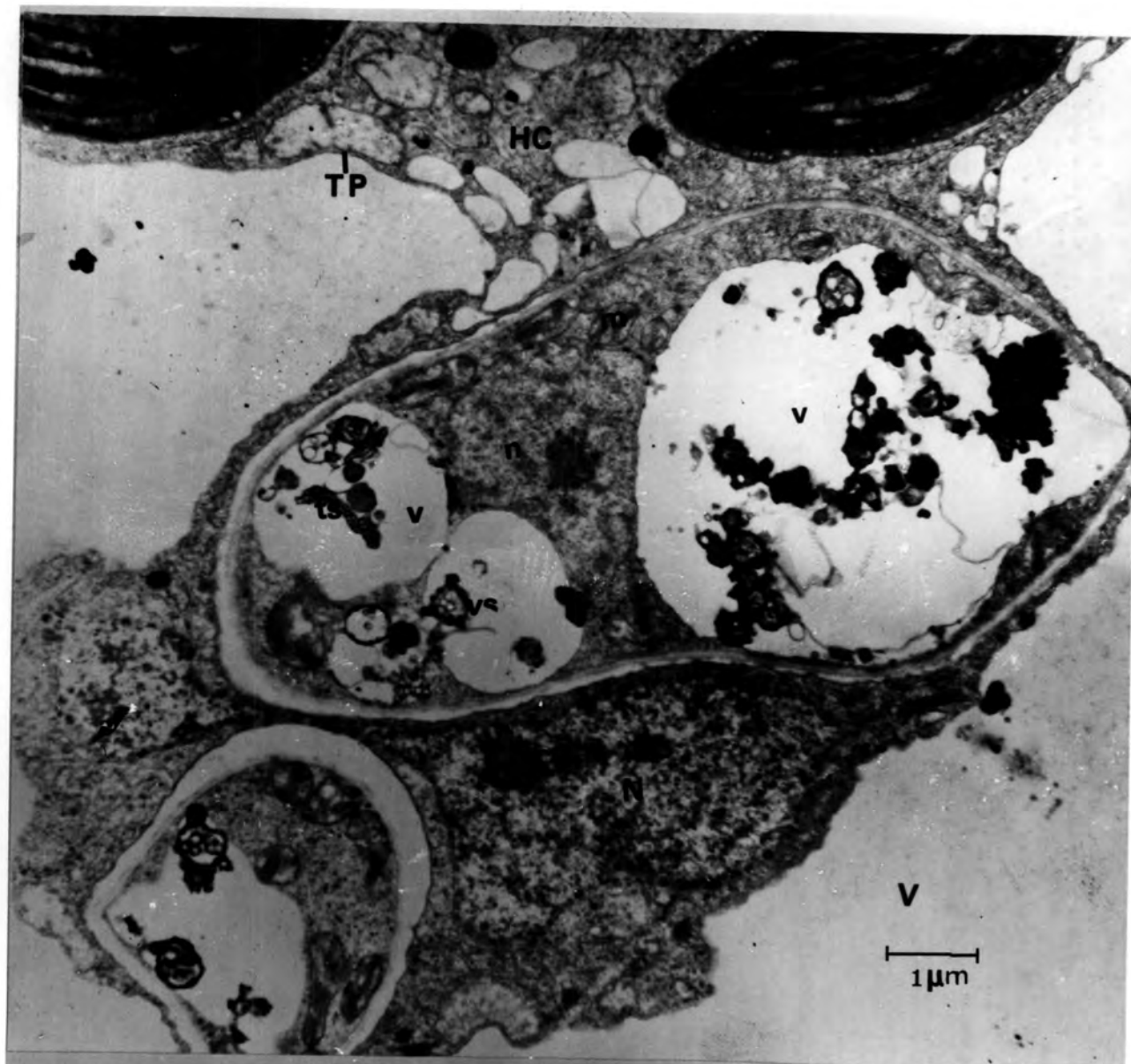
a



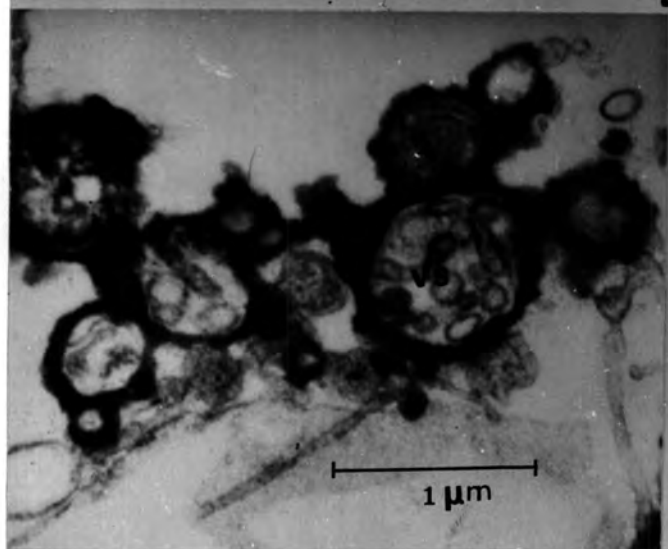
b

PLATE 34

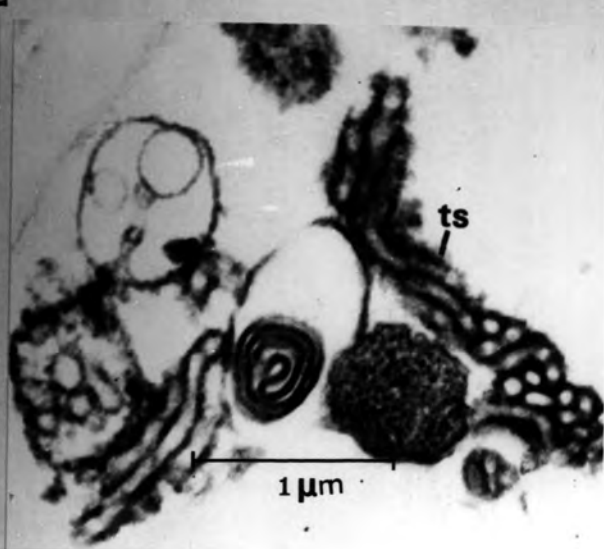
- (a) haustoria of *U. fabae* growing in the host cytoplasm (HC) showing the presence of vesicular and tubular structures in the vacuoles of these haustoria.
- (b) high power view of the vesicular type structure with electron dense coating
- (c) shows the tubular type of these structures.



a



b



c

PLATE 35

Electron micrograph of two mesophyll cells infected  
with U. fabae showing the physical relationship between  
the intercellular hyphae (ih) and the host cells



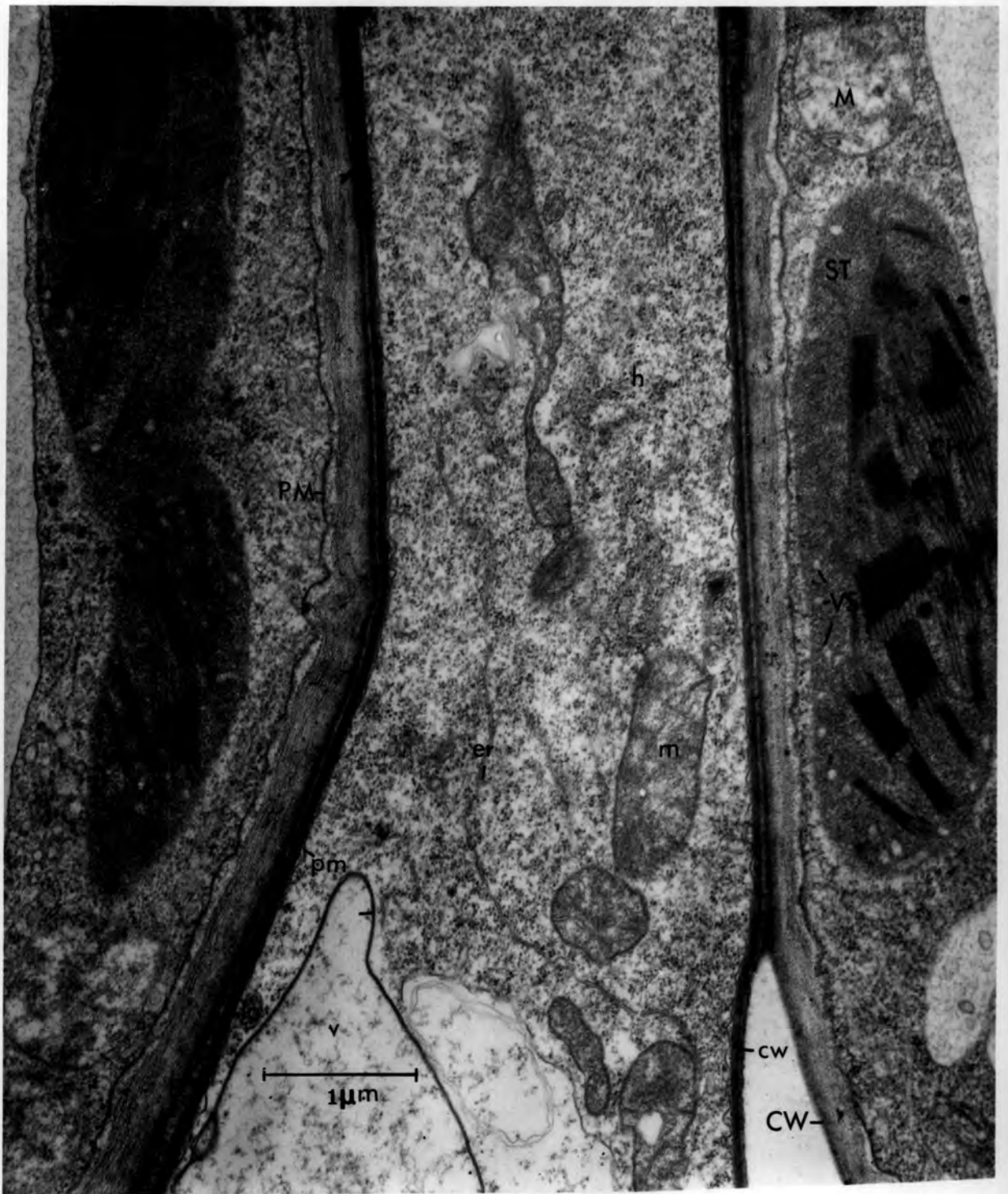


PLATE 36

Part of an intercellular mycelium of U. fabae  
showing the general organisation and the fine  
structure of the intercellular hyphae.

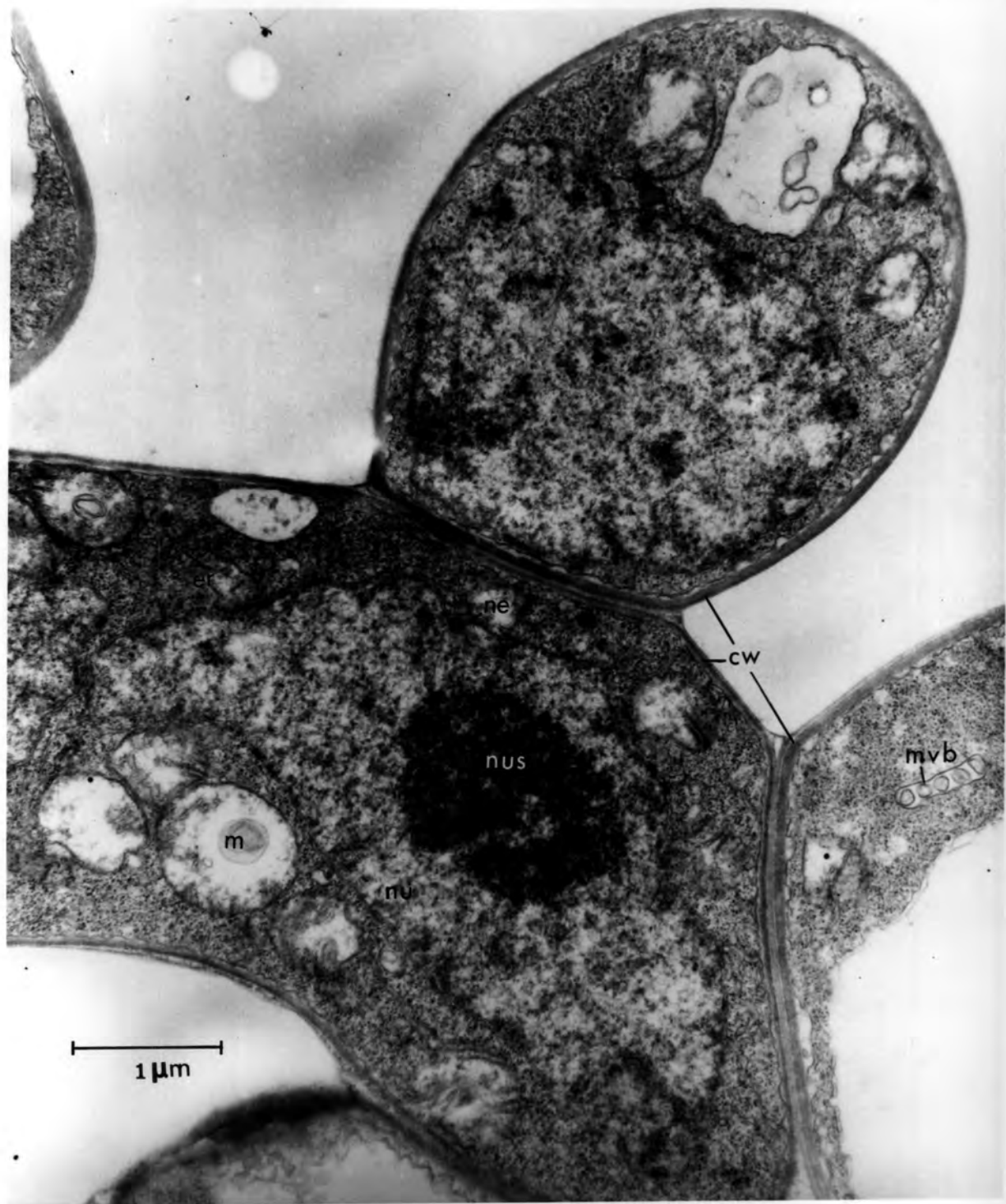


PLATE 37

Part of a mesophyll cell infected with B. fabae showing one of the earlier stages of host cell invasion. Note the invagination of host cell wall. Also the beginning of necrosis of the host cytoplasm.

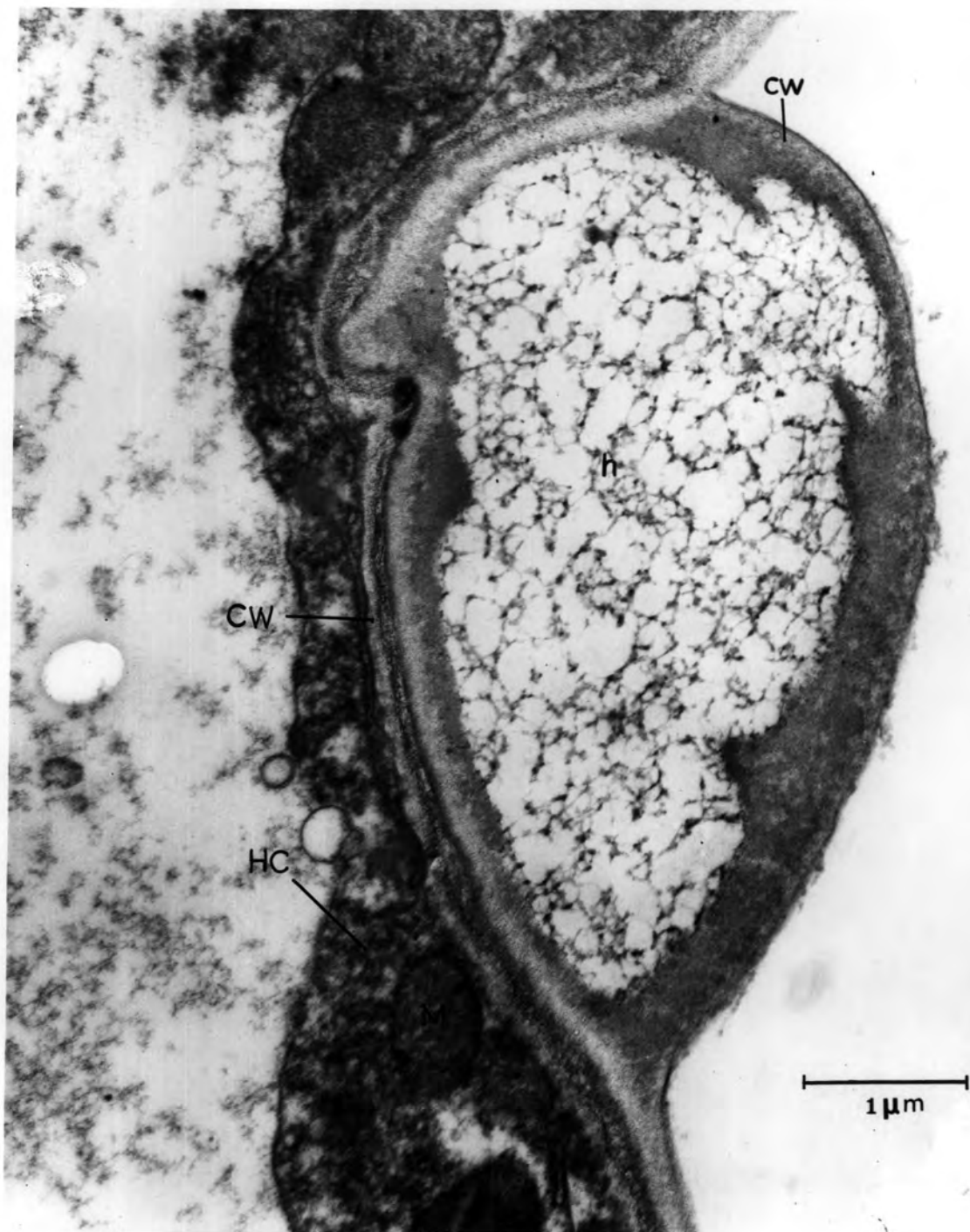


PLATE 38

Electron micrograph of a portion of bean mesophyll cell invaded by B. fabae mycelium. Observe the breakdown of host cell opposite to the fungal mycelium (ih) and the production of vesicles by the fungus presumably responsible for host cell wall hydrolysis.

