631

# ULTRASTRUCTURAL AND CYTOCHEMICAL STUDIES OF HIGHER PLANT-FUNGAL INTERFACES, WITH SPECIAL REFERENCE TO BIOTROPHY

Thesis presented for the degree of Ph.D. of the University of London

by

ALISON MARY WOODS

January, 1985

Department of Pure and Applied Biology,
Imperial College of Science and Technology,
London, SW7.

## Abstract

Haustorial infections by Albugo candida, the monokaryotic stage of Puccinia poarum and Bremia lactucae were investigated with regard to specialization of the region of the host plasmalemma invaginated around infecting structures and the presence of a neckband.

In sections treated by a procedure specific for plasma membranes, the staining of the uninvaginated region of the plasmalemma was normal and intense in all instances except in some cells of leaves infected by B. lactucae. In contrast, almost all the region invaginated around haustoria of A. candida and B. lactucae was only lightly to moderately stained, as was a variable extent of the host plasmalemma around part of the M-haustoria of P. poarum. However, the host PM adjacent to host wall-like material, which either enclosed the rest or all of haustoria of P. poarum, was intensely stained. Cytochemical localization of ATP-ase showed activity on the uninvaginated host plasmalemma, but none in the region around haustoria of A. candida or the fungal plasmalemma. A similar distribution of activity was found in cells infected by M-haustoria of P. poarum except the region of host plasmalemma adjacent to host wall-like material which showed normal activity.

Specific staining methods were also used to investigate the origin of the extrahaustorial membrane in cells infected by A. candida, but no definite conclusions could be drawn.

In A. candida infections the transition in characteristics between the domains of the host plasmalemma which had been distinguished occurred abruptly and consistently in a region adjacent to an abnormally staining part of the haustorial neck wall; in B. lactucae infections it occurred at the inflexion of the plasmalemma near the infection site. In contrast, in P. poarum, a gradual transition in characteristics of the host PM was observed at locations which varied according to the extent

of enclosure of haustoria. Thus, the abnormal haustorial neck wall region of A. candida shared characteristics with haustorial neckbands, but no comparable structure was observed around haustoria of P. poarum or B. lactucae. Therefore, other mechanisms for establishment of distinct plasmalemma domains were proposed.

The existence of a barrier within the haustorial neck wall of A. candida to diffusion of solutes was indicated by the absence of withdrawal of the host PM from haustoria in plasmolysed tissue. The possibility that the impermeability depends on deposition of silicon (as reported for rust neckbands) was disproved by the absence of this element from haustorial necks of A. candida and Erysiphe pisi examined by X-ray microanalysis.

In barley coleoptiles infected by Erysiphe graminis hordei, the distribution of ATPase, which was confined to the host wall-lining plasmalemma, and the results of fluorescence microscopy using acridine orange indicated that an electrochemical gradient exists between the haustorium and the exterior of the host cell.

The results of all the investigations have been discussed in relation to the mechanism of nutrient absorption by haustoria.

## Acknowledgements

I am deeply grateful to Dr. J.L. Gay for his continuous and very patient guidance and friendship during the course of this work. Our numerous discussions have stimulated my interest in electron microscopy and plant pathology. His supervision could not have been better.

I would also like to thank Miss Marion Martin and Mr. Norman Costa for their excellent technical assistance and helpful advice, my other friends and colleagues for their cheerful camaraderie and encouragement and Mrs. S. Westcott for efficiently typing this thesis.

In addition, I am grateful to Dr. R.J. Zeyen, University of Minnesota, for his advice and hospitality during my short period of research in his laboratory, and also to Mr. G.G. Ahlstrand for his technical assistance.

Finally, I thank my parents and friends outside college for their continued concern and interest without which this thesis could not have been completed.

TABLE OF CONTENTS


| | Page |
|---|---|
| ABSTRACT | 2 |
| ACKNOWLEDGEMENTS | 4 |
| TABLE OF CONTENTS | 5 |
| LIST OF ILLUSTRATIONS | 9 |
| SECTION 1 INTRODUCTION | 13 |
| SECTION 2 GENERAL MATERIALS AND METHODS | 19 |
| 2.1 Plants and Pathogens | 19 |
| 2.1.1 *Albugo candida* - *Cardamine hirsuta* | 19 |
| 2.1.2 *Erysiphe pisi* - *Pisum sativum* | 19 |
| 2.1.3 *Erysiphe graminis* - *Hordeum vulgare* | 19 |
| 2.1.4 *Puccinia poarum* - *Tussilago farfara* | 20 |
| 2.1.5 *Bremia lactucae* - *Lactuca sativa* | 20 |
| 2.2 Electron Microscopy | 20 |
| 2.2.1 General fixation procedure | 20 |
| 2.2.2 Sectioning and staining for routine observation | 21 |
| 2.2.3 Cytochemical staining procedures | 22 |
| 2.2.3.1 Phosphotungstic acid - Chromic acid technique (PACP) | 22 |
| 2.2.3.2 Periodic acid-thiocarbohydrazide-silver proteinate technique (PATCHSP) | 22 |
| 2.2.4 Localization of ATPase activity | 23 |
| SECTION 3 EVIDENCE FOR A NECKBAND DELIMITING STRUCTURAL AND PHYSIOLOGICAL REGIONS OF THE HOST PLASMA MEMBRANE ASSOCIATED WITH HAUSTORIA OF *ALBUGO CANDIDA* | 24 |
| 3.1 Introduction | 24 |
| 3.2 Materials and Methods | 25 |

| | | Page |
|---|---|---|
| | 3.2.1 Fixation and embedding of material for differential staining | 25 |
| | 3.2.2 Cytochemical staining methods | 25 |
| | 3.2.3 Localization of ATPase activity | 25 |
| | 3.3 Results | 26 |
| | 3.4 Discussion | 31 |
| | Plates 3:1 - 3:12 | 38-42 |
| SECTION 4 | THE RELATIONSHIP BETWEEN THE HOST ER AND THE EXTRAHAUSTORIAL MEMBRANE IN THE INFECTION OF C. HIRSUTA BY A. CANDIDA | 43 |
| | 4.1 Introduction | 43 |
| | 4.2 Materials and Methods | 43 |
| | 4.2.1 Osmium ferricyanide (OsFeCN) | 44 |
| | 4.2.2 Zinc iodide-osmium tetroxide (ZIO) | 44 |
| | 4.2.3 High voltage electron microscopy (HVEM) | 45 |
| | 4.3 Results | 45 |
| | 4.3.1 Osmium ferricyanide | 45 |
| | 4.3.2 Zinc iodide-osmium tetroxide | 46 |
| | 4.3.3 High voltage electron microscopy | 46 |
| | 4.4 Discussion | 47 |
| | Plates 4:1 - 4:7 | 50-52 |
| SECTION 5 | THE PERMEABILITY OF THE HAUSTORIAL NECK OF A. CANDIDA | 53 |
| | 5.1 Introduction | 53 |
| | 5.2 Materials and Methods | 53 |
| | 5.2.1 Experiments with a fluorescent apoplastic marker | 53 |
| | 5.2.2 Experiments with an electron dense apoplastic marker | 54 |
| | 5.2.3 Experiments with hypertonic treatment | 55 |
| | 5.3 Results | 57 |

Page

5.3.1 Experiments with a fluorescent apoplastic marker 57

5.3.2 Experiments with an electron dense apoplastic marker 57

5.3.3 Experiments with hypertonic treatment 57

5.4 Discussion 58

Plates 5:1 - 5:7 61-63

SECTION 6 X-RAY MICROANALYSIS OF HAUSTORIAL NECKBANDS AND INFECTED CELLS 64

6.1 Introduction 64

6.2 Materials and Methods 66

6.2.1 Preparation of *Albugo candida* infections of *Cardamine hirsuta* 66

6.2.2 Preparation of *Erysiphe pisi*-infected *Pisum sativum* 66

6.2.3 Isolation of haustorial complexes 67

6.2.4 Analytical procedure and treatment of data 67

6.3 Results 72

6.3.1 *Albugo candida* infecting *Cardamine hirsuta* 72

6.3.2 *Erysiphe pisi* infecting *Pisum sativum* 73

6.4 Discussion 80

Plates 6:1 and 6:2 83

SECTION 7 THE HOST-PATHOGEN INTERFACE OF *TUSSILAGO FARFARA* INFECTED BY *PUCCINIA POARUM* 84

7.1 Introduction 84

7.2 Materials and Methods 85

7.2.1 Fixation and embedding of material for differential staining 85

7.2.2 Cytochemical staining methods 85

| | | Page |
|---|---|---|
| | 7.2.3 Localization of ATPase activity | 85 |
| | 7.3 Results | 86 |
| | 7.4 Discussion | 89 |
| | Plates 7:1 - 7:25 | 100-112 |
| SECTION 8 | MODIFICATION OF THE HOST PM OF LACTUCA SATIVA INFECTED BY BREMIA LACTUCAE | 113 |
| | 8.1 Introduction | 113 |
| | 8.2 Materials and Methods | 113 |
| | 8.2.1 Fixation and embedding of materials for differential staining | 113 |
| | 8.2.2 Cytochemical staining methods | 113 |
| | 8.3 Results | 114 |
| | 8.4 Discussion | 115 |
| | Plates 8:1 - 8:7 | 119-121 |
| SECTION 9 | INVESTIGATIONS OF THE PHYSIOLOGY OF HAUSTORIA IN VIVO | 122 |
| | 9.1 Introduction | 122 |
| | 9.2 Materials and Methods | 123 |
| | 9.2.1 Preparation of coleoptiles | 123 |
| | 9.2.2 ATPase localization | 125 |
| | 9.2.3 Experiments with acridine orange | 125 |
| | 9.3 Results | 126 |
| | 9.3.1 ATPase localization | 126 |
| | 9.3.2 Experiments with acridine orange | 127 |
| | 9.4 Discussion | 129 |
| | Plates 9:1 - 9:5 | 136-137 |
| SECTION 10 | GENERAL SUMMARY AND CONCLUSIONS | 138 |
| REFERENCES | | 146 |