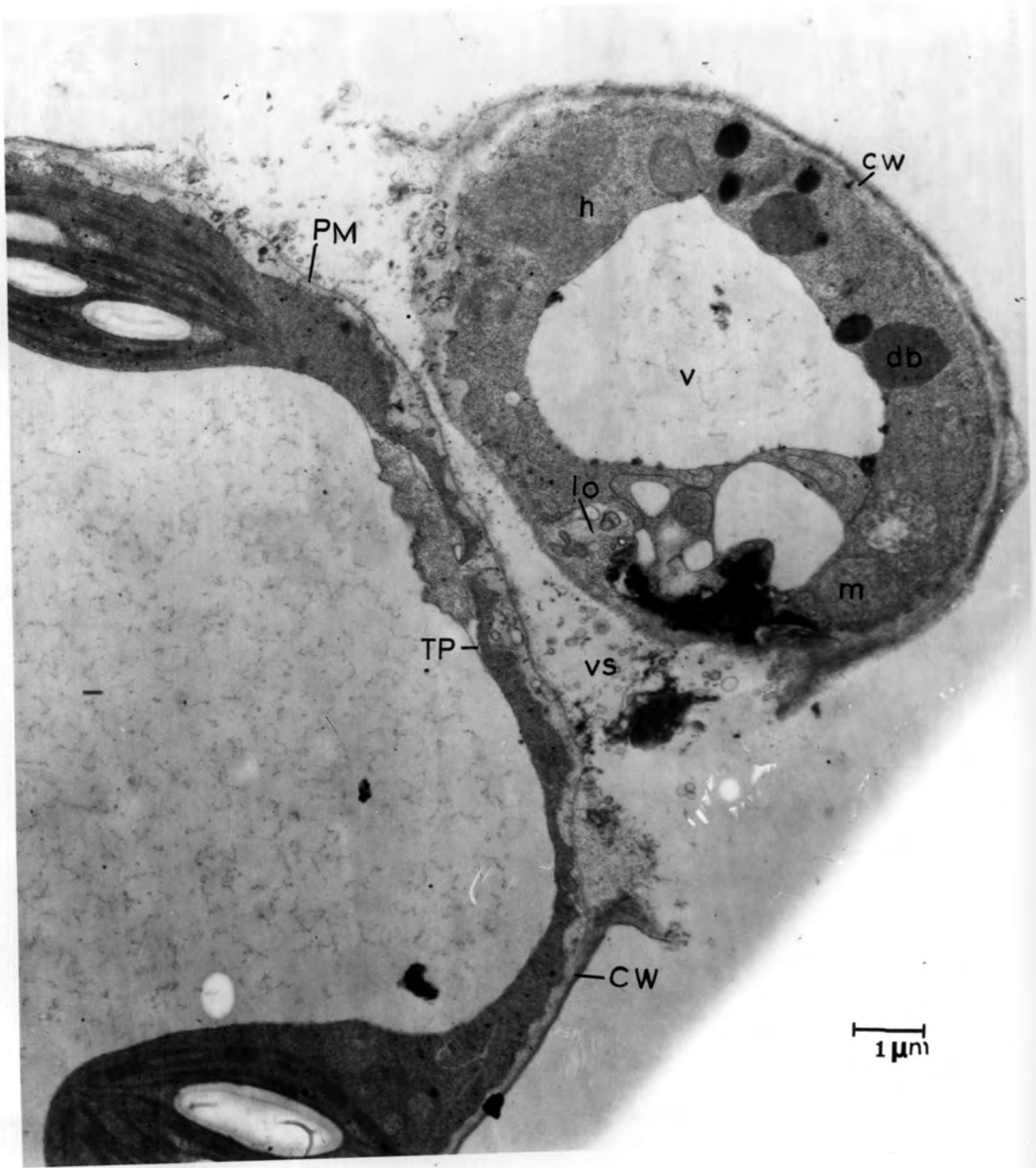
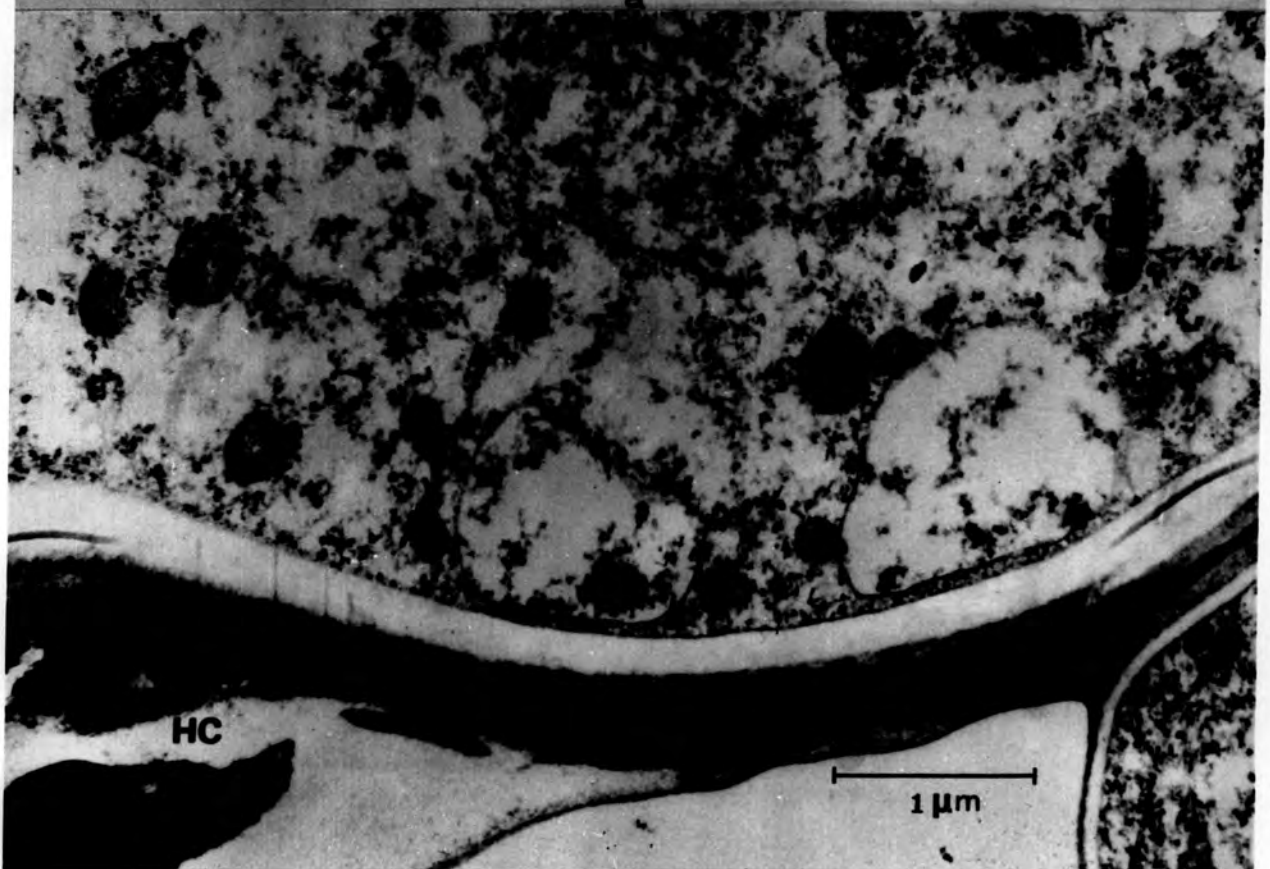
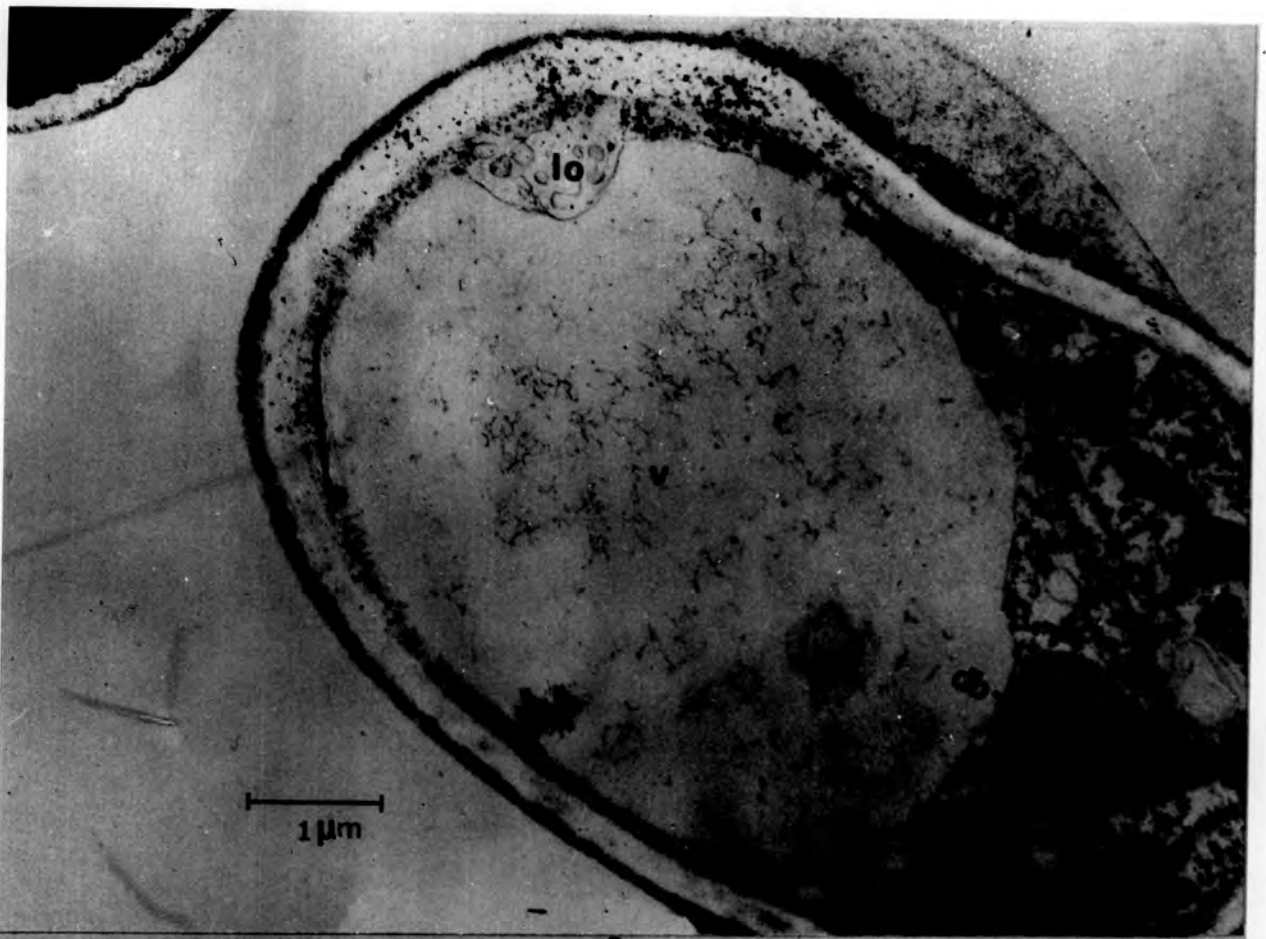


PLATE 39a,b

Electron micrograph of the intercellular mycelium of B. fabae growing on the host tissues. Note the presence of lomasome-like structures in Plate 39a and the killing and blackening of the host cytoplasm (HC) in Plate 39b.

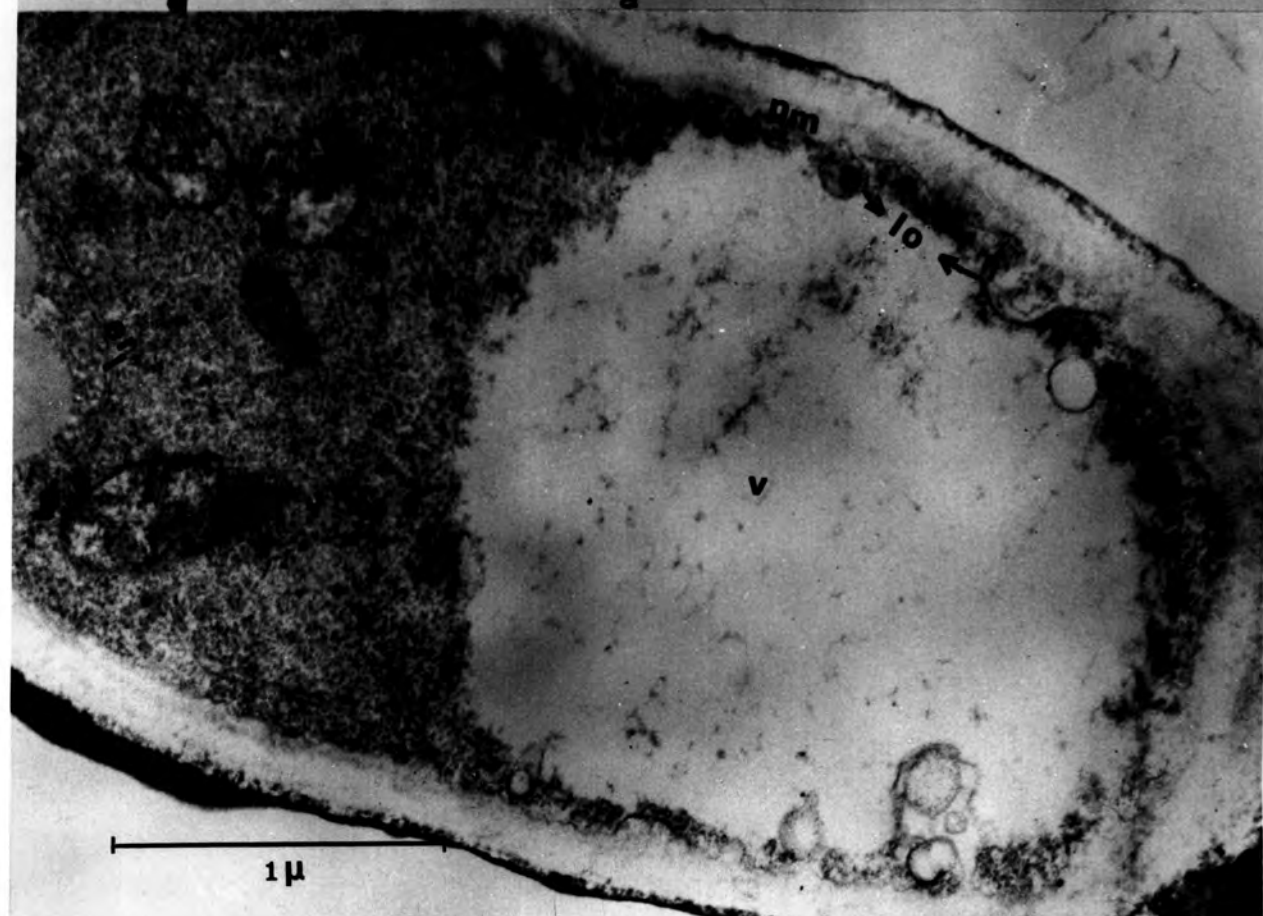
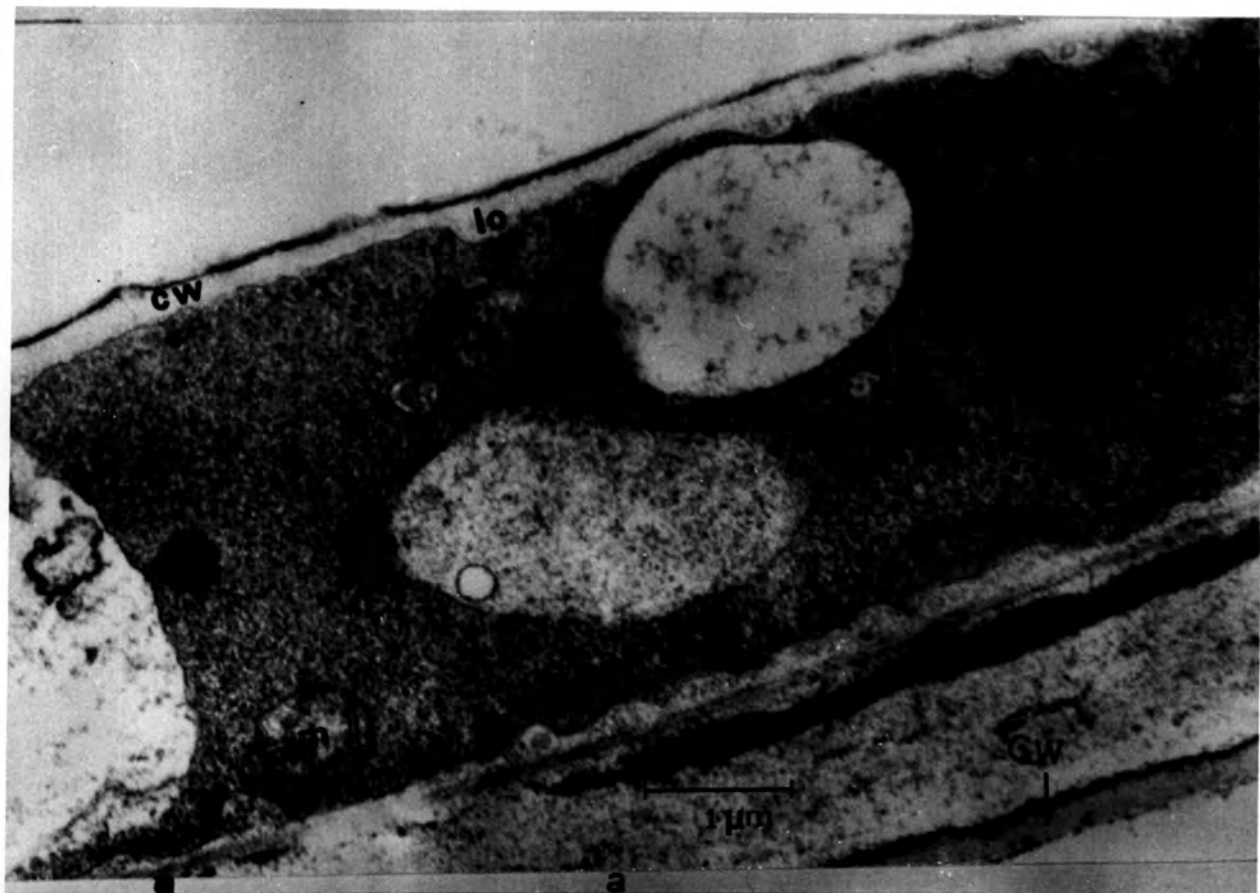




b

PLATE 40a,b

Electron micrograph of the intracellular mycelium of B. fabae. Note the blackening of host cytoplasm in Plate 40a, and the production of lomasome-like structures in hyphae in Plate 40a,b.