## List of Illustrations


| Plates | | Page |
|---|---|---|
| 3:1,2 | *Albugo candida* infecting *Cardamine hirsuta* incubated with ATP | 38 |
| 3:3 | Haustorium stained using the PACP technique | 39 |
| 3:4-6 | Haustorium and host Golgi body, ER and nuclear envelope stained by the PACP technique | 40 |
| 3:7,8 | Haustorium stained by the PATCHSP procedure | 41 |
| 3:9-12 | Serial sections of haustorium, host ER and nuclear envelope stained by the PATCHSP procedure | 42 |
| 4:1,2 | *A. candida* infecting *C. hirsuta* prepared by the OsFeCN procedure | 50 |
| 4:3 | Haustorium infecting mesophyll cell prepared by the OsFeCN procedure | 51 |
| 4:4,5 | *A. candida* infecting *C. hirsuta* and an uninfected cell prepared by the ZIO technique | 52 |
| 4:6,7 | Stereo pair of HVEM micrographs of thick section of *A. candida* infecting *C. hirsuta* stained by the PATCHSP procedure | 52 |
| 5:1-4 | Light micrographs and fluorescent light micrographs of *A. candida* infecting stem cortex of *C. hirsuta* infiltrated with Tinopal | 61 |
| 5:5 | *A. candida* infecting leaf of *C. hirsuta* injected with uranyl acetate solution | 62 |
| 5:6 | Haustorium in plasmolysed mesophyll cell | 63 |
| 5:7 | Encased haustorium in plasmolysed mesophyll cell | 63 |
| 6:1 | *A. candida* infecting *C. hirsuta* prepared from unosmicated material for subsequent X-ray microanalysis | 83 |
| 6:2 | Scanning electron micrograph of haustorium of *Erysiphe pisi* infecting epidermal cell of *Pisum sativum* | 83 |
| 7:1 | Monokaryotic stage of *Puccinia poarum* infecting *Tussilago farfara* | 100 |

| | | Page |
|---|---|---|
| 7:2 | M-haustoria infecting phloem parenchyma and transfer cells | 101 |
| 7:3 | Enclosed M-haustorium in vascular parenchyma cell | 101 |
| 7:4,5 | Enclosed M-haustorium stained by the PACP or the PATCHSP procedure | 102 |
| 7:6,7 | M-haustoria stained by the PACP or PATCHSP procedure | 103 |
| 7:8,9 | M-haustoria stained by the PACP or PATCHSP procedure | 104 |
| 7:10 | M-haustorium of P. poarum stained by the PACP procedure | 105 |
| 7:11,12 | Partially enclosed M-haustorium stained by the PACP procedure and transition in staining characteristics of extrahaustorial membrane | 106 |
| 7:13 | Transition in PACP staining characteristics of host PM invaginated around partially enclosed M-haustorium | 107 |
| 7:14 | Partially enclosed M-haustorium stained with uranyl acetate-lead citrate | 107 |
| 7:15,16 | Partially enclosed M-haustorium infecting T. farfara incubated with ATP, observed unstained or with PATCHSP staining | 108 |
| 7:17,18 | M-haustoria infecting xylem parenchyma cells, incubated with ATP | 109 |
| 7:19,20 | Enclosed M-haustorium infecting T. farfara incubated with ATP, observed unstained or with PATCHSP staining | 110 |
| 7:21 | Enclosed M-haustorium infecting transfer cell, incubated with ATP | 110 |
| 7:22 | M-haustoria infecting T. farfara, incubated with βGP | 111 |
| 7:23 | M-haustorium infecting T. farfara, incubated without substrate | 111 |
| 7:24 | Transfer cells of uninfected T. farfara, incubated with ATP | 112 |
| 7:25 | Transfer cells of uninfected T. farfara, incubated with ATP and sodium orthovanadate | 112 |

| | | Page |
|---|---|---|
| 8:1,2 | Bremia lactucae infecting Lactuca sativa, stained by the PACP technique | 119 |
| 8:3 | Haustorium infecting mesophyll cell, stained by the PACP technique | 120 |
| 8:4 | High magnification of haustorial PM and invaginated host PM | 120 |
| 8:5 | Encased haustorium infecting necrotic host cell | 120 |
| 8:6 | Haustorium infecting mesophyll cell, stained by the PACP technique | 121 |
| 8:7 | Uninfected mesophyll cell of L. sativa, stained by the PACP technique | 121 |
| 9:1,2 | Erysiphe graminis hordei infecting coleoptile epidermal cell of Hordeum vulgare, incubated with ATP | 136 |
| 9:3 | Haustorium infecting coleoptile epidermal cell, incubated with βGP | 137 |
| 9:4 | Haustorium infecting coleoptile epidermal cell, incubated with ATP and sodium orthovanadate | 137 |
| 9:5 | Haustorium infecting coleoptile epidermal cell, incubated without substrate | 137 |


Figures


| | | |
|---|---|---|
| 6:1 | Procedure to isolate haustorial complexes from infected leaves | 68-69 |
| 6:2,3 | X-ray spectra from thin sections of A. candida infecting C. hirsuta | 74 |
| 6:4-8 | X-ray spectra from epidermal cells of P. sativum infected by an haustorium of E. pisi | 74-75 |
| 6:9-16 | Histograms of the distribution of Si within epidermal cells of P. sativum infected by E. pisi | 76-79 |
| 9:1 | Diagram of mounts for coleoptiles of H. vulgare infected by E. graminis hordei | 124 |
| 9:2 | Diagrams of transverse sections of coleoptiles of H. vulgare to show their preparation for infection by E. graminis hordei | 124 |

| | Tables | Page |
|---|---|---|
| 3:1 | Reaction of host membranes and fungal wall of C. hirsuta infected by A. candida to specific treatments | 29 |
| 5:1 | Means and standard deviations of widths of extrahaustorial matrices around haustoria of A. candida infecting C. hirsuta in plasmolysed and unplasmolysed tissue | 56 |
| 9:1 | Response of cells of H. vulgare and haustoria of E. graminis hordei mounted in acridine orange to the application of inhibitors | 128 |
| 10:1 | The degree of specialization of haustoria of members of the Peronosporales, Uredinales and Erysiphales | 142-143 |

# 1. Introduction

Many fungal parasites penetrate host cells with specialised structures called haustoria. The term is derived from the Latin which means 'drawer of water' and, according to Rice (1927), the structure was first identified by Visiani in 1851. Detailed descriptions of haustoria have since been obtained by light microscopic studies by Smith (1900), Rice (1927) and others although most advances have been made since the advent of electron microscopy.

Bushnell (1972) defined the haustorium as 'a specialized organ which is formed inside a living host cell as a branch of an extracellular (or intercellular) hypha or thallus, which terminates in that host cell, and which probably has a role in the interchange of substances between host and fungus'. In attempts to understand the role of this specialized structure formed by biotrophic fungal pathogens, both structural and physiological characteristics of the haustorium and associated components of the invaded host cell have been examined.

Evidence has accumulated which demonstrates the importance of haustoria in nutrient uptake by powdery mildews, rusts and downy mildews. In experiments with a microculture system consisting of young colonies of Erysiphe graminis f. sp. hordei growing on a single layer of barley epidermis in specially prepared coleoptiles (Bushnell, Dueck & Rowell, 1967), infected cells were incised using a microneedle and haustoria were excised. It was found that although the surface hyphae remained alive and could grow slightly, normal growth rates were not possible without an haustorium. In addition, Ellingboe and co-workers (Mount & Ellingboe, 1969; Slesinski & Ellingboe, 1971) showed that the establishment of a primary haustorium by cereal powdery mildews is essential for the transport of $^{32}P$- and $^{35}S$-labelled compounds from host to pathogen.

In autoradiographic studies with crown rust-infected oat (Onoe, Tani & Haito, 1973 cited in Manners & Gay, 1983) tritiated pyrimidine nucleosides supplied to the host were only utilized after haustorium formation, although $^{14}CO_2$ and $^{3}H$ glucose were incorporated previously. The utilization of tritiated leucine by Bremia lactucae was shown by Andrews (1975) to occur only after haustorium formation, although tritiated glucose was utilized before penetration as well as throughout growth. All these experiments indicate that the haustorium has a role in nutrient transport to the fungus. More direct evidence of nutrient uptake by haustoria was obtained by Manners & Gay (1978, 1980) who isolated haustorial complexes of E. pisi from infected plants. Haustorial complexes comprise the haustorium surrounded by the extrahaustorial matrix and extrahaustorial membrane. When isolated from pea leaves which had been photosynthesising in the presence of $^{14}CO_2$ the isotope was detected within the haustorial complex. Subsequently $^{14}CO_2$ was released by respiration by the complexes in vitro which indicated strongly that the haustorial cytoplasm, not just the extrahaustorial matrix, contained the isotope.

Evidence of the haustorium as an organ for the passage of nutrients from host to pathogen has therefore been obtained for powdery mildew , rust and downy mildew infections. However, in order to formulate an hypothesis for the mechanism of nutrient transport, the structural characteristics of haustoria and the associated host components have also to be considered.