**b**

PLATE 41

Electron micrograph representing the final stage in host cell penetration by B. fabae. Note the complete killing and blackening of the host cytoplasm. Also note the vasculature of the invading fungus.

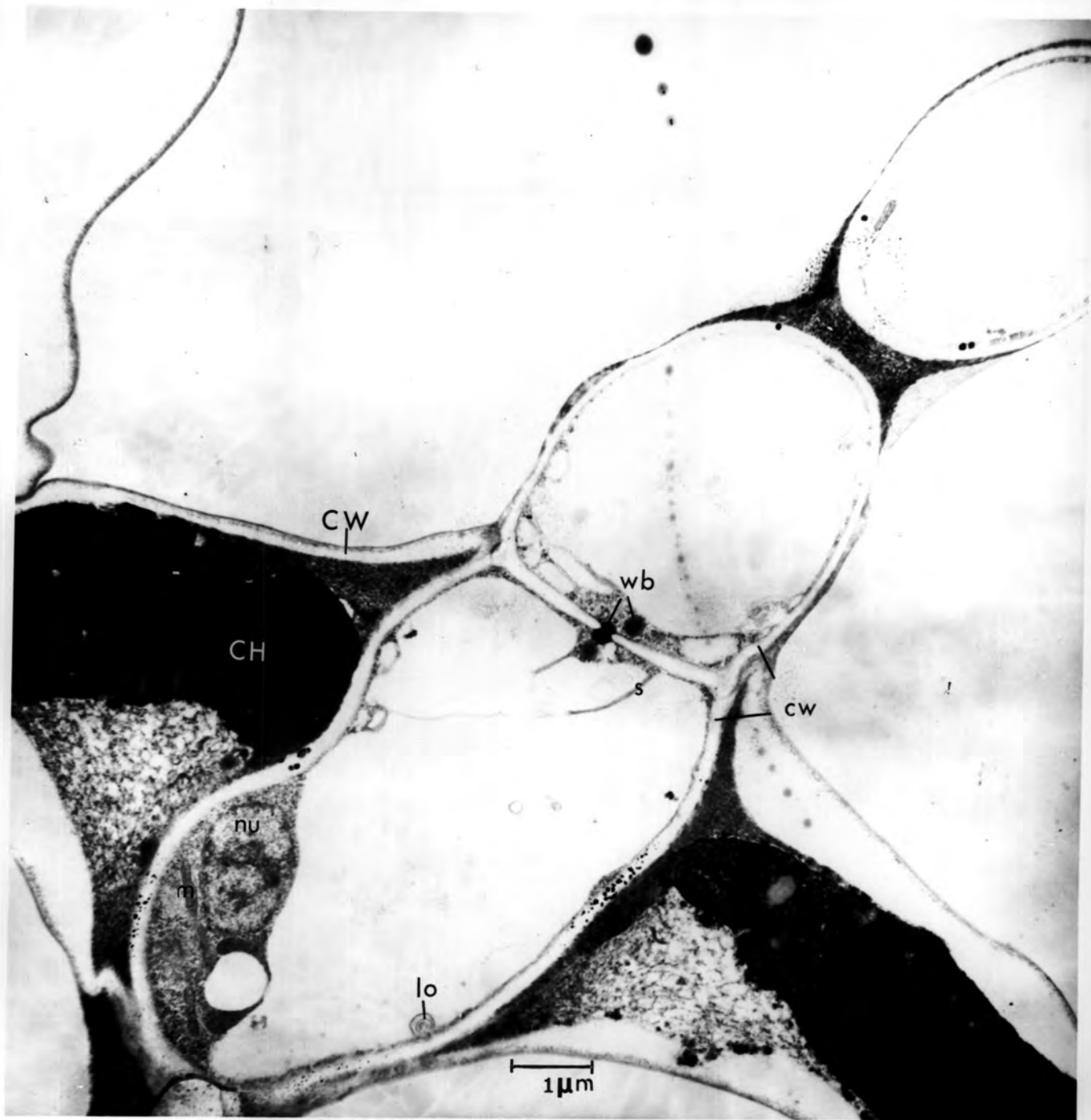


PLATE 42

Electron micrograph of a parasitised bean cell in the necrotic lesion showing the effect of B. fabae infection on the host cytoplasm and the chloroplastic membranes. Observe the vaculation and the formation of lomasomes (lo) in the intercellular hyphae (ich)

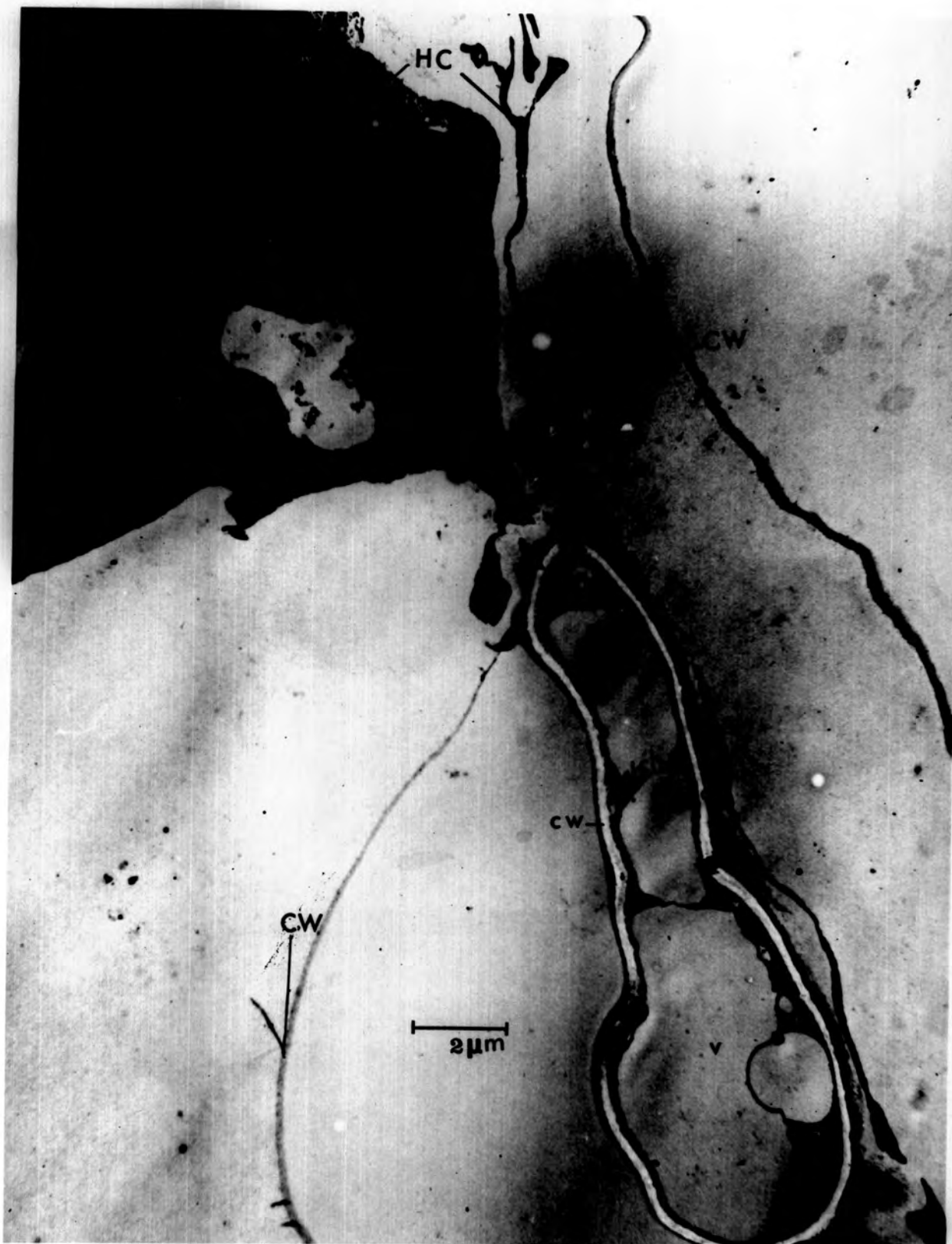


PLATE 43

Electron micrograph of an invaded bean cell  
showing the presence of the intercellular mycelium  
of B. fabae. Observe the blackened host cytoplasm (BHC)



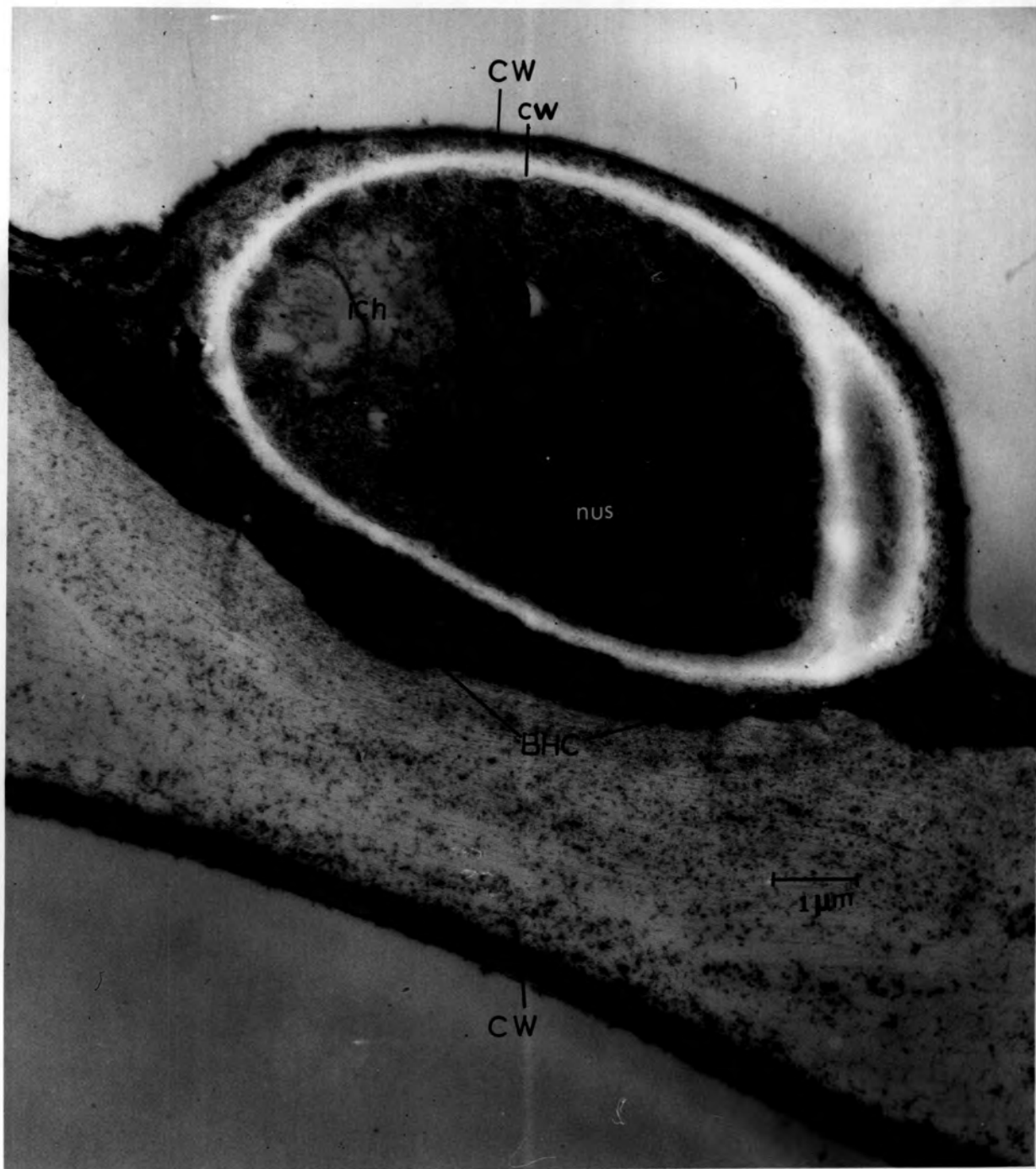


PLATE 44a,b,c

Diagrammatic representation of the method of penetration of B. fabae to the beans cells. Vesicles shown in Plate 44a and 44b presumably are responsible for the hydrolysis of the host cell wall seen in Plate 44b.

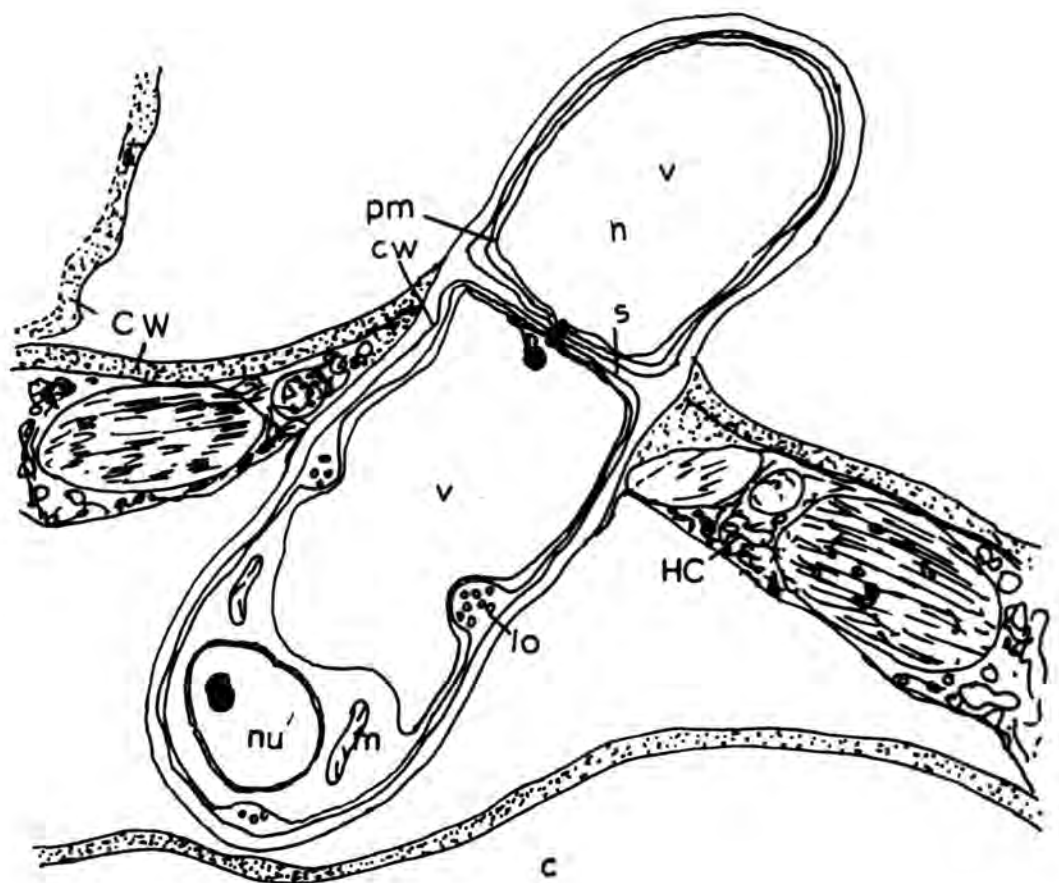
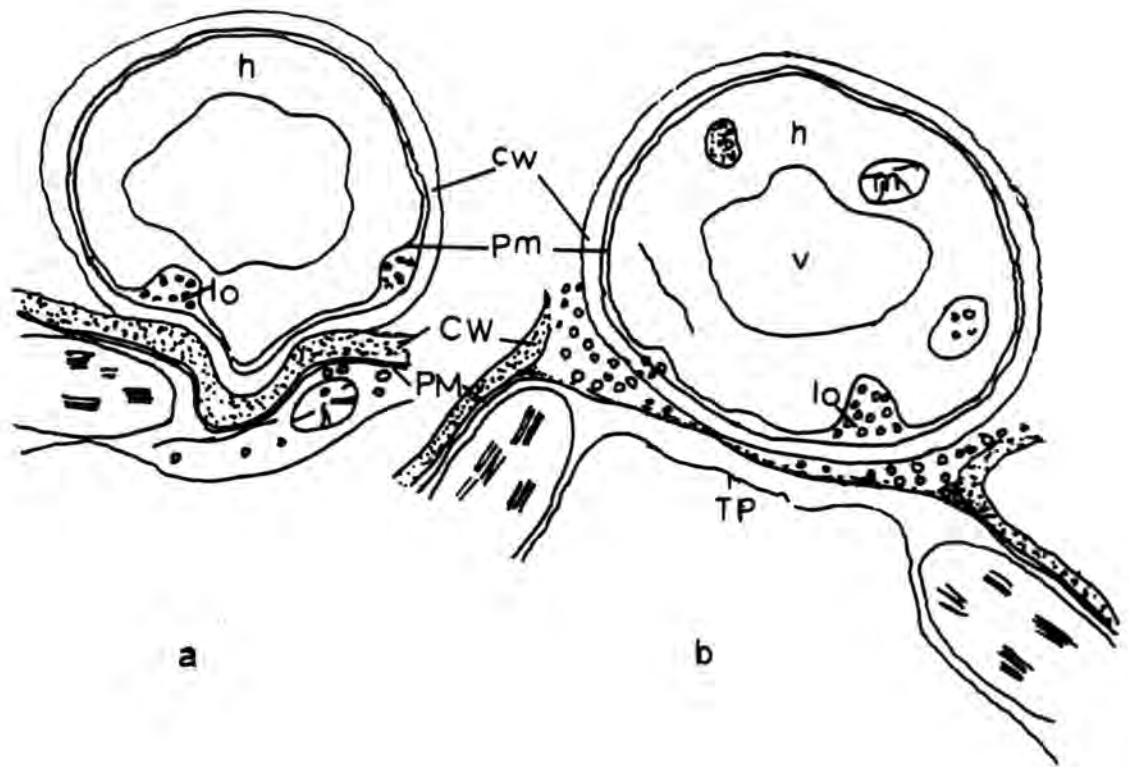


PLATE 45

Microphotograph of portion of two mesophyll cells infected with B. fabae showing the various effects of infection on host cells in the immediate vicinity of the necrotic lesion. Note that chloroplasts integrity in the cell at right hand side is still preserved despite the killing of the rest of the cytoplasm.

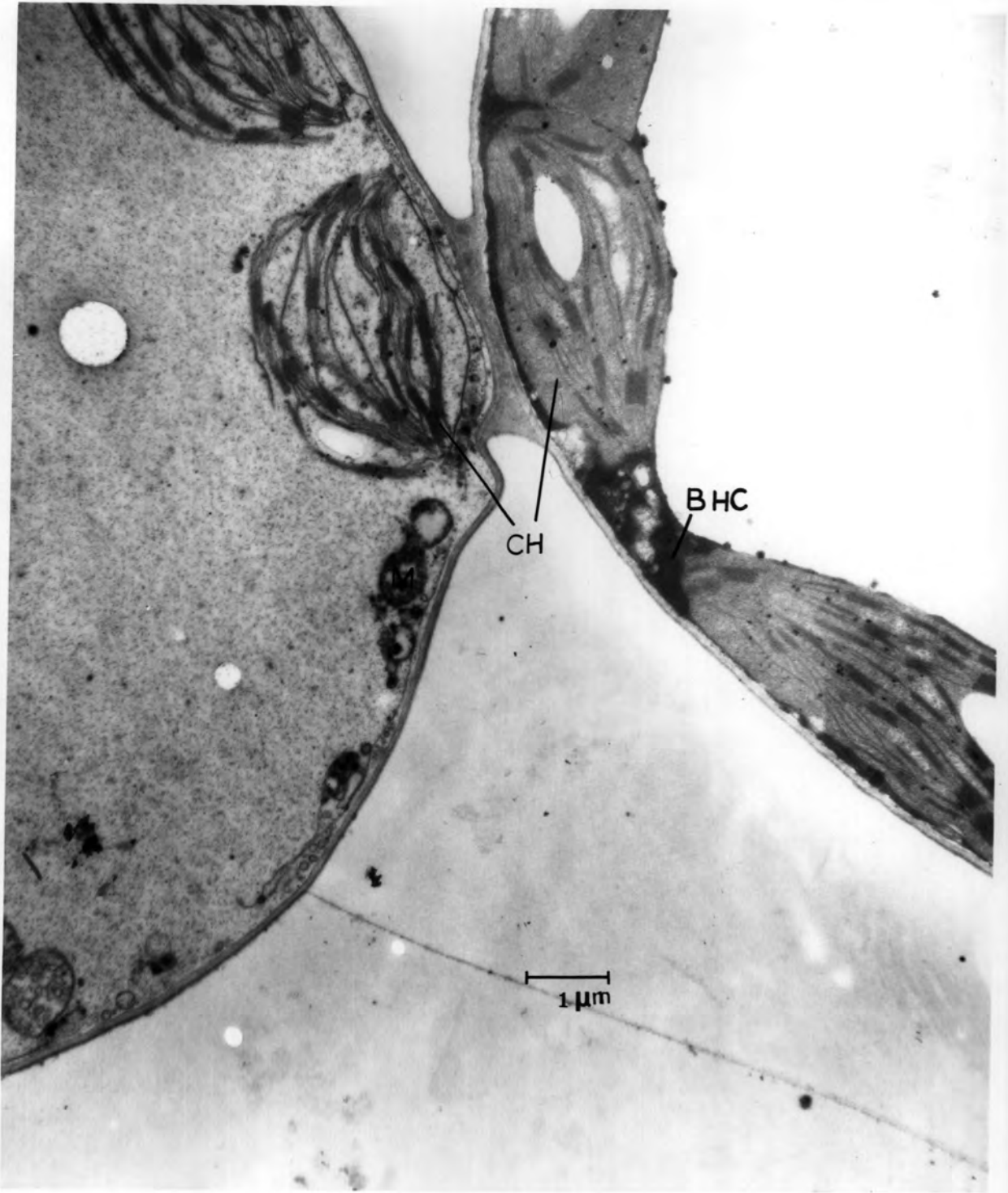


PLATE 46

An overall view of the tissues of bean leaf in the area of the necrotic lesion (5 days after infection). Observe the blackening of the cytoplasm of the cells at the right hand side and the presence of considerably vacuolated fungal hyphae at the left hand side. Also note the invagination of the wall of the latter cell.



PLATE 47

Portion from a cell in the necrotic lesion caused by B. fabae (24 hours after infection). Note the granularity and disorganisation of the host cell wall opposite to the fungal hyphae (h).





PLATE 48

General view showing the organisation and the fine structure of the cells at the periphery of the necrotic lesion caused by the infection with B. fabae. Observe the necrotic cell (NC) at the upper right hand side corner of the Plate.

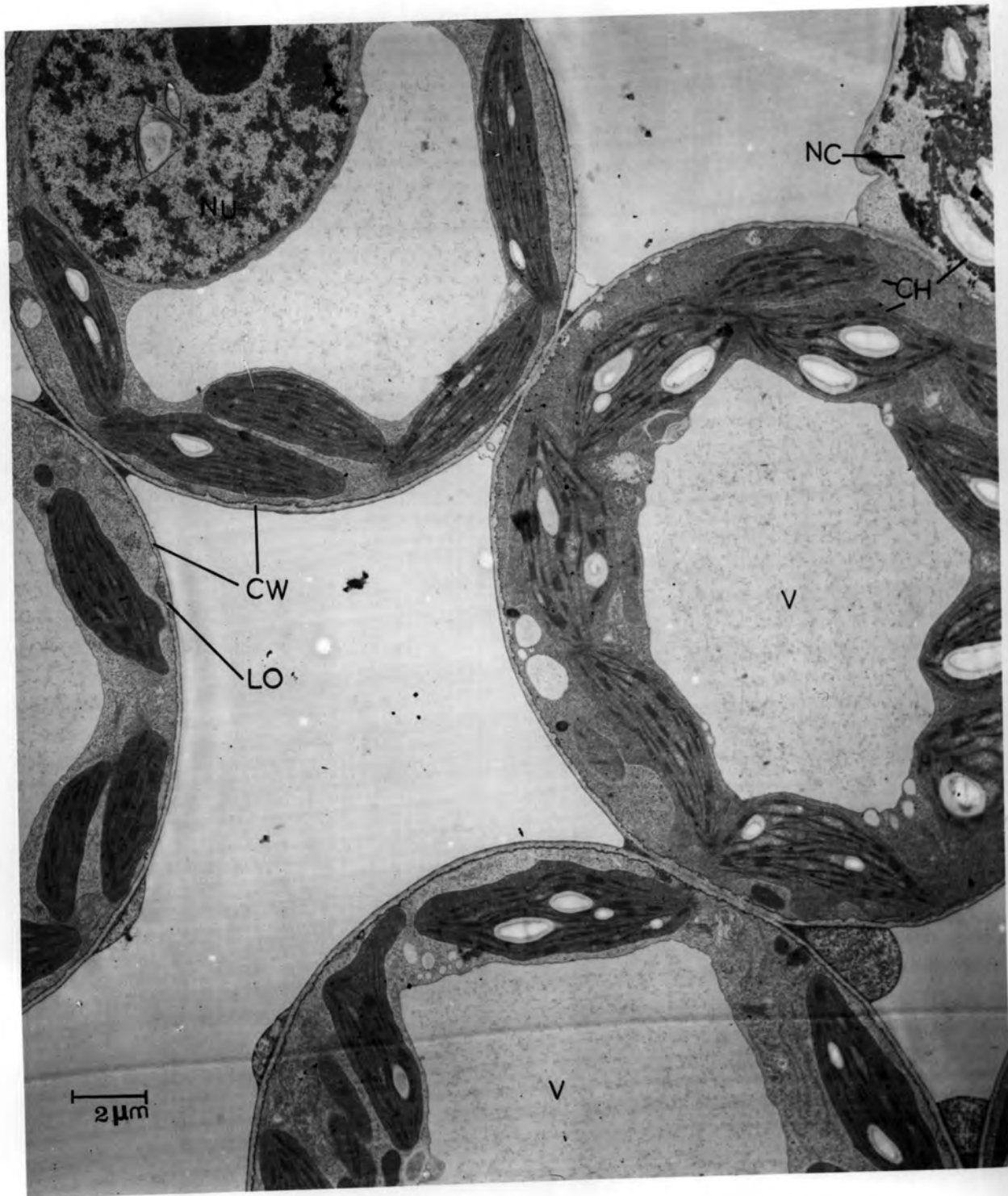


PLATE 49

Cross section of some mesophyll cells at the periphery of the necrotic lesion caused by B. fabae showing the increase in the volume of the cytoplasm and the concentration of the endoplasmic reticulum (ER). Also showing the lomasome (LO) like structure produced as a result of infection.

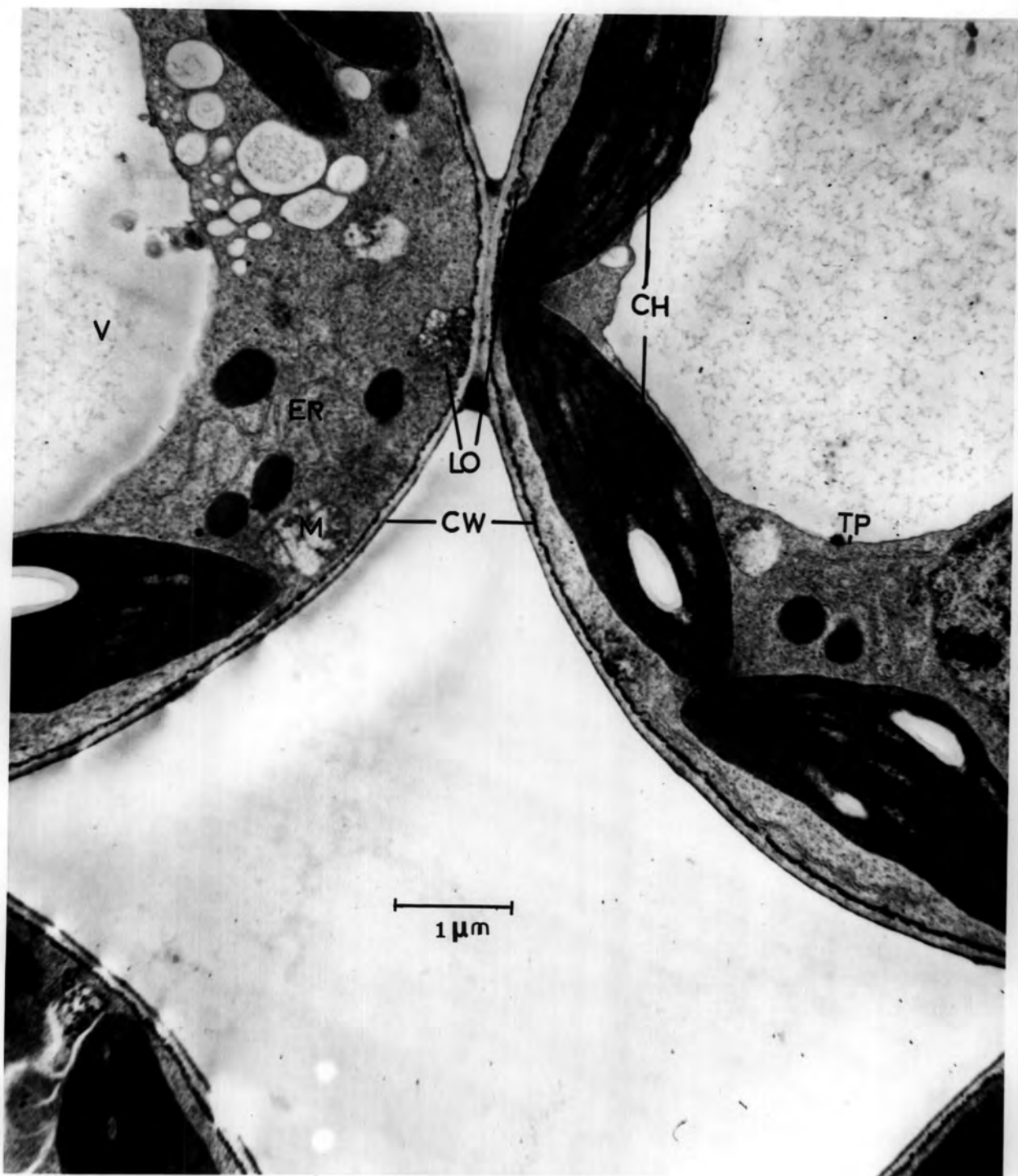


PLATE 50

Enlarged portion of a cell in tissues at the periphery of the necrotic lesion showing some of the fine structural response of such cells. The structure in the extracellular spaces has not been identified.

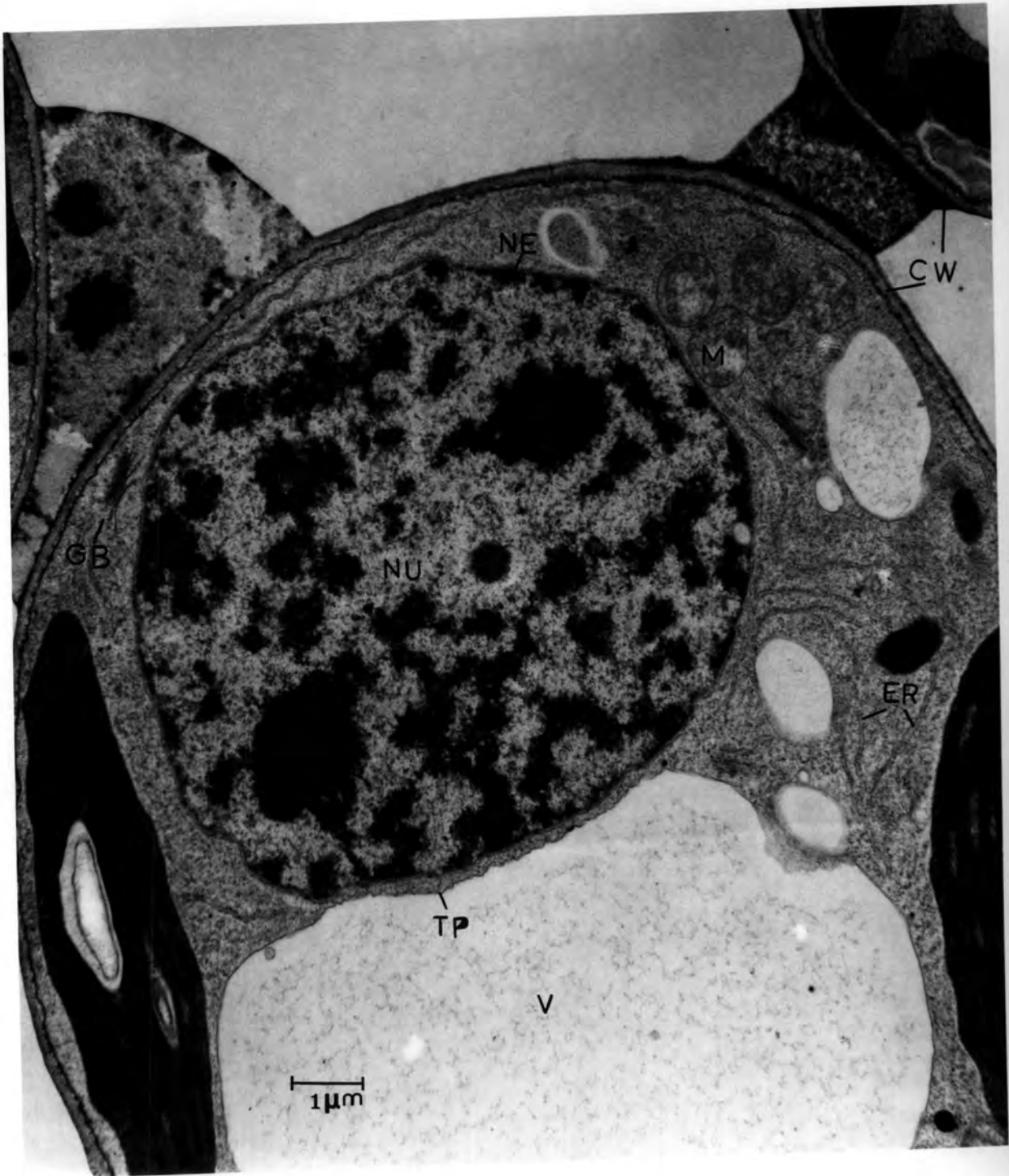


PLATE 51

High power Electron micrograph of a part of the enlarged nuclei of cells at the periphēry of the necrotic lesion caused by B. fabae.



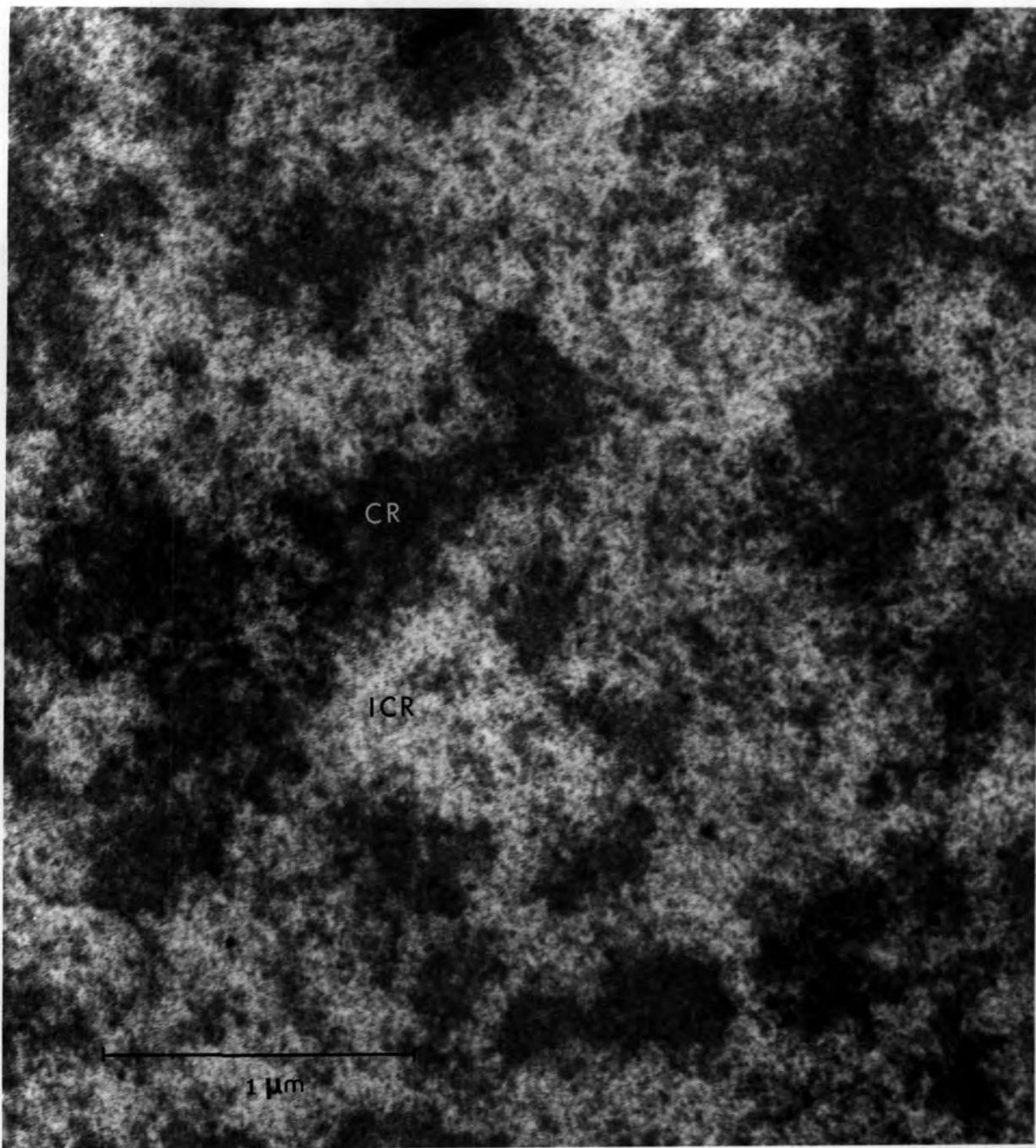


PLATE 52

Parts of two mesophyll cells at the periphery of the B. fabae necrotic lesion showing the increased proliferation of ER and the movement of vesicles from the host cell to the extracellular spaces where an unidentified structure (presumably a disintegrating fungal mycelium) is situated.