It has been established by electron microscopy that the host PM is continuous around the haustoria of powdery mildews (Gil & Gay, 1977), rusts (Coffey, Palevitz & Allen, 1972; Hardwick, Greenwood & Wood, 1971; Littlefield & Bracker, 1970; Littlefield & Bracker, 1972) and downy mildews (Peyton & Bowen, 1963; Berlin & Bowen, 1964; Ehrlich & Ehrlich,

1966; Hanchey & Wheeler, 1971; Pares & Greenwood, 1979). However, the region invaginated around both powdery mildew and rust haustoria has been shown to be structurally distinct from the host wall lining region. The extrahaustorial membrane in powdery mildew infections has been shown to be thicker than the normal host plasma membrane (Bracker, 1968; Gil & Gay, 1977) and is highly resistant to osmotic stress (Bracker, 1968; Gil & Gay, 1977). A difference in staining reaction between the invaginated and uninvaginated regions has also been found in studies of powdery mildew (Gil, 1976), rust (Littlefield & Bracker, 1972; Heath, 1979) and downy mildew (Hickey & Coffey, 1978; Pares & Greenwood, 1979) infections. With both the rust and downy mildews the invaginated part of the host plasmalemma did not stain with the PACP procedure whereas the uninvaginated part stained normally. Investigation of powdery mildew infections with the silver methanamine technique, specific for polysaccharide, showed that the extrahaustorial membrane contained much more polysaccharide than the wall-lining host plasmalemma (Gil, 1976). In addition, both powdery mildew (Gil & Gay, 1977) and rust (Littlefield & Bracker, 1972) extrahaustorial membranes lacked intra-membrane particles which occur in freeze fracture surfaces of the uninvaginated plasma membrane.

An important physiological difference was demonstrated between the two regions of host PM in recent enzyme cytochemical investigations of powdery mildew and rust infections. ATPase activity is absent from the membrane surrounding the haustorium but present in normal amounts on the wall-lining PM (Spencer-Phillips & Gay, 1981).

In all cases, the transition in characteristics of the two regions of plasmalemma occurs where electron dense structures, termed the Neckbands or Neckrings are present in and around the haustorial neck wall. A neckband was first observed by light microscopy by Rice in 1927 and was re-discovered in 1971 by Hardwick, Greenwood and Wood using electron microscopy. It was suggested by Gil and Gay (1977) that neckbands are

stabilizing structures maintaining the two distinct domains of the host plasma membrane, the molecules of which would otherwise, according to the fluid mosaic model of Singer & Nicholson (1972), be expected to diffuse laterally and so mix freely.

Evidence has accumulated to suggest that the neckband also forms an apoplastic seal separating the extrahaustorial matrix from the general apoplast. This has been most clearly demonstrated by experiments with powdery mildew infections. When cells infected by powdery mildew haustoria were plasmolysed, the protoplast did not retract from the haustorium as it did from the host cell wall and seemed to be attached to the haustorium at what has since been identified as the neckband (Hoskin, 1969 cited in Bushnell & Gay, 1978). The existence of a seal around necks of powdery mildew haustoria has also been indicated by the swelling and contraction of the extrahaustorial matrix when cells infected by Erysiphe graminis f. sp. hordei in a microculture system were incised. This procedure was thought to expose the membrane invaginated around the haustorium (some host cytoplasm probably remained (Sullivan, Bushnell & Rowell, 1974) which was then subjected to hypotonic and hypertonic media (Hoskin, 1969 cited in Bushnell & Gay, 1978). A similar expansion and contraction of the extrahaustorial matrix in response to different osmotica was observed by Hirata & Kojima (1962) (cited in Bushnell & Gay, 1978) after they had pulled haustorial complexes (then described as a haustorium in a sack) from host cucumber cells, thus demonstrating that this complex formed a sealed compartment. Powdery mildew haustorial complexes have since been isolated by Bracker (1968) and in far larger numbers by Dekhuijzen (1966), Gil & Gay (1977) and Manners & Gay (1977). Gil & Gay identified two neckbands, A and B, around necks of Erysiphe pisi haustoria. When haustorial complexes were placed in hypotonic media the extrahaustorial matrix swelled, the

extrahaustorial membrane remaining tightly bound to the haustorial neck wall in the B neckband region. Electron microscopy showed the host PM closely adherent to the haustorial wall at both A and B neckbands. The seal was further confirmed by Gay and Manners (in preparation) when haustorial complexes were treated with the apoplastic marker uranyl acetate and examined by electron microscopy. After precipitation with $PO_4^{3-}$, uranyl crystals were absent from haustorial complexes with intact extrahaustorial membranes.

Experiments with other pathogens have given conflicting evidence for a seal at the neckband. Plasmolysis of rust and downy mildew infected cells (Fraymouth, 1956; Thatcher, 1942) succeeded in separating the protoplast from the haustorium suggesting that the host plasmalemma is not attached to the haustorium at the neckband. However, a permeability experiment carried out by Heath (1976) with Puccinia sorghi infecting maize indicated that the neckband does form an apoplastic seal. After infected leaves had transpired a solution of uranyl acetate for 24h the material was prepared for electron microscopy and the distribution of uranyl crystals was observed. In the haustorial neck wall crystals were found up to, but not beyond, the neckband. This result was confirmed by light microscopic studies of rusted cowpea leaves infiltrated with a fluorescent reagent which does not readily penetrate protoplasts. Although intercellular hyphae and host cell walls fluoresced, mature haustoria in intact host cells did not.

Thus, the essential features of the haustorial infections which have been examined extensively are that the part of the host plasmalemma enveloping haustoria is differentiated whereas the remainder is normal. The two domains are separated and maintained by recognizable neckbands which are also apoplastic barriers to solute diffusion. It has been proposed by Spencer-Phillips & Gay (1981) that this couples the transport

systems of an haustorium and the cell which it infects, and the absence of ATPase activity from the region of host plasmalemma adjacent to the haustorium polarizes the transport towards the fungus. The neckband seems to be a key feature of this transport hypothesis and therefore it was decided to investigate the generality of plasmalemma differentiation within cells infected by haustoria with no recognizable neckbands, which can still maintain a biotrophic relationship. The composition and physiology of neckbands and analogous structures were also investigated and an attempt was made to determine the origin and development of the extrahaustorial membrane. Finally, in an attempt to gain direct physiological evidence for a solute transport mechanism, preliminary experiments were carried out on living, infected cells, using fluorescent probes.

# 2. General Materials and Methods

## 2.1 Plants and Pathogens

### 2.1.1 Albugo candida - Cardamine hirsuta

Albugo candida (Pers.) Kuntze was obtained as a natural infection on Cardamine hirsuta L. This was maintained in a greenhouse at a temperature of 15-20°C with normal winter daylength (approximately 9h light, 15h dark). New infections were established by spraying a mixture of infected and uninfected plants at dusk with distilled water until dripping occurred. Alternatively, mature blisters on leaves and stems were broken open under distilled water to release the sporangia. The sporangial suspension was chilled for 3-4h at 10°C to encourage zoospore formation when droplets were placed on the leaves of C. hirsuta using a pasteur pipette. After 7-8 days white blisters were usually visible.

### 2.1.2 Erysiphe pisi - Pisum sativum

Erysiphe pisi D.C. ex St. Amans inoculum was the strain used in successive investigations in the department following Gil & Gay (1977). Young plants of Pisum sativum L. cv. Onward supplied by the Botanical Supply Unit of the University of London and grown for 10 days in a growth cabinet with 12h light (24°C) and 12h dark (18°C) periods were inoculated using a settling tower and re-incubated in a growth cabinet providing the same conditions.

### 2.1.3 Erysiphe graminis - Hordeum vulgare

Erysiphe graminis DC. ex Merat f. sp. hordei Em. Marchal ($V_o$, originally obtained from Plant Breeding Institute, Cambridge) infecting Hordeum vulgare L. cv Proctor ($R_o$) was maintained in a growth cabinet at 20°C with 12h light and 12h dark. Plants were infected by shaking

heavily infected plants over them. By 7 days sporulating lesions had developed and were then used to inoculate coleoptiles of Hordeum vulgare L. cv Atlas ($R_o$).

Plants were raised for coleoptiles using a modified method from Professor W.R. Bushnell's laboratory in St. Paul, Minnesota. Plant pots, 100mm square, were filled to the ledge with a mixture of Fisons potting compost (50%) and medium coarse sand (50%) and watered with 215ml tap water. Seeds were then sown in 4 rows of 5 and the pots filled with the soil-sand mixture. After damping the soil surface, the pots were placed in a tray in a growth cabinet at 20°C. On the third day a 1:100 dilution of a concentrate prepared by mixing 58g $NH_4NO_3$, 58g KCl and 58g $NH_4H_2PO_4$ in 1L water was added to the tray at the rate of 125ml per pot. Subsequently, daily additions of tap water, at the rate of 63ml per pot were made to each tray.

### 2.1.4 Puccinia poarum - Tussilago farfara

An approximately 2 week-old infection by the pycnial-aecial phase of Puccinia poarum (Niels.) on Tussilago farfara L. was kindly supplied by Dr. D.M. Lösel from the University of Sheffield.

### 2.1.5 Bremia lactucae - Lactuca sativa

Fixed and embedded pieces of leaves of Lactuca sativa L. either uninfected or with a 4 day infection by Bremia lactucae Regel were kindly supplied by Dr. J. Mansfield from Wye College, University of London.

## 2.2 Electron Microscopy

### 2.2.1 General Fixation Procedure

Leaves of the plant under examination were cut into 1mm squares under a fixative solution of 2.5% glutaraldehyde and 0.1M sodium

cacodylate buffer at pH 7. The pieces were left in the fixative for 24h at room temperature (20-25°C) and then washed in the buffer 3 times, each for 20 min. The specimens were post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate (pH 7) for 1-2h at room temperature. (This stage of the procedure was omitted when un-osmicated tissue was required.) The material was dehydrated in 10 min stages using a 10% graded series of ethanol. Two changes in 95% and three changes in 100% were made and then the material was processed for embedding as follows: successive 10 min periods in ethanol/epoxypropane (2:1), ethanol/epoxypropane (1:2) and 100% epoxypropane. The specimens were then transferred to epoxypropane/epon (70:30) for 1h in a closed container which was then left open overnight at room temperature to allow the epoxypropane to evaporate. The embedding medium, prepared to give a resin of medium hardness, was made according to Luft's (1961) 1A:7B mixture. Epon 812 was obtained from TAAB Laboratories and DDSA from Searle diagnostic. The specimens were then transferred to fresh resin on four occasions over 2 days before polymerization at 60°C for 60h in a flat PVC embedding dish.