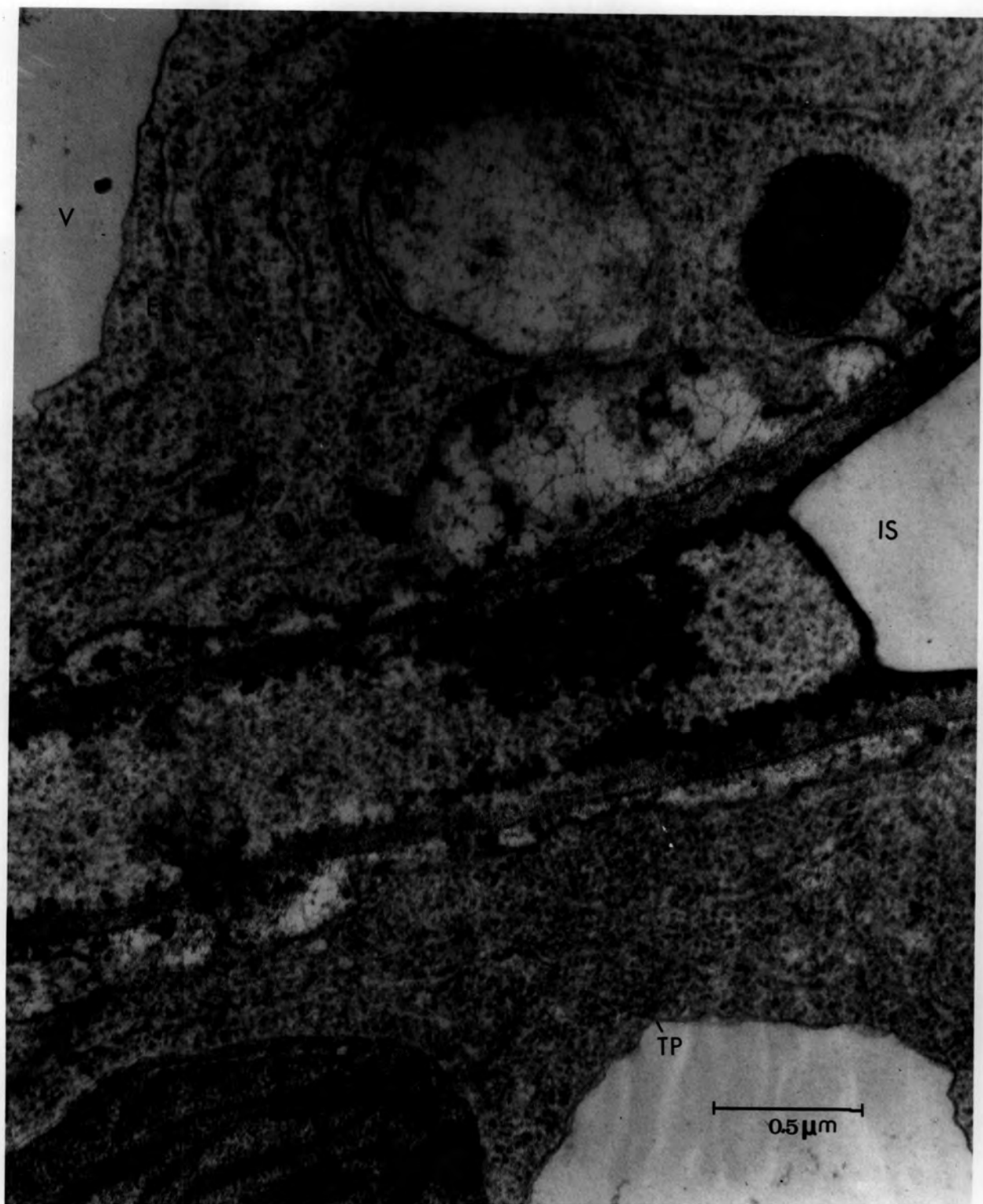


PLATE 53

1B. Electron micrograph of a part of a mesophyll cell infected with U. fabae showing two of the electron dense bodies (EB) attached to the outer part of the host cell wall. Electron dense band (DB) can also be seen underneath the cell wall to which these bodies are attached.

PLATE 54

High power electron micrograph for one of the electron dense bodies - showing the lack of any cytoplasmic organelles.

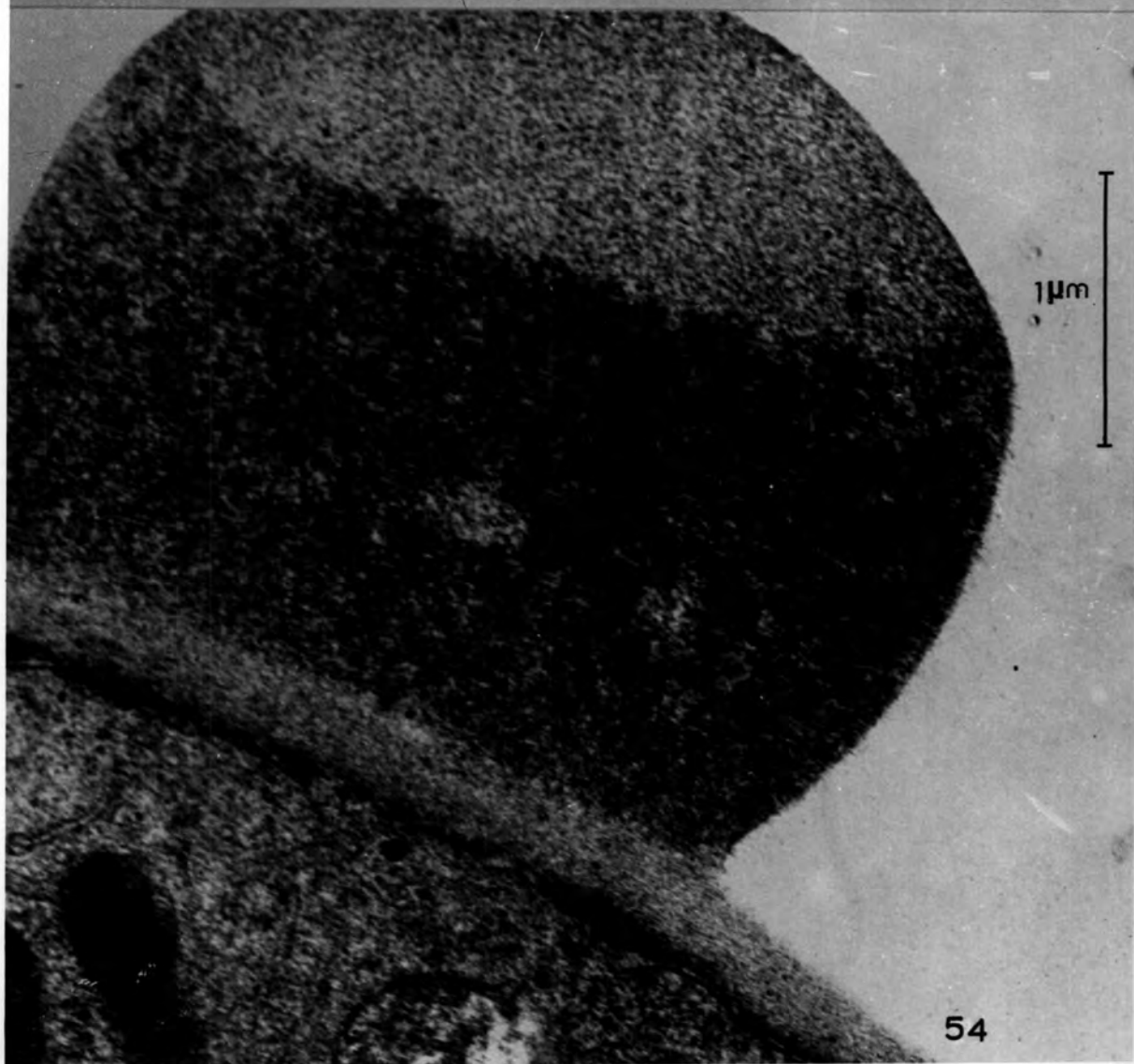
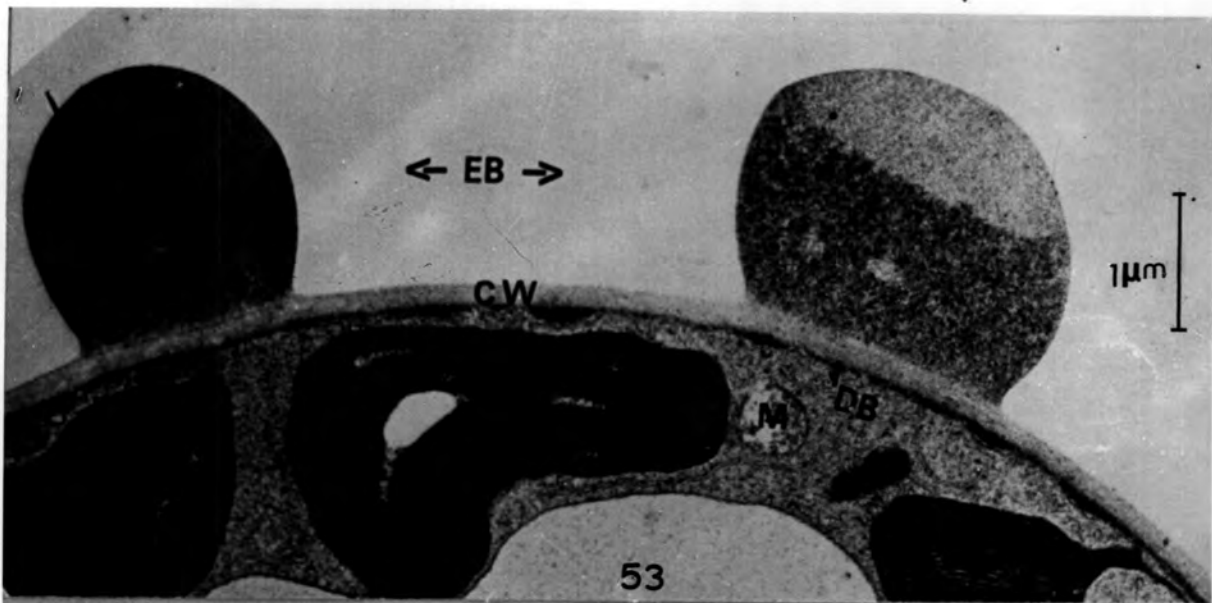
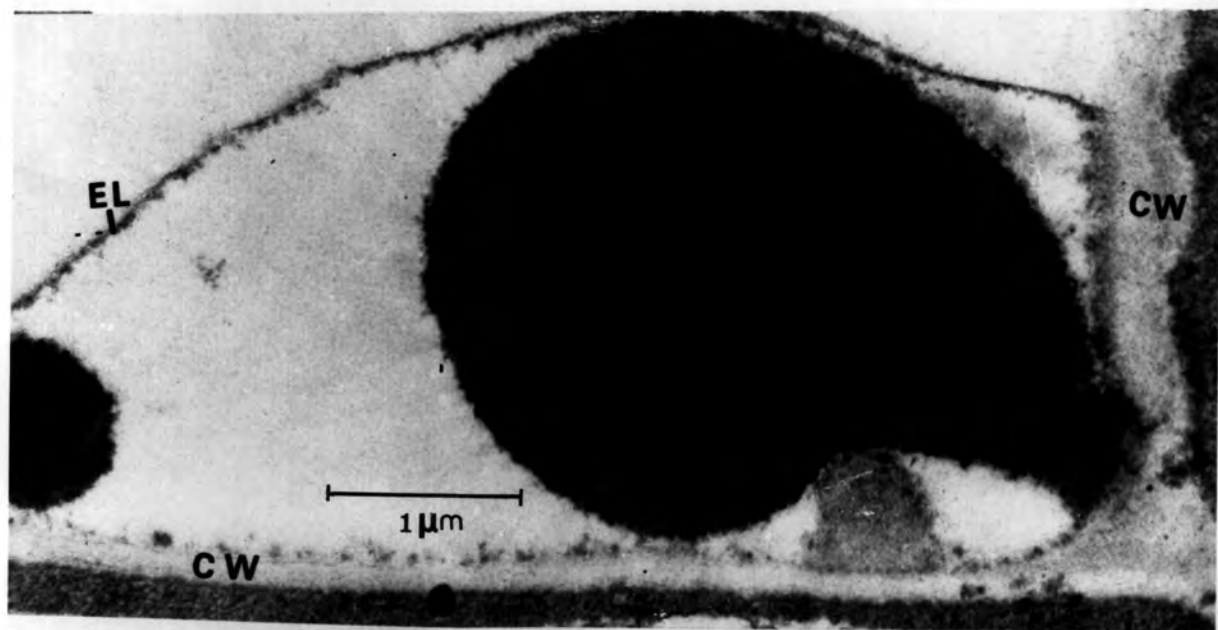


PLATE 55a,b,c

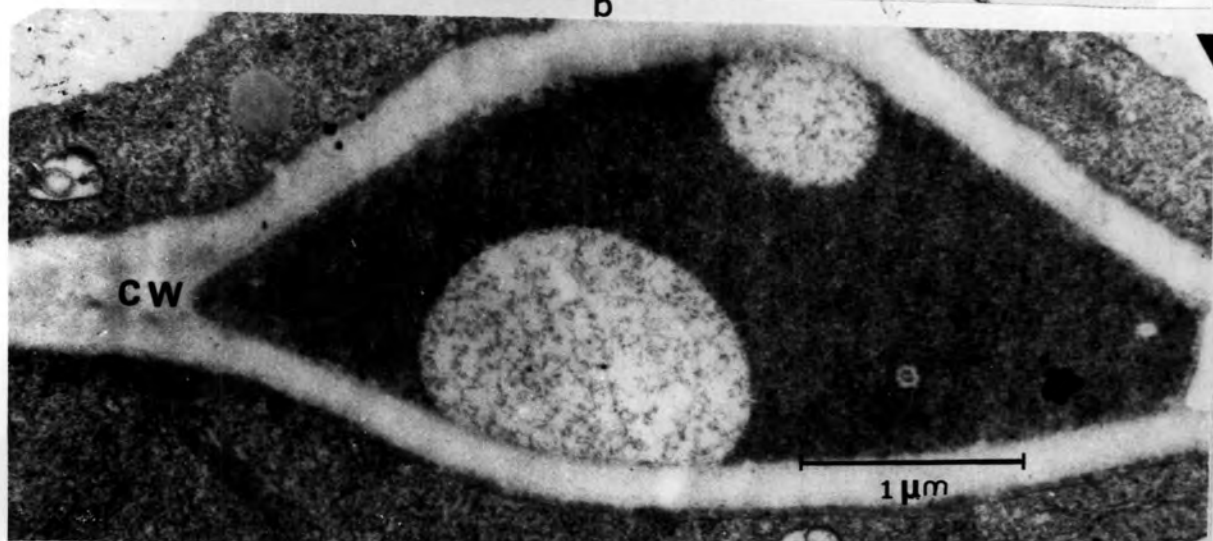
Microphotographs showing another type of unidentified body found either attached to the outer host cell wall (55a,b) or situated between two mesophyll cells (54c). (Note the presence of ornamental covering around bodies in Plate 55a,b).



a



b



c

PLATE 56

Electron micrograph of two mesophyll cells infected with B. fabae showing a fungal hyphae (h) growing in the extracellular spaces between the two host cells. Note the production of vesicles at the point of contact between the wall of the cell at the right hand side and the fungal hyphae.



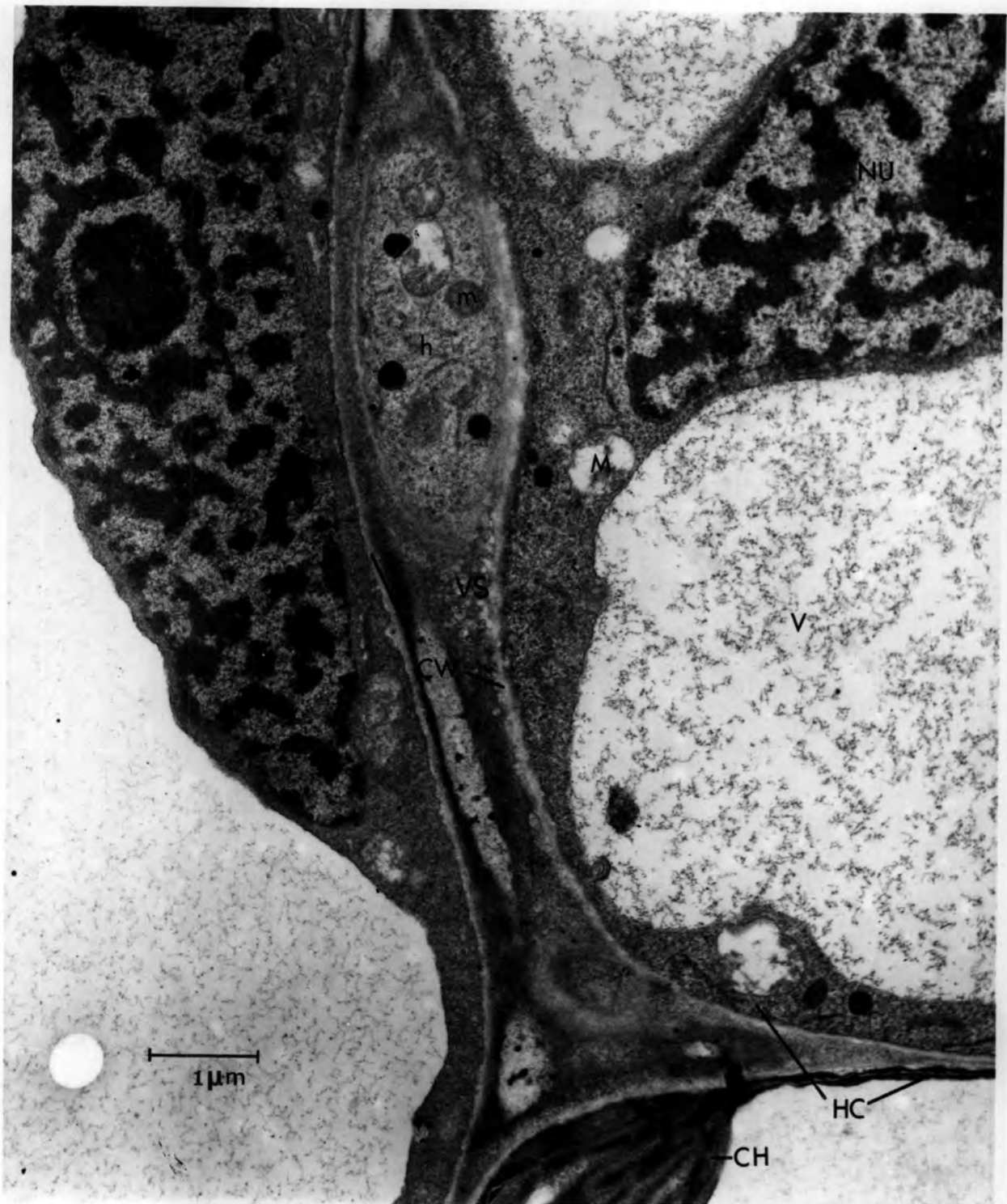
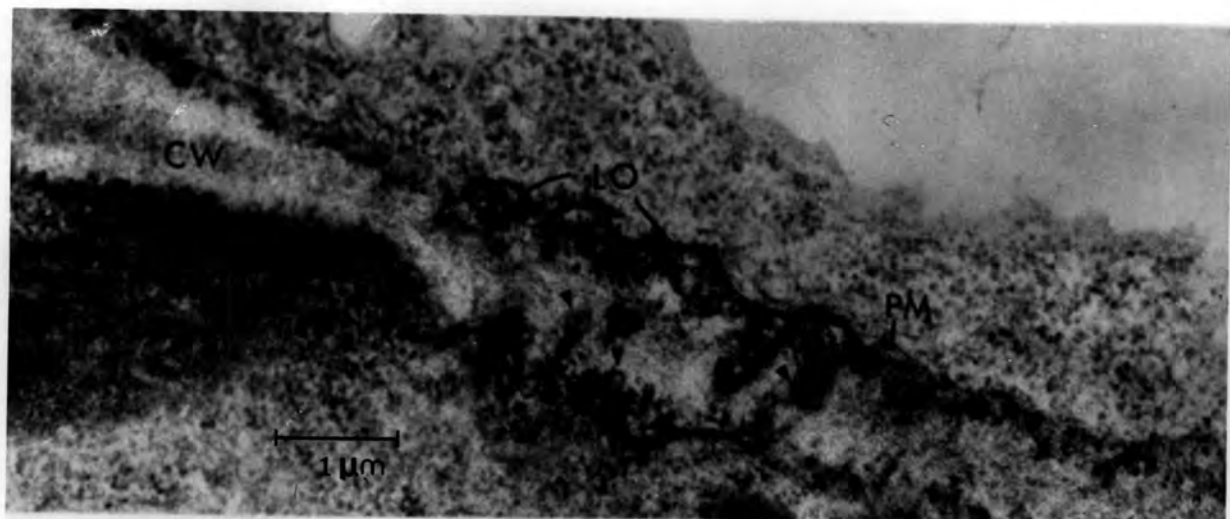
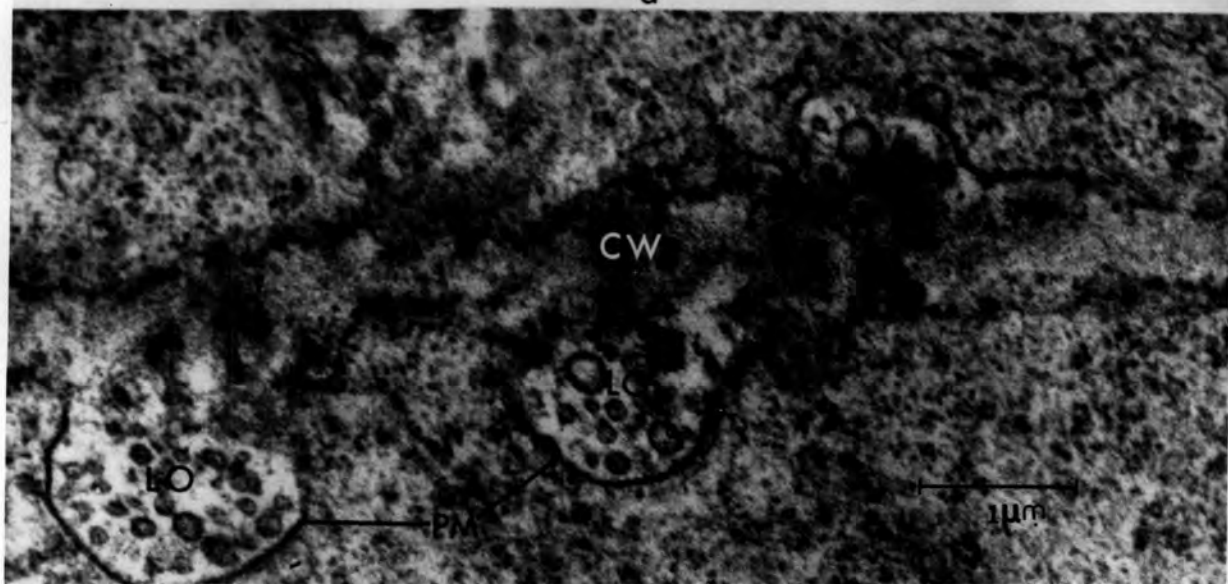


PLATE 57 a,b,c

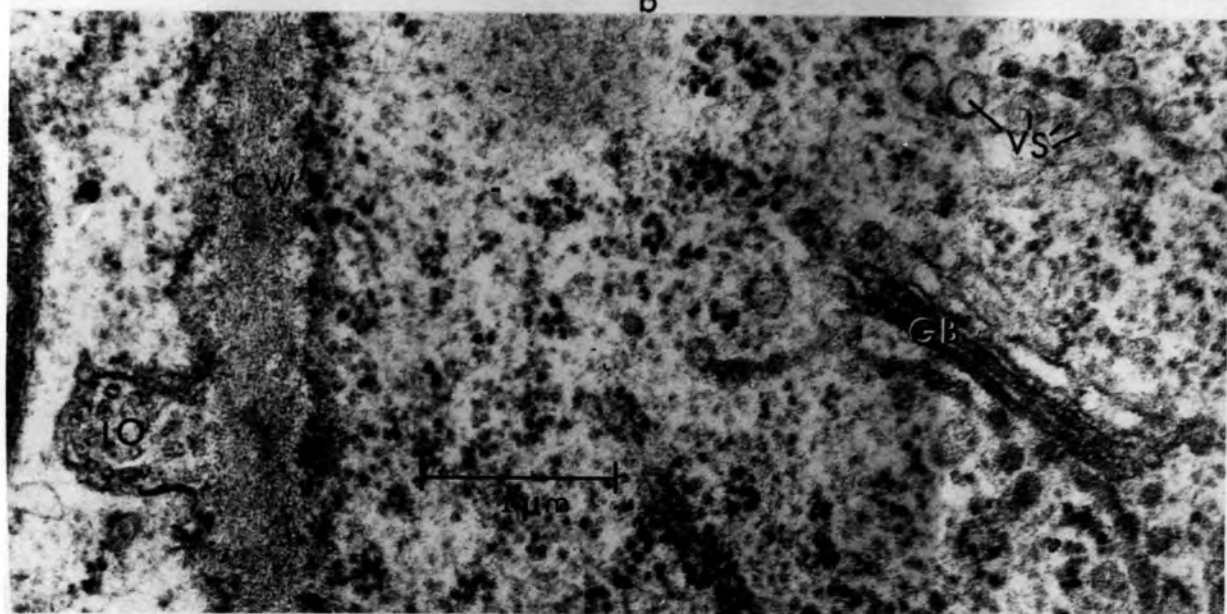
Electron micrograph showing the lomasome-like structures found in the mesophyll cells neighbouring the necrotic lesion caused by B. fabae. Observe the presence of some vesicles in the cell wall areas (Arrows plate 56a,b) and association of vesicles with the Golgi bodies (Plate 56c.)



a



b



c

PLATE 58

High power Electron micrograph showing the presence of aggregates of vesicles found in the extracellular spaces of cells near to the necrotic lesion caused by B. fabae.

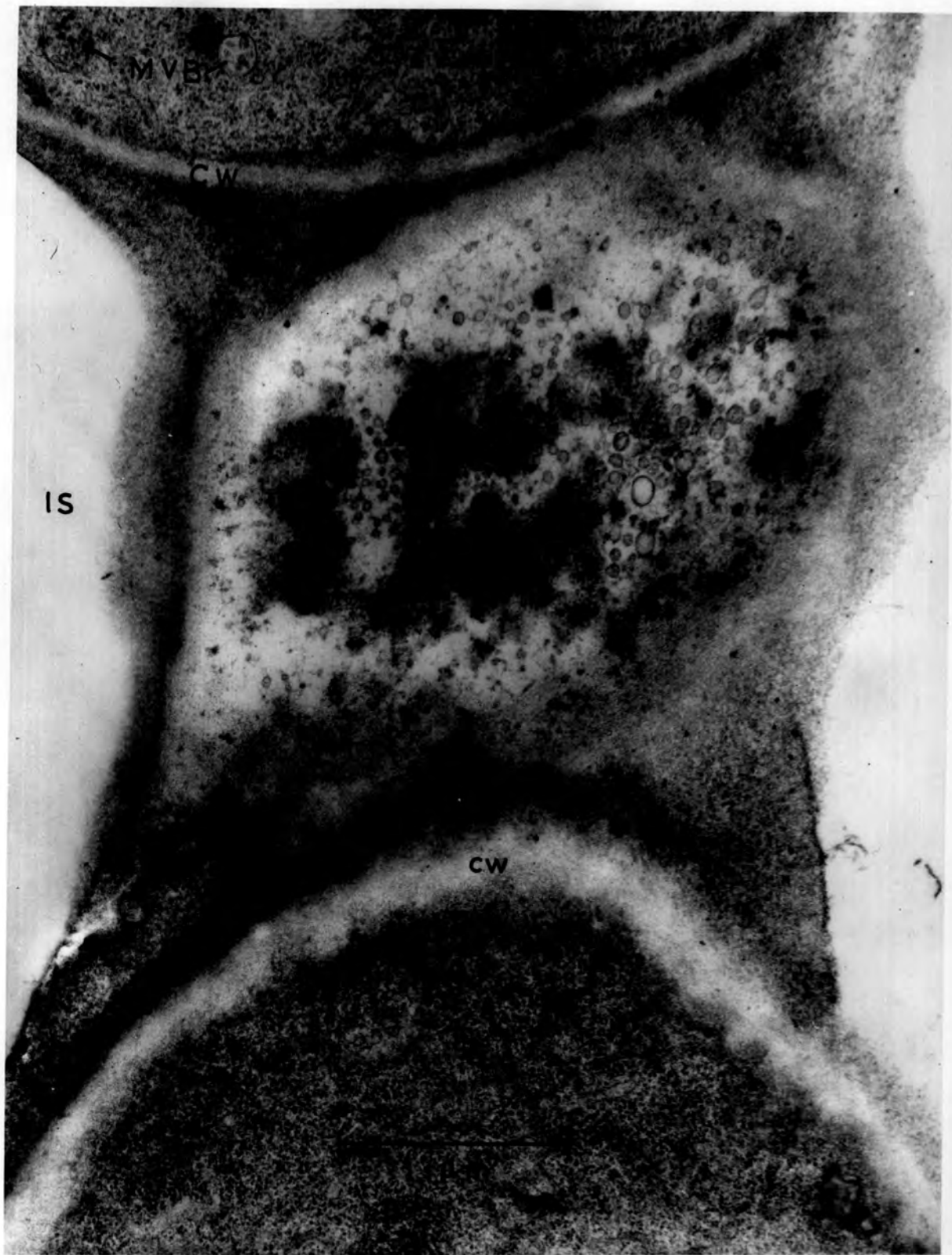


PLATE 59

Electron micrograph showing the vesicular inclusions found in the cytoplasm and the vacuoles of mesophyll cells of V. faba leaves inoculated with B. fabae (4 days after infection). (59a) Vesicular inclusion embedded in the cytoplasm but separated with an electron transparent layer. (59b). Two vesicular inclusions one still bound by the host tonoplast and the other (lower) was separated from the cytoplasm of the host and discharged to the vacuole. (59c) another inclusion bound to the host tonoplast.

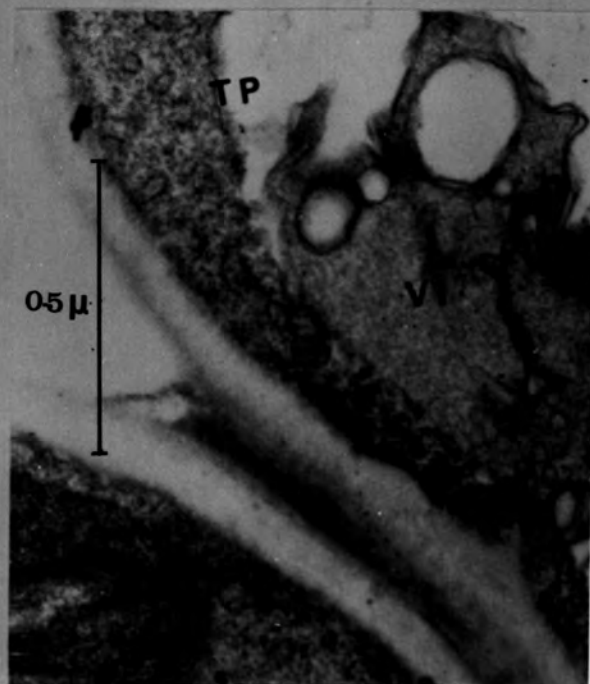
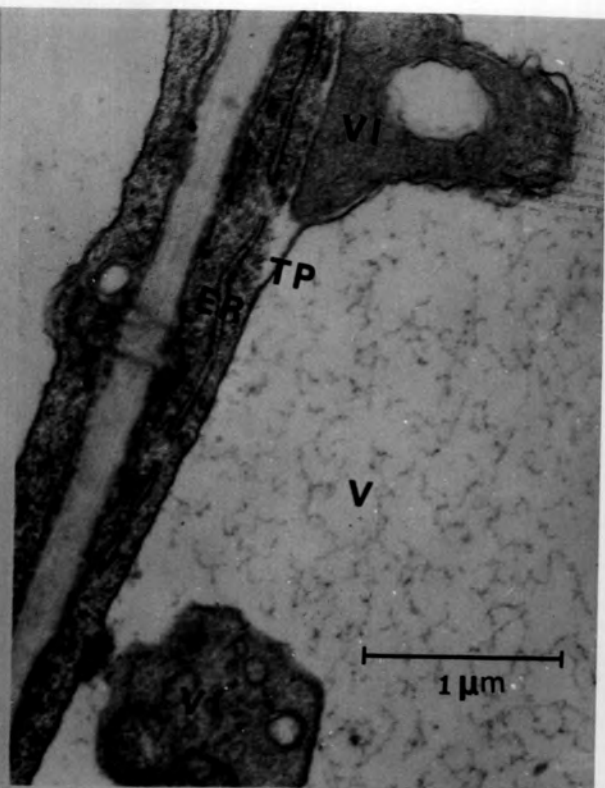
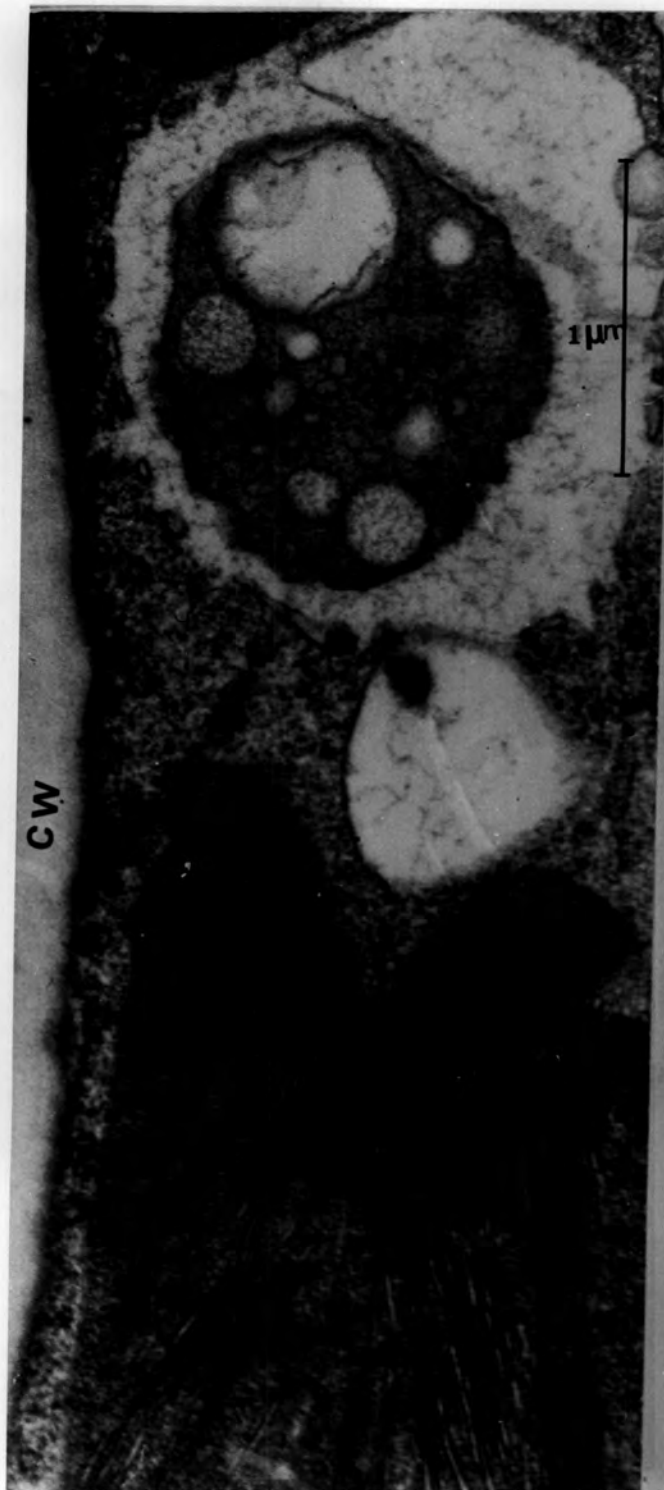


PLATE 60

Electron micrograph of a part of B. fabae  
showing the general fine structure of the  
fungal mycelium growing on bean leaf malt agar.