2.2.2 Sectioning and Staining for Routine Observation

The specimen to be sectioned was sawn from the polymerized disc of resin so that it was situated near the apex of a pyramid with a base of 3-4mm. This was then mounted with Durofix on a wooden dowell rod, approximately 10mm long. The resin was then trimmed using Wilkinson's razor blades to form a flat topped four-sided pyramid around the specimen, the rectangular block face measuring 0.5-1.0mm x 0.5mm. Sections were then cut on either a Reichert OM U2 or an LKB 4802A ultramicrotome using a diamond knife (IVIC). The sections were then mounted on Formvar coated 100 mesh copper grids. Mounted sections with silver interference colour were stained in drops of 2% aqueous uranyl acetate for 30 min at 60°C, rinsed with distilled water, and further stained in drops of

Reynold's lead citrate which were placed in a covered petri dish containing a very strong solution of sodium hydroxide to absorb the carbon dioxide from the air, thereby preventing lead precipitates forming on the sections. After 10 min the mounted sections were rinsed thoroughly with distilled water, dried using triangles of filter paper and the heat from a lamp and then stored in capsule cards.

Sections were viewed with either a Philips 301G or an AEI 6B transmission electron microscope at 80 keV.

### 2.2.3 Cytochemical Staining Procedures

Sections with a pale gold interference colour were cut as described above and mounted on either unfilmed 200 mesh gold grids or Formvar coated 100 mesh gold grids.

#### 2.2.3.1 Phosphotungstic Acid - Chromic Acid Technique (PACP)

This method was adapted from Roland, Lembi & Morré (1972). Sections on 200 mesh gold grids were immersed in 1% periodic acid for 30 min, washed in double distilled water, stained for 15 min in an aqueous solution of 10% chromic acid and 1% phosphotungstic acid (PTA) and then again washed in double distilled water.

For a control, treatment with chromic acid and PTA was omitted.

#### 2.2.3.2 Periodic Acid - Thiocarbohydrazide-Silver Proteinate Technique (PATCHSP)

This method was adapted from Thiéry (1967). Sections on Formvar coated 100 mesh gold grids were immersed in 1% periodic acid for 30 min, washed in distilled water and then transferred to 0.2% thiocarbohydrazide (TCH) in 20% acetic acid for 3 days. After washing in a series of acetic acid solutions (20, 15, 10 and 5%) for 5 min at each concentration and finally in double distilled water, the grids were transferred

to an aqueous solution of 1% silver proteinate for 30 min in the dark. Freshly-prepared solutions of TCH and silver proteinate were always used.

Control treatments, proposed by Courtoy & Simar (1974), omitted either periodic acid, TCH or silver proteinate treatments.

2.2.4 Localization of ATPase Activity

The method employed was a modification of that used by Spencer-Phillips & Gay (1981) which had been adapted from Bentwood & Cronshaw (1978). Infected and uninfected leaves were immersed in fixative and pieces 0.5-1.0$mm^2$ were excised. The fixative was a mixture of purified glutaraldehyde (Polaron) (0.5% v/v) and formaldehyde (4% w/v) buffered at pH 7.2 with sodium cacodylate (0.1M). The formaldehyde was freshly prepared from a solution of paraformaldehyde in distilled water (dissolved at 60-70$^{o}$C) to which drops of NaOH (1N) were added until clear. After 1.5-2h the specimens were washed at 4$^{o}$C for 2-3h in 3-4 changes of tris-maleate buffer (0.1M) at pH 7.2. They were then immersed for 30 min in the medium employed in the subsequent incubation, but without substrate. The specimens were transferred to a mixture of either ATP (2mM), magnesium nitrate (2mM) and lead nitrate (2mM) in tris-maleate buffer (0.1M) at pH 7.2, or, for control treatments, with β-glycerophosphate (2mM) replacing ATP, with ATP and an inhibitor (either 10mM NaF or 150μM Sodium orthovanadate or without any substrate in the medium. After incubation at room temperature for 2.5h, the specimens were washed for 1h with 3-4 changes of sodium cacodylate buffer (0.1M) at pH 7.2, post-fixed with osmium tetroxide (1% w/v) in the same buffer for 1h and dehydrated, embedded and sectioned according to the procedure described in sections 2.2.1 and 2.2.2. Sections mounted on Formvar-coated 100 mesh copper grids were examined either unstained or after uranyl acetate and lead citrate staining.

## 3. Evidence for a Neckband Delimiting Structural and Physiological Regions of the Host Plasma Membrane Associated with Haustoria of Albugo candida

Published as a paper by A.M. Woods and J.L. Gay (1983) Phys. Plant Path. 23, 73-88.

### 3.1 Introduction

Considerable evidence has accumulated to show continuity of the host plasma membrane around haustoria of rusts (Coffey, Palevitz & Allen, 1972; Ehrlich & Ehrlich, 1971; Hardwick, Greenwood & Wood, 1971; Littlefield & Bracker, 1970; Littlefield & Bracker, 1972), powdery mildews (Gil & Gay, 1977), and downy mildews (Berlin & Bowen, 1964; Ehrlich & Ehrlich, 1966; Hanchey & Wheeler, 1971; Pares & Greenwood, 1979; Peyton & Bowen, 1963). However, the invaginated region of the membrane has been found to be structurally distinct from the wall-lining region in both powdery mildew (Gil & Gay, 1977; Manners & Gay, 1977) and rust (Littlefield & Bracker, 1972) infections. In addition, recent research has demonstrated a physiological difference between the two domains of the host plasma membrane in both types of infection, and this has provided a basis for an hypothesis for nutrient transport into haustoria (Spencer-Phillips & Gay, 1981). Wherever distinct domains have been observed the transition regions are marked by structures, termed neckbands, visible in and around the haustorial neck wall. Since, according to the fluid mosaic model for membranes (Singer & Nicholson, 1972), the molecules of two regions of a plasmalemma are free to diffuse laterally and so mix freely, the evidence indicates that the neckband forms a stabilizing structure which maintains the two distinct domains of the host plasma membrane. This accounts for the lack of modification shown by the wall-lining region of the host plasma membrane and it has been proposed that this feature is essential for the transport of nutrients into haustoria (Spencer-Phillips & Gay, 1981).

In Ooomycete infections only Hickey & Coffey (1978) with Peronospora pisi, and Pares & Greenwood (1979) with Pseudoperonospora humuli have demonstrated structural distinctness of the invaginated from the uninvaginated regions of host plasma membranes, but neither physiological differences nor structures corresponding to neckbands have been described. Therefore, a thorough investigation of an Albugo candida infection has been made using three techniques to distinguish structural and physiological characteristics of the membranes, and special structures associated with the transition.

Albugo candida is an Oomycete classified in the Peronosporales and was chosen for this study since it is one of the most biotrophic of this group and therefore seemed likely to have structures comparable to those of rust and powdery mildew infections.

## 3.2 Materials and Methods

### 3.2.1 Fixation and Embedding of Material for Differential Staining

Pieces of naturally infected leaves and stems of Cardamine hirsuta bearing young blisters of Albugo candida were fixed, post-fixed in $OsO_4$, dehydrated, embedded and sectioned according to the procedure described in Sections 2.2.1 and 2.2.2.

### 3.2.2 Cytochemical Staining Methods

Sections mounted on gold grids were stained by either the PACP or PATCHSP techniques as described in Sections 2.2.3.1 and 2.2.3.2.

### 3.2.3 Localization of ATP-ase Activity

The method employed was the same as used by Spencer-Phillips & Gay (1981) which was as described in Section 2.2.4, but omitting the 30 min immersion in medium without substrate prior to the 2.5h incubation in

the reaction medium with substrate. Sodium fluoride (10mM) was used in the ATP + inhibitor control treatment.

## 3.3 Results

Observation with the electron microscope showed the collar, penetration jacket, haustorium and extrahaustorial matrix within the infected cells, as described in previous investigations of A. candida (Berlin & Bowen, 1964; Coffey, 1975), except that the penetration jacket was often ill-defined and variable in extent making it impossible to detect its limits with certainty. Continuity of the wall-lining region of the host plasma membrane with invaginations around individual haustoria was traced and shown to follow the contours of the collar, the haustorial neck distal to the collar, and the haustorial body (Plate 3:3). Both host and fungal plasma membranes were very closely associated with the fungal wall in a part of the neck which is referred to as region B (Plates 3: 1,2,3,7,8). Many vesicles and membranous cisternae could be seen in the host cytoplasm around the bodies of mature haustoria (Plates 3: 3-7). Since their contents always appeared identical to the extrahaustorial matrix and connections could often be seen between their membranes and the extrahaustorial membrane, so that an extensive network was sometimes present, they probably represent extensions of the matrix. Also, cisternae with contents staining differently from the matrix, and representing host endoplasmic reticulum were often seen in very close association with the extrahaustorial membranes, but few definite connections were seen (Plates 3: 5,6,9-12).

The special staining and enzyme localization techniques employed, selectively stained structures of the host-parasite interface to reveal various domains in the host and fungal plasma membranes and regions of the fungal cell wall.

In the experiments where ATP was used as a substrate, precipitates ranging from slightly granular, to granular, to flocculent were observed and indicated different degrees of ATP-ase activity. Deposits heavier than those obtained in the controls without substrate and the β-GP controls were confined to the wall-lining region of the host plasma membrane and were absent from the invagination (Plates 3: 1,2). β-GP gave precipitates at the host tonoplast. The sodium fluoride controls sometimes gave deposits on the wall-lining plasma membrane very similar to those obtained without sodium fluoride in the medium. The mesophyll cells usually showed relatively low ATP-ase activities on their plasma membranes (Plate 3: 2) but occasionally high levels were observed (Plate 3: 1). Infection did not alter the level of ATP-ase activity of the wall lining plasma membrane. No activity above the levels in any controls was observed on the fungal plasma membrane in haustoria (Plates 3: 1,2) but occasionally a reaction was shown in intercellular hyphae.

Additional evidence of differences between invaginated and wall-lining regions of the host plasma membrane was obtained when their reaction to the PACP staining technique was observed (Plates 3: 3-6). The host plasma membrane of uninfected cells and the wall-lining region of infected cells were usually heavily stained although there was some variation of intensity within the same cell (Plates 3: 3-6). However, the extrahaustorial region was only lightly to moderately stained (Plates 3: 3-6). The only exceptions were the membranes of the cisternae and the periphery of the matrical network around mature haustoria which were frequently as electron dense as the wall-lining region (Plate 3: 3-6). Thus, two distinct types of invaginated plasmalemma can be identified - the normal (modified) and the cisternal-network periphery. In both the intercellular hyphae and the haustoria, the fungal plasma membrane was more intensely stained than any part of the host plasma membrane.

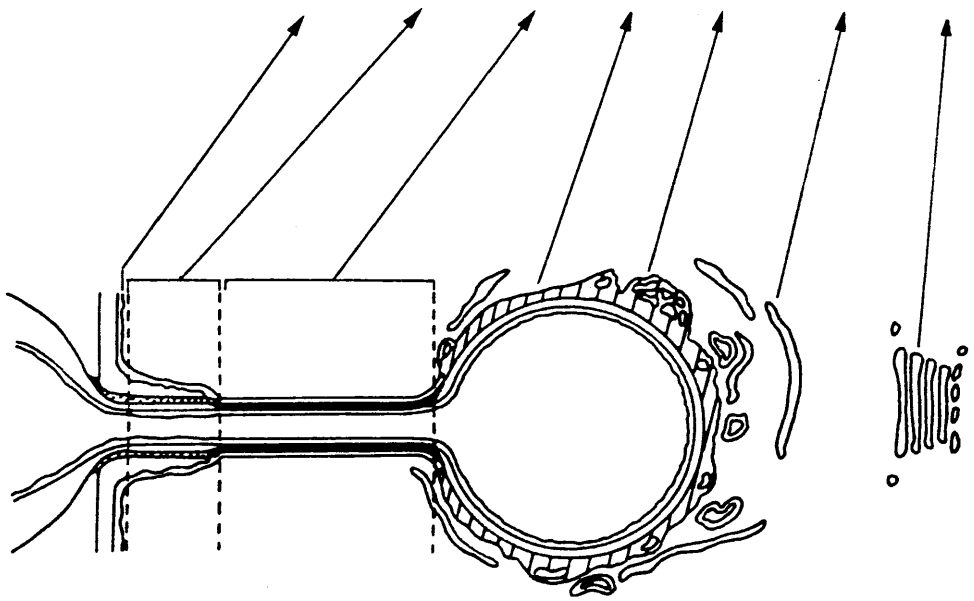
When material had been treated with the PATCHSP method both the wall-lining and most of the invaginated host plasma membrane appeared moderately granular (Plates 3: 7-12). However, the part of the host plasmalemma in the neck region was distinguished by being only lightly stained (Plates 3: 7,8). The fungal plasma membrane within this region was unstained in contrast to its intense granular staining elsewhere (Plates 3: 7,8).

Thus, distinctions between different regions of both host and fungal plasma membranes have been observed with all methods. In each case the transition occurred in region B. The ATP-ase activity of the host plasma membrane ends abruptly where the membrane comes into close contact with the wall of the haustorial neck (Plates 3: 1,2), the transition in staining characteristics of the host plasmalemma observed with the PACP technique occurs at the other edge of this wall-associated band (Plate 3: 3); and both the host and fungal plasma membranes in this annular region are distinguished by the PATCHSP technique (Plate 3: 8). In addition, within region B the fungal wall stains differently with both the PACP and PATCHSP methods. This region of special staining tapers towards its edges at the outer surface of the fungal wall. One edge is about one-third of the way along the neck from the penetration site and the other is just beyond the junction of the neck with the haustorial body. After treatment with the PACP technique, region B of the fungal wall is more densely stained than in other regions (Plate 3: 3). However, with the PATCHSP method the wall in region B appeared electron-transparent in contrast to the lightly stained walls of the intercellular hypha and haustorial body (Plates 3: 7,8). In uranyl acetate-lead citrate-stained material, this part of the neck wall can just be detected as slightly darker than normal (Plates 3: 1,2). All these observations are summarized in Table 3:1).

TABLE 3:1

*Reaction of host membranes and fungal wall to specific treatments*

| Treatment | | Wall lining region of host plasmalemma | Haustorial neck region | | Haustorial body region | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | B | Normal | Cisterna/ network periphery | Endoplasmic reticulum | Golgi body |
| ATP-ase | Host membrane | +/++ | +/++ | − | − | − | − | − |
| PACP | Host membrane | +++ | +++ | +++ | +/++ | +++ | +/++ | + |
| | Fungal wall | NA | + | +++ | + | + | NA | NA |
| PATCHSP | Host membrane | ++ | ++ | −[a] | ++ | ++ | ++ | ++ |
| | Fungal wall | NA | + | − | + | + | NA | NA |
| Uranyl acetate lead citrate | Host membrane | + | + | + | + | + | + | + |
| | Fungal wall | NA | + | +/++ | + | + | NA | NA |



[a] The fungal plasmalemma lacks stain in this region only.

−, No reaction; +, slight reaction; ++, moderate reaction; +++, intense reaction; NA, not applicable.

Diagram indicates regions examined.

Other features of infected cells were also differentially stained. After treatment by the PACP method the membranes of the ER and the nuclear envelope were lightly to moderately stained, similar to the normal type of extrahaustorial membrane with which they were closely associated (Plate 3: 5). All the membranes of the Golgi stack were only lightly stained (Plate 3: 4). The PATCHSP technique, however, did not distinguish between the endoplasmic reticulum, nuclear envelope or Golgi membranes, all being moderately to intensely stained as described above for the invaginated and wall-lining host plasma membrane.

The extrahaustorial matrix was moderately electron-dense after both the PACP and PATCHSP procedures, as were the contents of the extrahaustorial extensions and similar profiles for which connections with the extrahaustorial matrix could not be seen (Plates 3: 3-7). Usually, the contents of the endoplasmic reticulum were only lightly stained with both procedures, although some portions which were in very close association with the haustorium were more electron-dense (Plates 3: 9-12). With both the PACP and PATCHSP methods the Golgi stack showed a gradation in staining with unstained contents at one face and contents of moderate electron density, similar to the extrahaustorial matrix, at the other (Plate 3: 4).

Controls for PACP and PATCHSP Staining

All membranes were unstained in control sections of the PACP technique where chromic acid and PTA were omitted, illustrating the removal of osmium by the periodic acid. When periodic acid was omitted from the PATCHSP procedure, silver was deposited wherever osmium was present, due to the argentophilic nature of osmium. When either TCH or silver proteinate was omitted there was no deposition of silver.

## 3.4 Discussion

The types of deposits obtained in the ATP-ase localization experiments on A. candida were the same as those obtained in previous investigations (Browning, Hall & Baker, 1980; Spencer-Phillips & Gay, 1981). Sometimes the addition of sodium fluoride to the incubation medium did not alter the level of deposit on the wall-lining region of the host plasmalemma. However, it has been shown that sodium fluoride is neither a specific nor an efficient inhibitor of ATP-ase activity (Sexton & Hall, 1978). Furthermore, it is to be expected that non-enzymic hydrolysis of the substrate by lead would result in similar deposits with ATP and β-GP as substrates. This was not found and therefore the activity shown on the wall-lining plasma membrane is almost certainly due to a specific ATP-ase. The lack of enzymic activity at the extrahaustorial membrane cannot be attributed to the non-penetration of the lead trapping agent because, with β-GP, precipitates were observed at the host tonoplast. The types of staining obtained with the PACP and PATCHSP techniques and their controls agree with those previously observed on comparable material (Littlefield & Bracker, 1972; Hickey & Coffey, 1978).

The application of these methods has demonstrated both physiological and structural differences between the wall-lining and the invaginated regions of the host plasma membrane in cells infected by A. candida with abrupt transitions occurring at the haustorial neck (region B ). Thus, the results of this investigation indicate the presence of a stabilizing structure maintaining the two distinct domains of a continuous host plasma membrane. Region B may therefore be comparable to the neck bands of powdery mildews and rusts. The neckbands of powdery mildews and rusts do not show precisely the same morphology and differences even exist between the neckbands of individual rust species. Also, powdery mildews possess two neckbands, A and B (Gil & Gay, 1977), while rusts generally possess only one, although two adjacent bands, α and β, have

been observed in Puccinia coronata var. avenae (Chong & Harder, 1980). However, common characteristics are shared by rust and powdery mildew neckbands as listed below, and the properties of region B of the haustorial neck wall of A. candida will be discussed in relation to each of them:

(i) The composition of the fungal wall is abnormal within the neckband (or neck ring) as indicated by its different staining properties (Chong & Harder, 1980; Coffey, Palevitz & Allen, 1972; Ehrlich & Ehrlich, 1971; Gil & Gay, 1977; Hardwick, Greenwood & Wood, 1971; Heath, 1972; Heath & Heath, 1971; Manners & Gay, 1977; Perera & Gay, 1976; Van Dyke & Hooker, 1969).