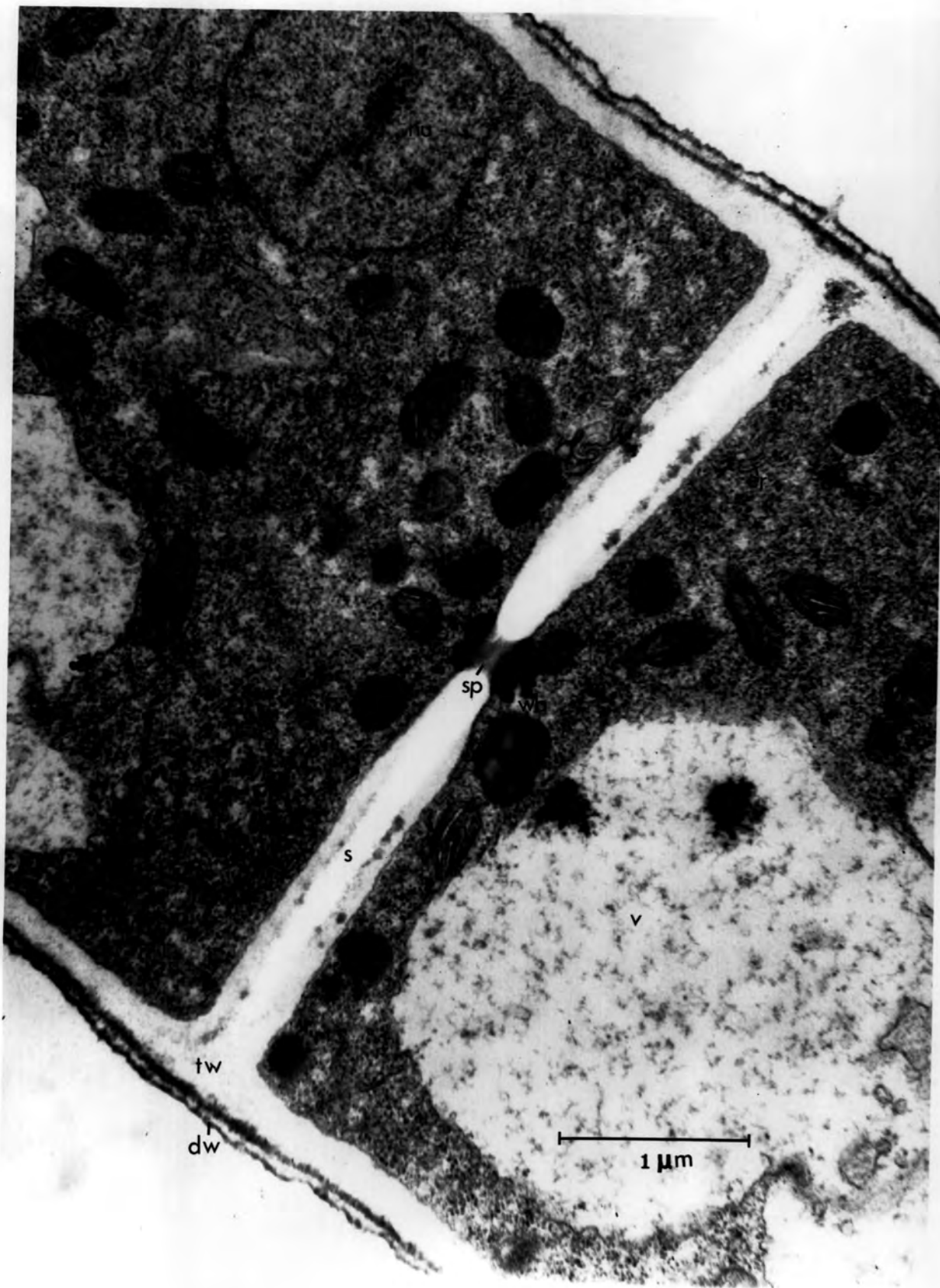


PLATE 61

Higher power Electron micrograph of a part of B. fabae  
mycelium grown on artificial medium (bean leaf malt agar)  
showing the detailed fine structure of the septum region.

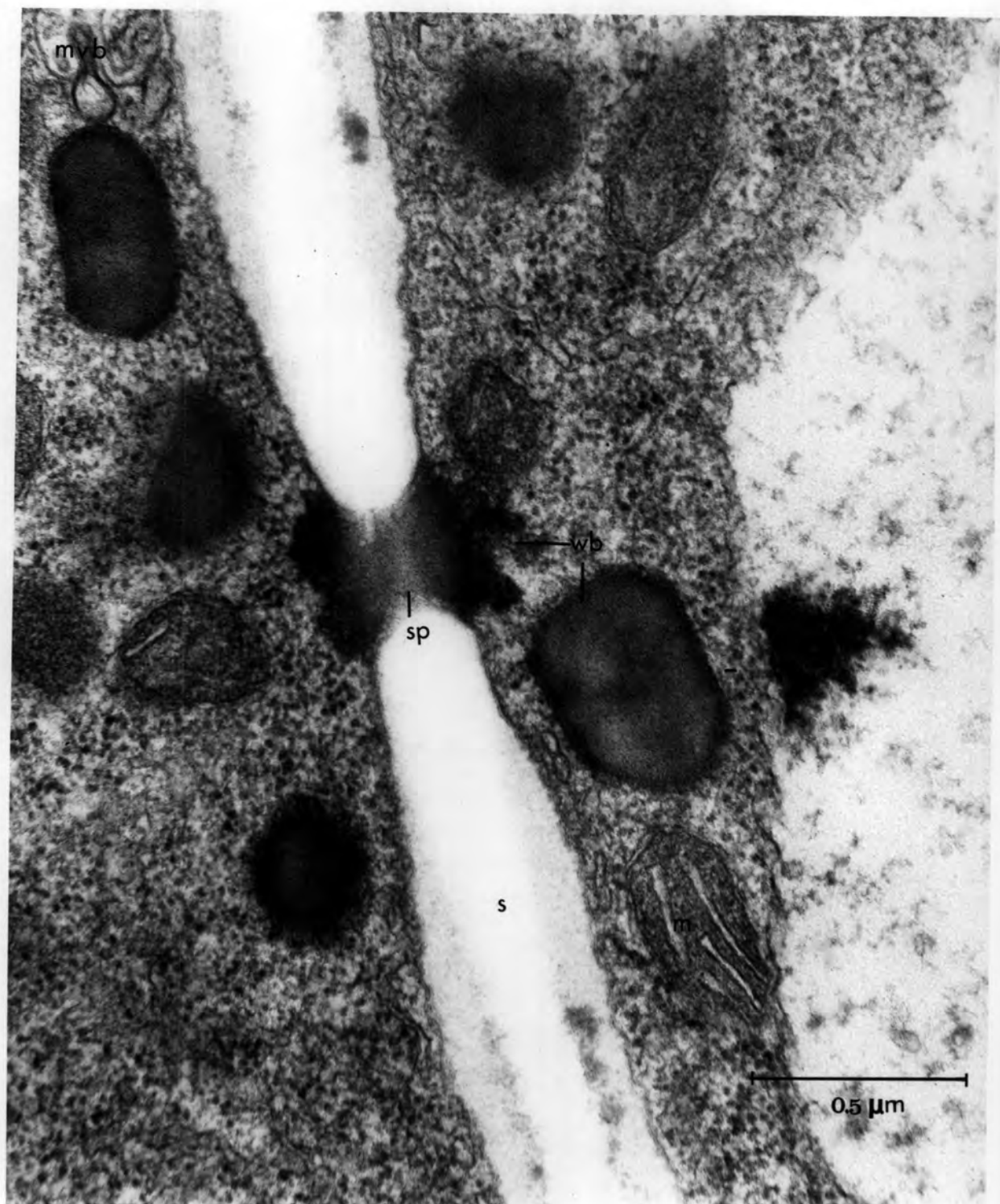
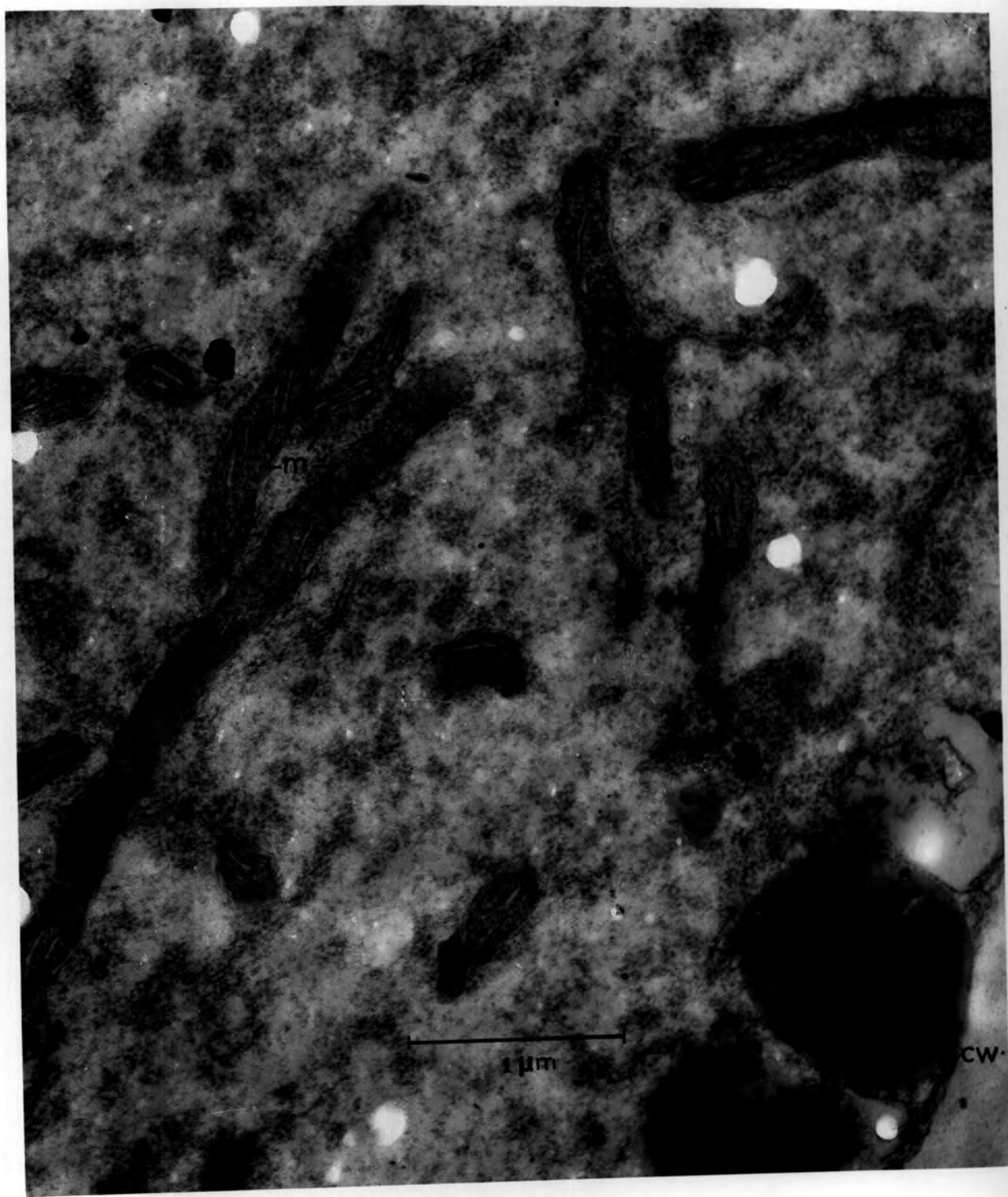


PLATE 62

High power Electron micrograph of a part of B. fabae cytoplasm showing the structure and length of mitochondria of the fungus growing on artificial medium (bean leaf malt agar).



PLATES 63-66

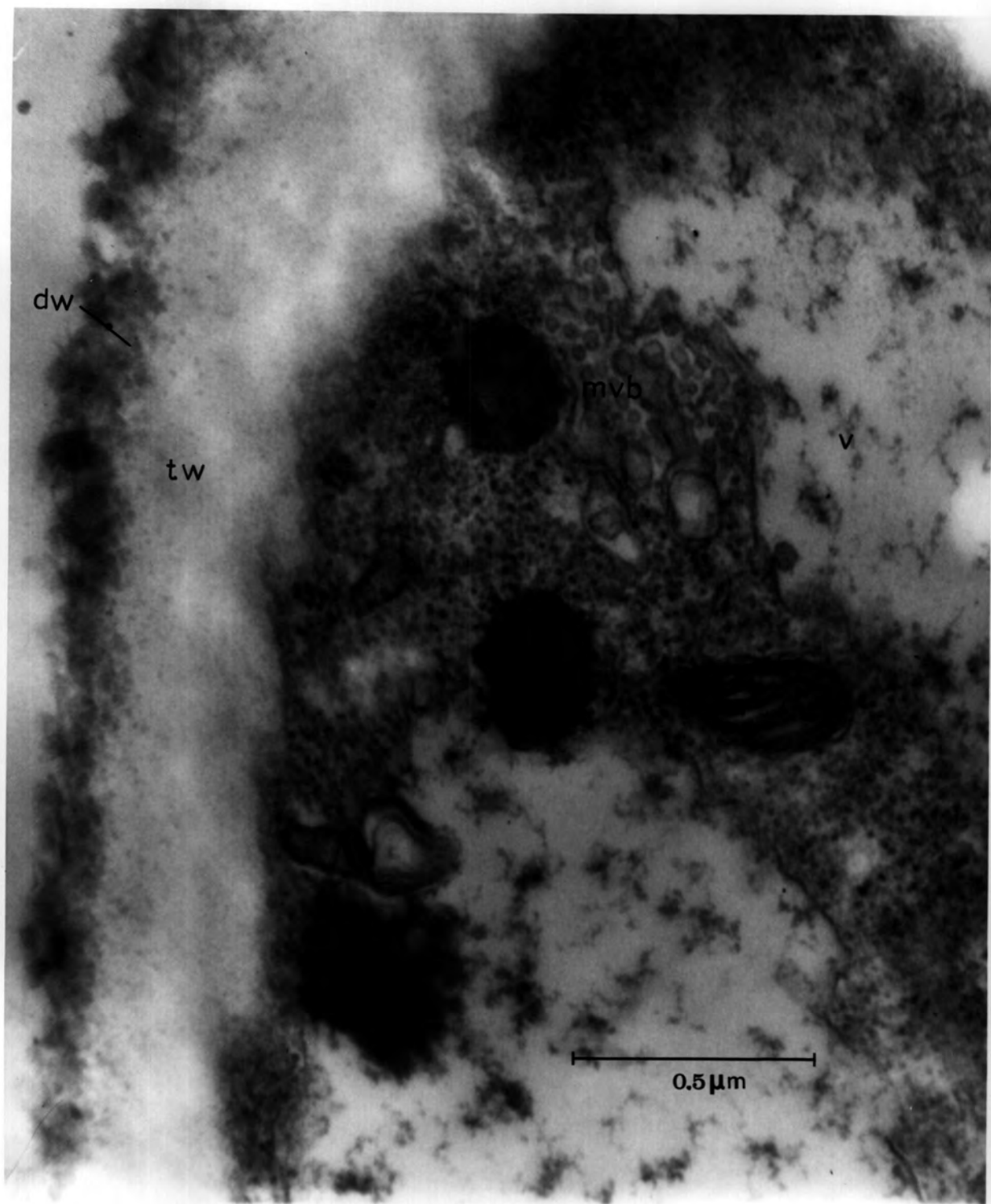
Series of Electron micrographs showing the correlation of the presence of vesicular structures and lomasomes with the nature of the complex substances present in the media on which the fungus was growing.

Plate 63 B. fabae grown on Bean leaf malt agar

Plate 64 B. fabae grown on medium containing pectin.