Both the A and B bands of the powdery mildews and the neckband(s) of rusts are more electron-dense than elsewhere in the fungal wall after fixation including osmium tetroxide treatment and staining with uranyl and lead salts. Region B of the haustorial neck wall of A. candida is not especially electron-dense after uranyl acetate-lead citrate staining of osmium fixed material, but staining above the level in other regions of the fungal wall can just be discerned.

The neck ring of rusts (or the β band when two bands are observed) is preferentially etched away by periodic acid, sometimes leaving a hole in the section, to give an electron-transparent region when stained by the PACP (Littlefield & Bracker, 1972) or PATCHSP and PACP methods (Chong & Harder, 1980). Region B of A. candida also appeared electron-transparent after staining with PATCHSP, but was electron-dense after PACP. Thus, unlike the rust neck ring, this region of the wall was not completely removed by periodic acid. These results indicate that during the PATCHSP procedure osmium was removed by the periodic acid to give electron transparency, but aldehyde groups were not generated; and that PTA and chromic acid preferentially stain substances which are present in region B but absent elsewhere.

Thus, although region B is like the rust and powdery mildew neckbands in being structurally different from the normal fungal wall, it differs from them in composition.

(ii) Only in the region of the neckband are both the host and fungal plasma membranes closely associated, without undulation, with the surfaces of the neck wall (Gil & Gay, 1977; Hardwick, Greenwood & Wood, 1971; Littlefield & Bracker, 1972).

The host and fungal plasma membranes of A. candida infections are more closely associated with region B of the neck wall than in other regions. However, there is some undulation of the membranes indicating that the junction may not be as "tight" as in the other pathogens.

(iii) The neckband marks the point of transition in the characteristics of the host plasma membrane (Gil & Gay, 1977; Littlefield & Bracker, 1972; Spencer-Phillips & Gay, 1981).

The transitions of ATP-ase activity, PACP- and PATCHSP-staining occurred at one or other edge of region B of the haustorial neck wall of A. candida as described in the results.

(iv) The neckband forms an impermeable barrier preventing direct diffusion of solutes between the extrahaustorial matrix and the apoplast (Gay & Manners, in preparation; Heath, 1976).

This is an important criterion of a neck band which has yet to be investigated in A. candida infections (see Section 5).

The above comparisons provide strong evidence that region B in the haustorial neck wall of A. candida represents a neckband.

The findings of the present work agree with results of earlier investigations of A. candida (Berlin & Bowen, 1964; Coffey, 1975) although the interpretations differ. Berlin & Bowen (1964) noted the change of staining characteristics of the fungal wall at region B, but considered this to indicate the total absence of fungal wall. They suggested that the material present in its place was continuous with regions which are

now commonly referred to as the extrahaustorial matrix and the penetration jacket. Coffey (1975) established that the fungal wall was continuous around the haustorium, but the differently stained band was not reported. However, it can just be detected in his micrographs.

The electron transparency of region B observed after PATCHSP-staining may be due to the absence of polysaccharides or to their impregnation by additional substances which prevent them from reacting. The latter phenomenon was indicated in barley when, after staining for polysaccharide, clear halos were seen in epidermal cell walls in contact with Erysiphe graminis (Sargent & Gay, 1977). This was due to the deposition of silicon and cuticular lipids within the wall. Similarly, X-ray microanalysis has demonstrated the presence of silicon, phosphorous, chlorine and iron in the PATCHSP-negative β neckband of Puccinia coronata var. avenae (Chong & Harder, 1980). However, the α neckband of P. coronata was PATCHSP-positive despite containing silicon.

No evidence has yet emerged indicating the cause of differences between the invaginated and wall-lining regions of the host plasma membrane in biotrophic infections. Possibly the fungus exerts some direct influence on the host plasma membrane with which it is associated, so changing its structure and function. Alternatively, the invaginated plasma membrane may be formed in a different way from the normal host plasma membrane. According to the endomembrane concept (Morré & Mollenhauer, 1973), membrane derived from the nuclear envelope passes to the endoplasmic reticulum, then to transition vesicles which coalesce to form the cisternae of the Golgi body. Subsequently, secretory vesicles separate from the mature face of the Golgi and travel through the cytoplasm to fuse with the plasma membrane. However, in A. candida the frequently observed close association of the endoplasmic reticulum with the extrahaustorial membrane and the similar staining reaction of these two types of membrane with the PACP technique suggest that the extra-

haustorial membrane may be formed directly from the endoplasmic reticulum. A similar suggestion has been made for rust infections of wheat (Harder, Rohringer, Samborski, Kim & Chong, 1978), where frequent close associations and occasional connections between the endoplasmic reticulum and the invaginated plasma membrane have been observed. There, a much more extensive endoplasmic reticulum network (termed the endoplasmic reticulum complex) was present and it was suggested that it represented a modified Golgi-like structure. However, this hypothesis does not explain the intense staining of regions of the extrahaustorial membrane with PACP. Since these portions of the membrane react with PACP in the same way as the wall-lining plasma membrane, unlike any other host membrane, they may be derived either from the wall-lining plasma membrane or the same source as the latter. The common source explanation seems more likely since close associations of the membrane around the haustorial body with Golgi were more frequent than associations with the wall-lining plasma membrane. In addition, the PACP-positive regions were not present within the extrahaustorial membrane around young (as defined by Coffey (1975)) haustoria and therefore were unlikely to be derived from the original invagination of the host plasma membrane. The presence around the haustorial body of two different types of membrane which are continuous indicates that restriction is imposed on the fluidity within the extrahaustorial membrane. However, unlike in the neck region, a structure associated with the distinction is not apparent.

The localization of ATP-ase activity on the wall-lining plasma membrane suggests the presence of an ATP-energized proton pump such as that proposed by Baker (1978). The hydrolysis of ATP at the membrane is thought to result in the extrusion of protons from the cell, thus maintaining an electrochemical gradient of protons across the membrane, down which organic solutes are transported into the cell. The level of activity in infected and uninfected mesophyll cells was usually low but

similar to that observed in this tissue in pea (Bentwood & Cronshaw, 1978; Spencer-Phillips & Gay, 1981). This is probably because they contain chloroplasts and so manufacture their own photoassimilates, unlike epidermal cells which possess higher levels of ATP-ase activity on their plasma membrane. This presumably enables them to scavenge the apoplast for photosynthates.

The distribution of ATP-ase in plasma membranes of haustorium-infected cells of Cardamine hirsuta is similar to that described for powdery mildew and rust infections (Spencer-Phillips & Gay, 1981). The Albugo and rust infections investigated are also similar in the absence of ATP-ase activity from the haustorial plasma membranes, but differ from haustoria of Erysiphe pisi in this respect. Thus, the mechanism proposed for solute uptake into haustoria of E. pisi (Spencer-Phillips & Gay, 1981) cannot be directly applied to haustoria of A. candida and Uromyces appendiculatus. In these infections the possible forces which may be implicated in solute influx into haustoria are:

(i) influx into infected cells by the ATP-ase driven pumps in the wall-lining region of the host plasma membrane;

(ii) increase of solute concentration by photosynthesis in infected cells;

(iii) decrease in concentration of soluble translocates by their incorporation into non-diffusable compounds, e.g. polysaccharide and protein, within the haustoria and intercellular mycelium.

In the Albugo and Uromyces infections haustorial uptake is thus likely to depend largely on active processes of the host and, for these to be effective, the absence of ATP-ase pumps from the extrahaustorial membrane and a neckband seal preventing loss from the extrahaustorial matrix to the leaf apoplast of solutes undergoing transport are essential. As pointed out above, the neckband, or its equivalent in A. candida is also essential in preventing the intermixing of components of the

invaginated and wall-lining domains of the plasma membrane. The mixing would probably be detrimental to the host cell. Thus, the presence of an efficient and permanent neckband is likely to be a key factor determining biotrophy and its period of operation in haustorial infections.

## 3.5 Addendum Discussion

The results of the PACP and PATCHSP staining procedures are in agreement with the findings of Coffey (1983) in a similar study of *A. candida* published while this paper was in press. In addition, this author showed that the distal haustorial wall is not degraded by cellulase or zymolyase. These enzymes digested cellulose and β1-3 glucans within the walls of intercellular hyphae and the proximal haustorial neck. Thus, either the distal haustorial wall does not contain β1-3 and β1-4 glucans, or materials are laid down within this region which protect cell wall polysaccharides from degradation, or an apoplastic barrier within the haustorial neck wall prevents access of enzymes to the distal region. The latter is least likely since the specimens had been fixed prior to digestion and should therefore be permeable. However, this was not pointed out by the author.

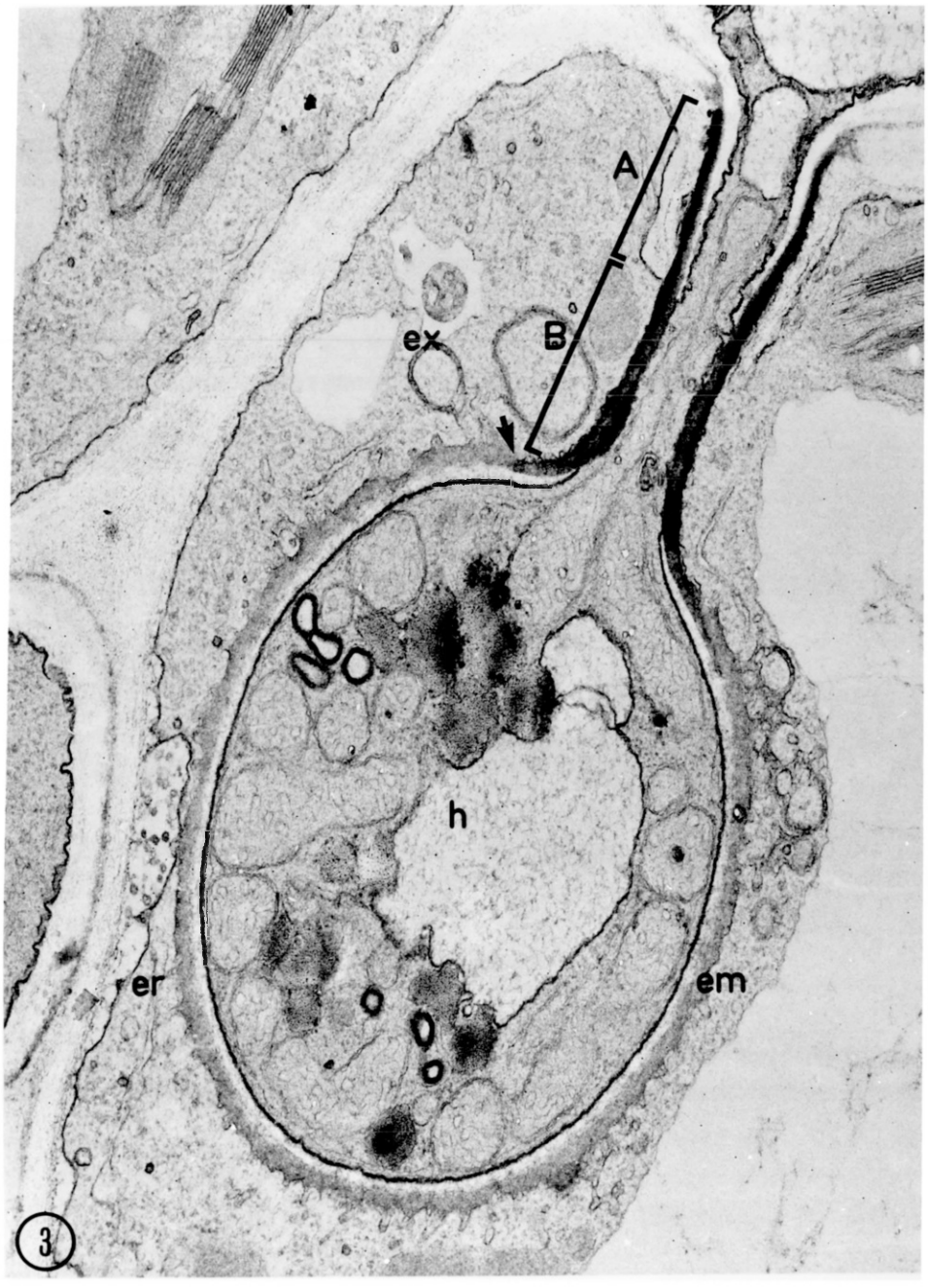
## Plates 3: 1,2

Sections of mesophyll cells of Cardamine hirsuta infected with haustoria (h) of Albugo candida prepared from material incubated with ATP and magnesium ions at pH 7.2. The sections were stained with uranyl acetate and lead citrate. Precipitates indicating ATP-ase activity are present on the wall-lining host plasma membrane of the infected and adjacent cells; these are unusually dense in(3:1). Precipitates are absent from the invaginated host plasma membrane and the fungal plasma membrane. The transition in activity on the host plasmalemma occurs where the membrane comes into close contact with the fungal wall one-third to half-way along the haustorial neck (arrowed). Region B (bracketed in(3:2))of the haustorial neck wall is only just discernable as slightly darker than other regions of the fungal wall after staining with uranyl acetate and lead citrate. ih, intercellular hypha; m, mesophyll cell. (3:1)x11800; (3:2) x15700.


m
ih
h
m
h
m
ih
B
h
m
1
2

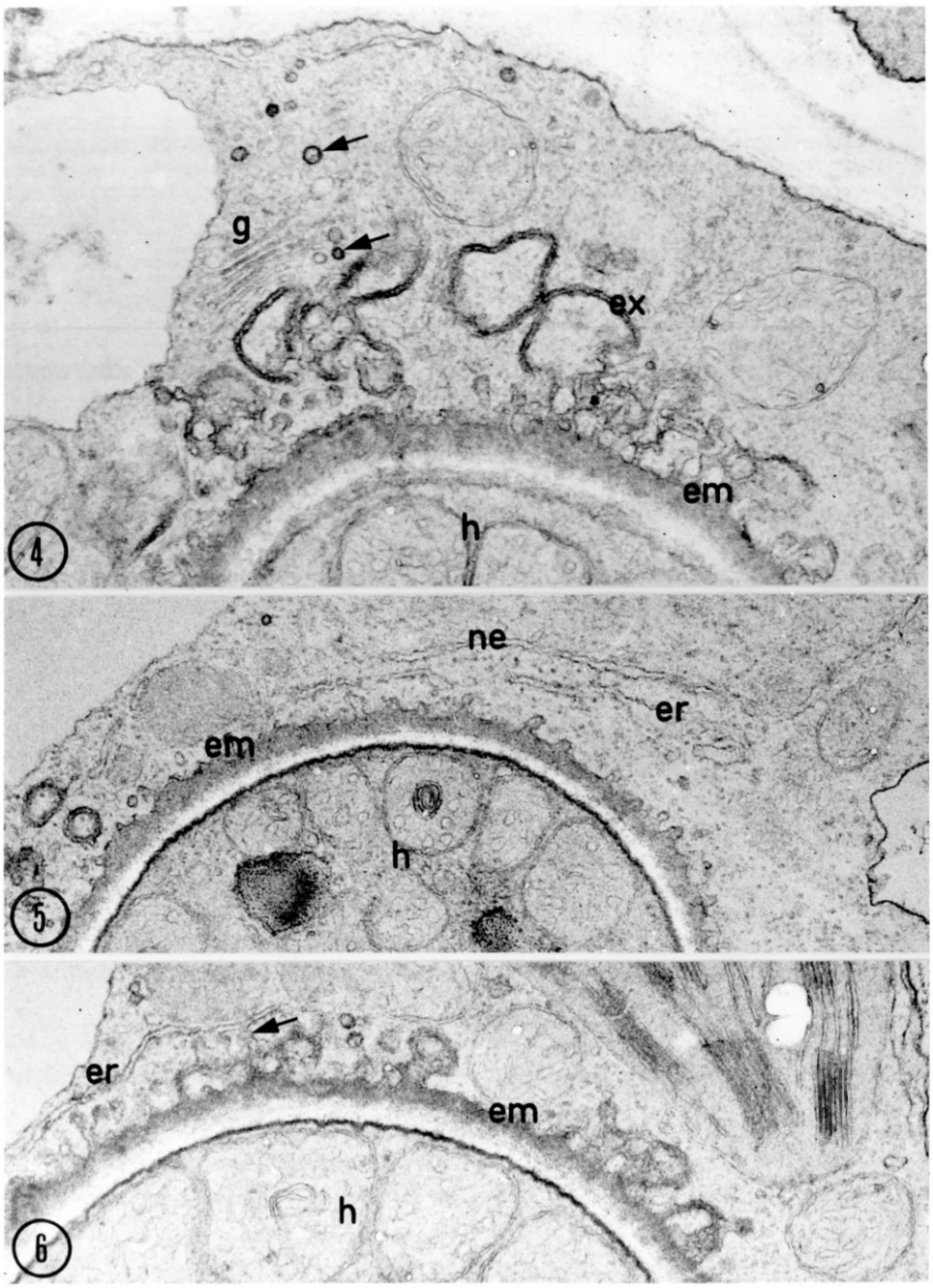
Plate 3: 3

Median section of haustorium (h) stained using the PACP technique. The haustorial neck wall shows region A, which is lightly stained as is the wall of the intercellular hypha (ih) and haustorial body, and B, which is intensely stained. The host plasmalemma is intensely electron-dense where it lies adjacent to the host cell wall and the haustorial neck region B, but is mostly only lightly stained around the haustorial body. The transition in the staining occurs at the edge of region B (arrowed). Extensions (ex) of the extrahaustorial matrix (em) are limited by darkly stained membranes, unlike most of the membrane surrounding the haustorial body. Smooth and rough endoplasmic reticulum (er) cisternae have lightly stained membranes x27200.