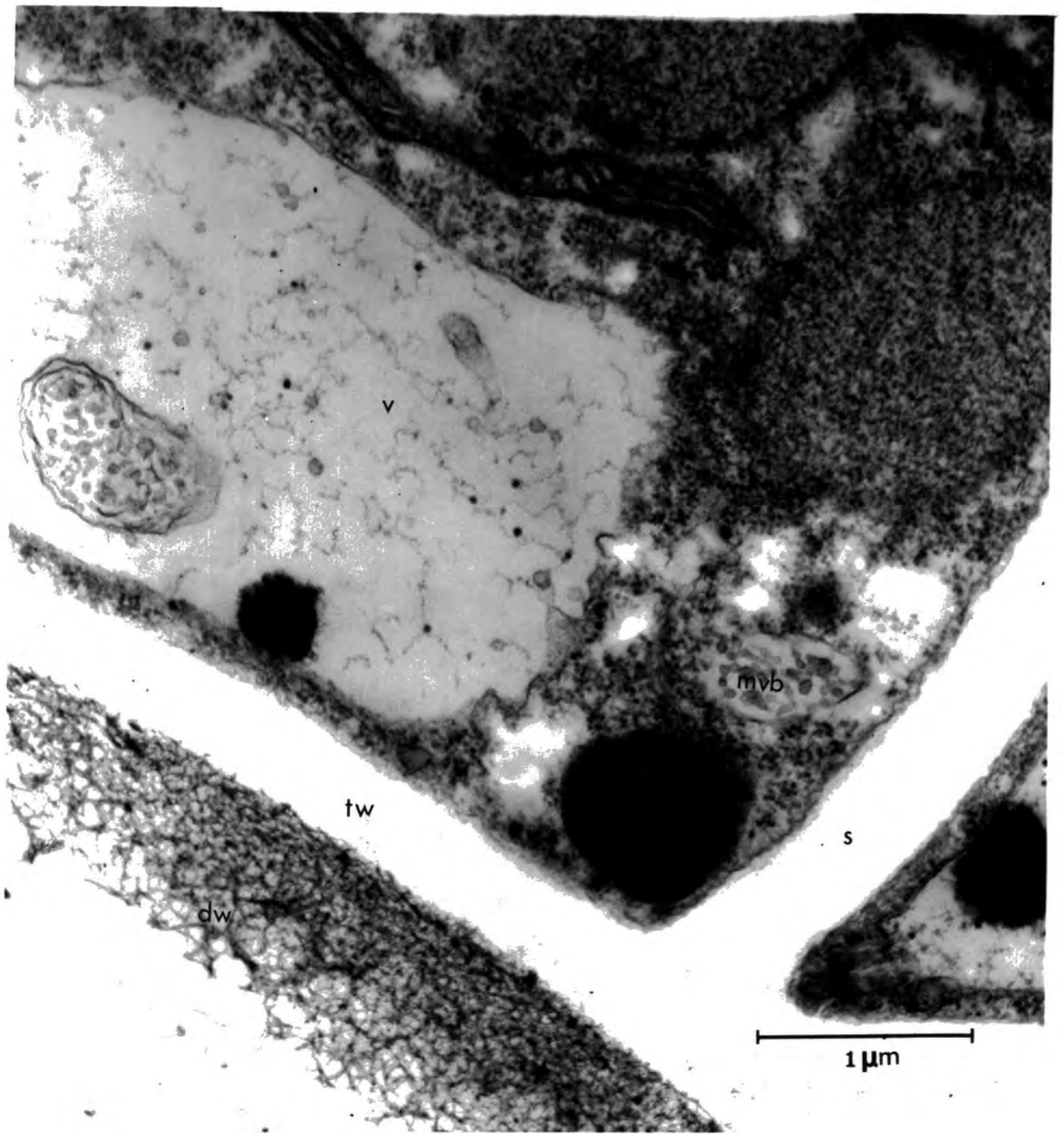
Plate 65 B. fabae grown on medium containing cellulose

Plate 66 B. fabae grown in medium containing starch









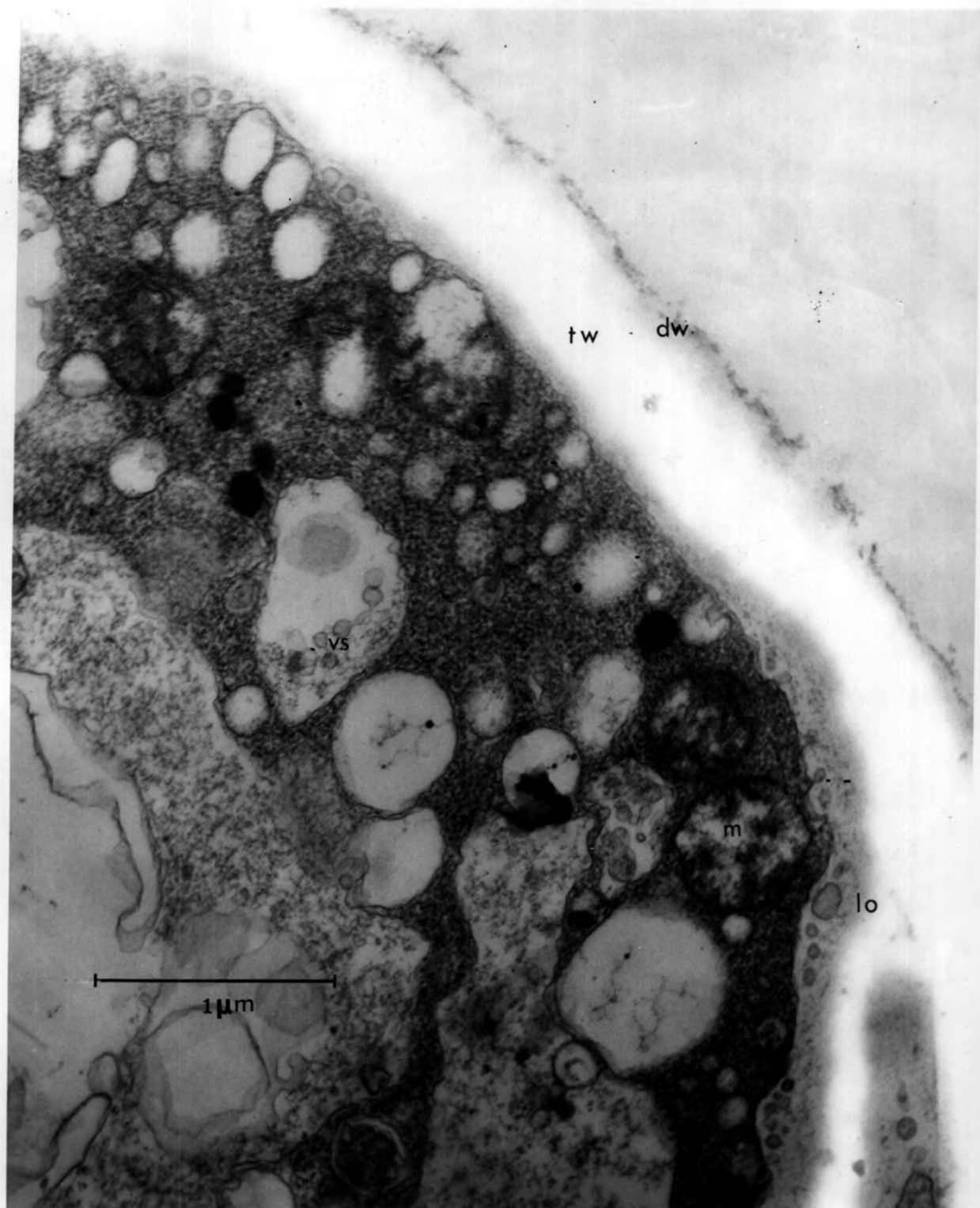


PLATE 67

Electron micrograph of a longitudinal Section in  
B. fabae mycelium incubated for 24 hours in a water  
extract of leaves infected with B. fabae, showing  
the disintegration of the fungal cytoplasm.

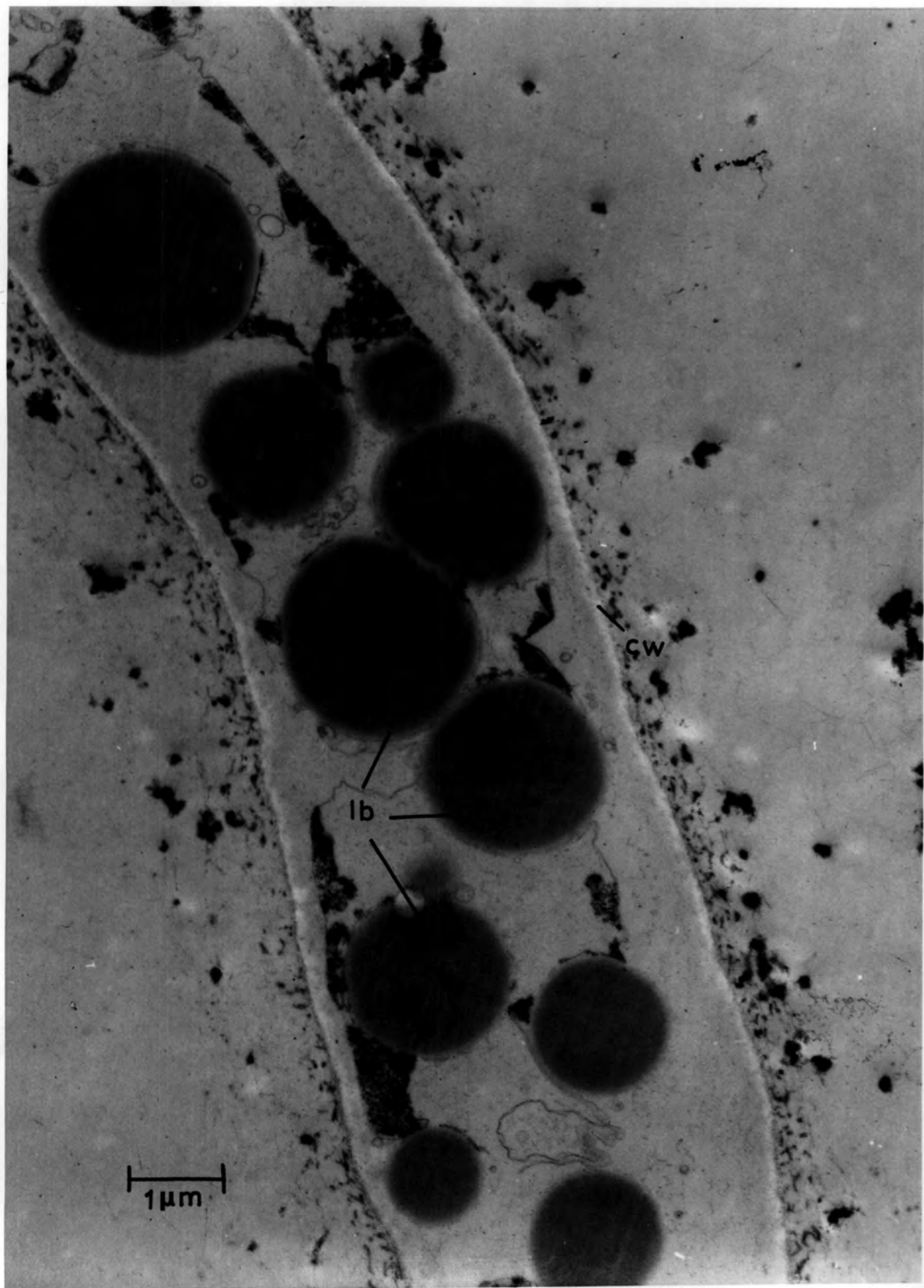


PLATE 68

A phase contrast microscope view showing  
the type of chloroplasts preparation normally used  
in the biochemical assay.

ML = chloroplasts without the outer membranes

LT = intact chloroplasts

Mag 100X

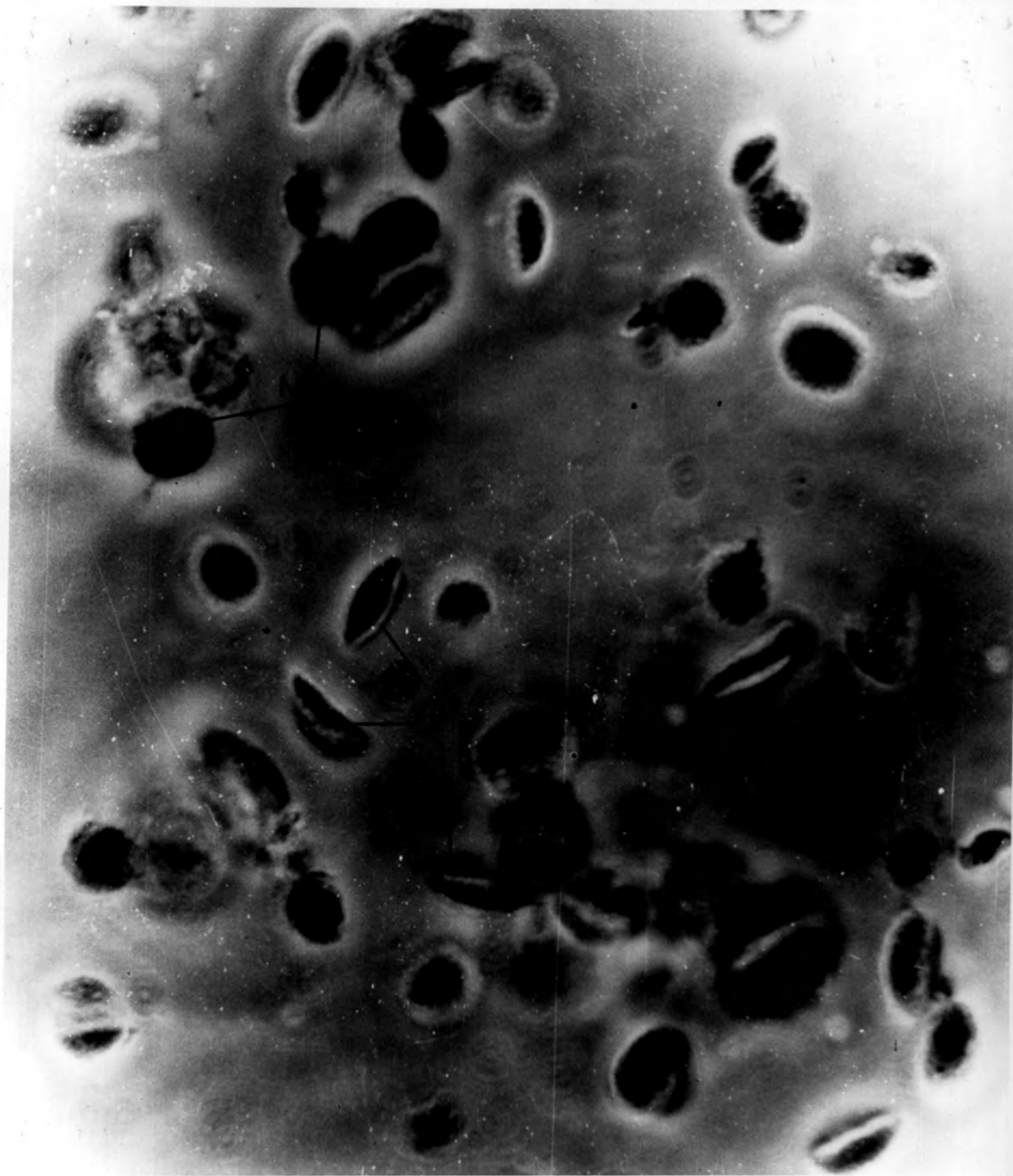
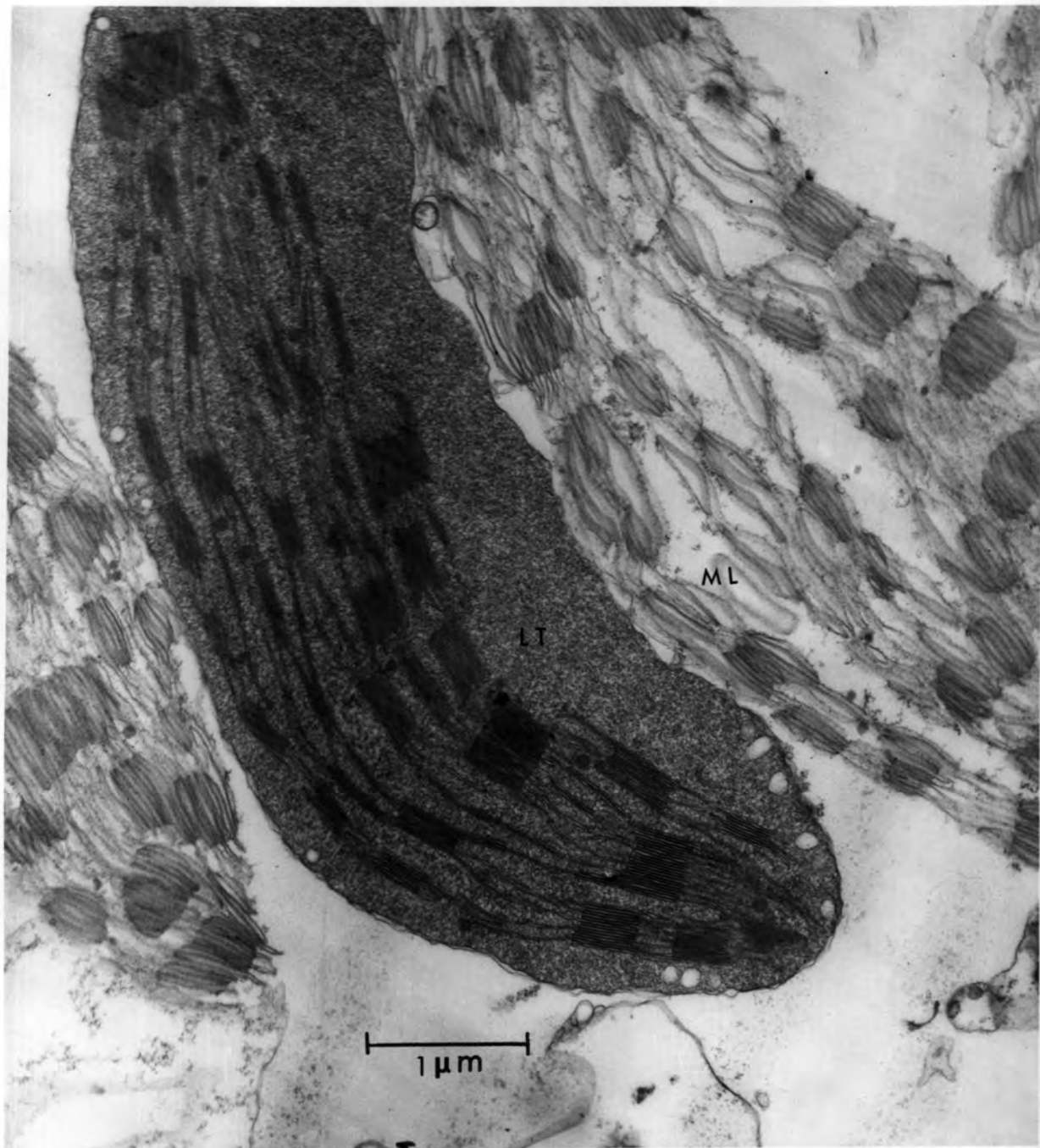


PLATE 69

High power Electron micrograph showing both an  
intact and membraneless V. faba chloroplasts.

ML = chloroplasts without the outer membrane

LT = intact chloroplasts.





PLATES 70-72

Series of microphotographs of chloroplasts preparation from healthy leaves (Plate 70) U. fabae infected leaves (Plate 71) and B. fabae infected leaves (Plate 72) (12 days after infection). All preparations were fixed in gluteraldehyde ~~post~~ followed by post fixation in osmic acid.

ML = Chloroplast without the outer membrane

LT = Intact chloroplast.