A
B
ex
h
er
em
3

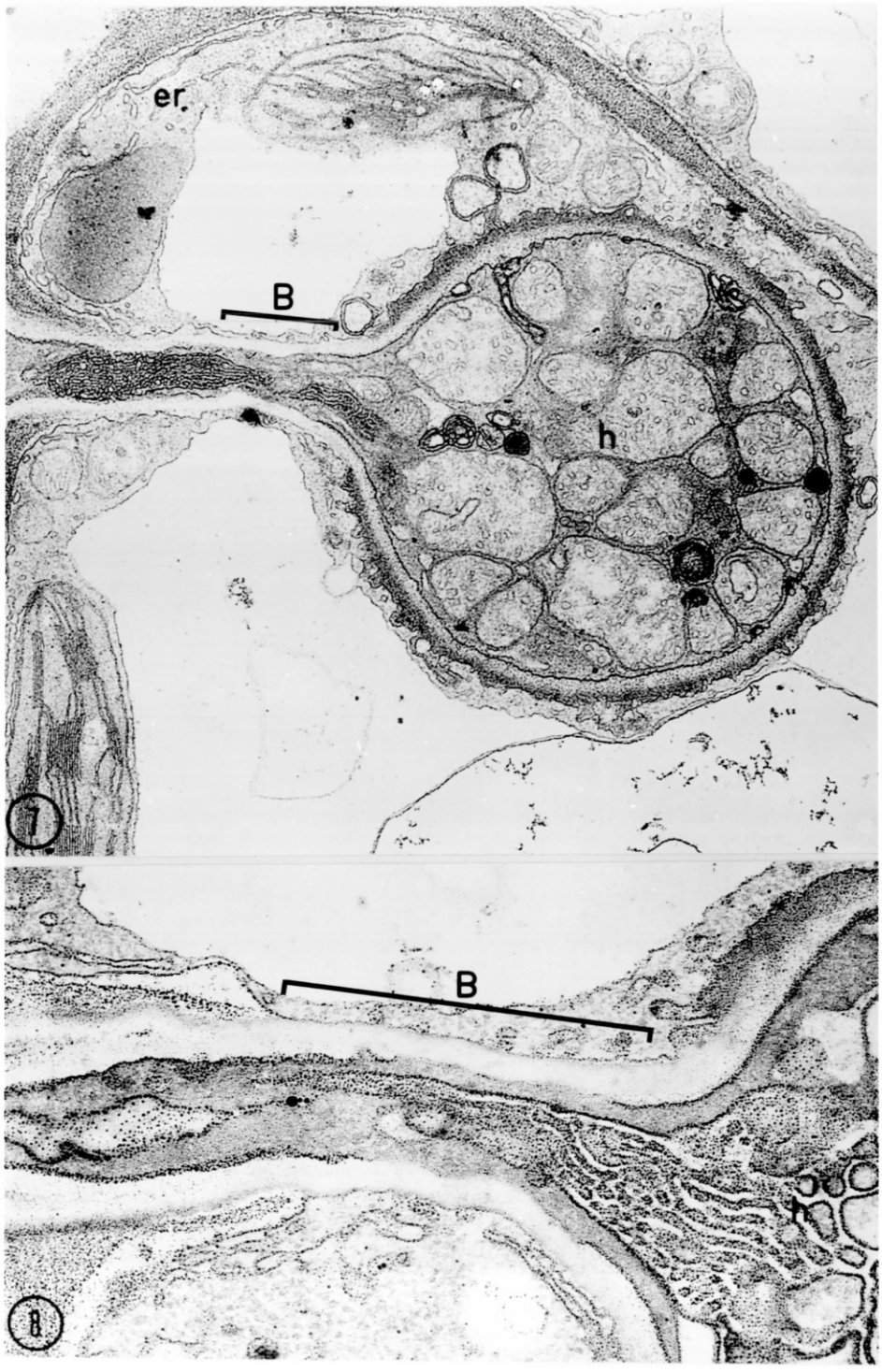
## Plates 3: 4,5,6

Sections of margins of haustoria (h) stained using the PACP procedure. (3:4) shows intensely stained membraneous cisternae (ex) connected to the light to moderately electron-dense parts (em) of the extrahaustorial membrane. The membranes of the Golgi body (g) within the host cytoplasm are very lightly stained although vesicles (arrowed) which may have been derived from the Golgi are bounded by membranes with similar electron density to the membraneous cisternae and the wall lining host plasmalemma. X32400.(3:5) illustrates the similarity of staining shown by the normal regions of the extrahaustorial membrane (em), the host endoplasmic reticulum (er) and the nuclear envelope (ne). X27200.(3:6) shows a probable connection (arrowed) between the normal extrahaustorial membrane (em) and the smooth host endoplasmic reticulum (er). X36950.


g
ex
em
h
4
ne
er
em
h
5
er
em
h
6

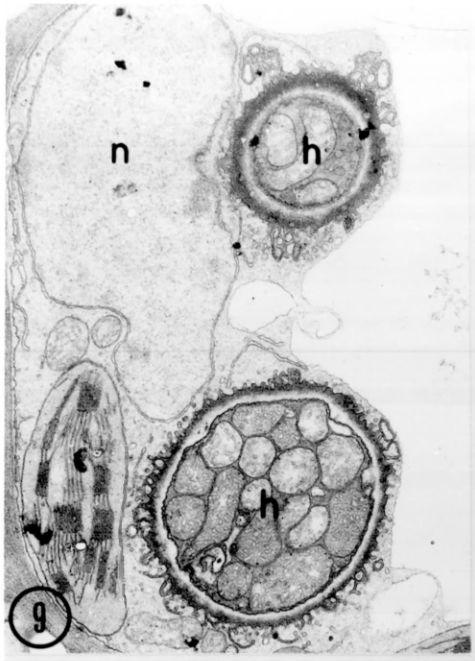
Plates 3: 7,8

Median sections of haustoria (h) stained by the PATCHSP method. Region B of the haustorial neck wall is virtually unstained in contrast to the granularity of the fungal wall elsewhere. Both host and fungal plasmalemmas are almost unstained in this region; this is shown especially clearly in (3:8). Elsewhere, the host plasmalemma is moderately granular, as is the host endoplasmic reticulum (er) and the fungal plasma membrane shows intense granularity. (3:7)x19850; (3:8) x48600.

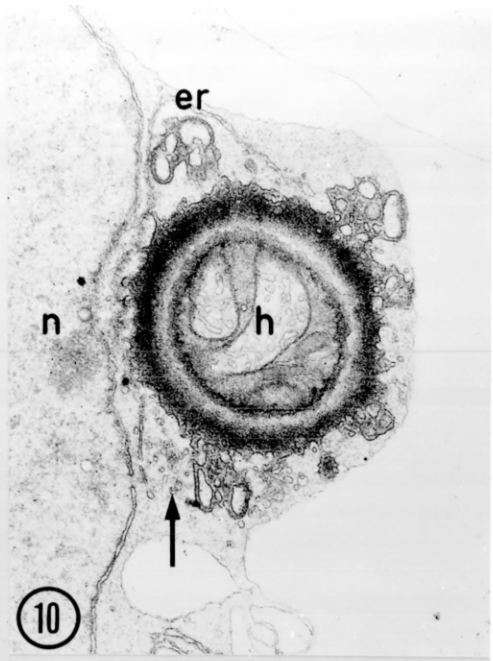

er
B
h
7
B
8

Plates 3: 9,10,11,12

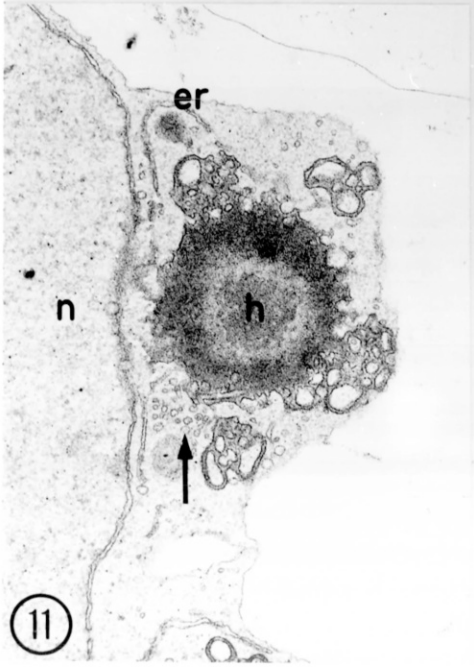
Serial sections, stained by the PATCHSP method, of an haustorium (h) adjacent to the host nucleus (n), showing the very close relationship between the nuclear envelope, endoplasmic reticulum (er) and extrahaustorial membrane (em), and also the sponge-like elaborations of the latter. Profiles resembling transition vesicles can be seen between the endoplasmic reticulum and the extrahaustorial membrane (arrowed). (3:9)x9900; (3:10-3:12) x18500.



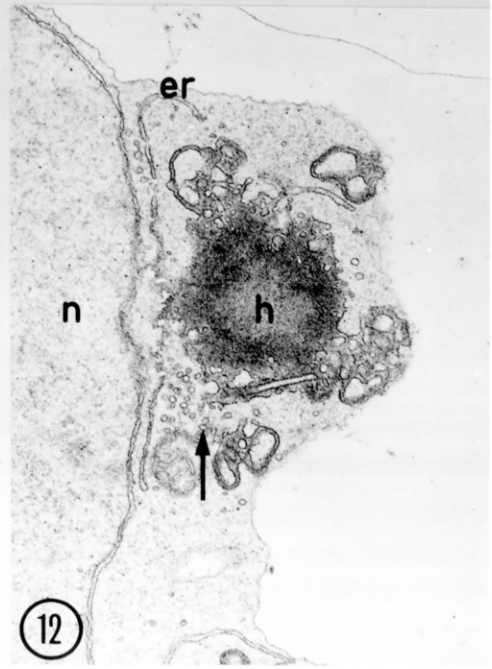

9




10




11




12

# 4. The Relationship Between the Host ER and the Extrahaustorial Membrane in the Infection of C. hirsuta by A. candida

## 4.1 Introduction

The host PM invaginated around haustoria of A. candida has been shown to be both structurally and physiologically distinct from the uninvaginated region (Woods & Gay, 1983), as in haustorial infections by other groups of fungi (Littlefield & Bracker, 1972; Gil & Gay, 1977; Manners& Gay, 1977; Spencer-Phillips & Gay, 1981). The origin of the specialisation of this region of the host PM is uncertain and it has been suggested that it may be due to modification of normal PM by the fungus or formation of PM from an abnormal source (Woods & Gay, 1983). An investigation of A. candida infecting Raphanus sativus (Berlin & Bowen, 1964) showed what were termed secretory bodies within the host cytoplasm, some of which were continuous with the extrahaustorial membrane. However, the close association of the invaginated host PM with the host ER and the light staining with PACP of both these membranes, in contrast to the intense staining of the uninvaginated region of the host PM, suggest that the extrahaustorial membrane may be derived directly from the ER (Woods & Gay, 1983). The following experiments were therefore carried out to investigate the relationship between the two types of membrane.

## 4.2 Materials and Methods

Leaves of Cardamine hirsuta infected by Albugo candida were treated by the following methods.

### 4.2.1 Osmium Ferricyanide (OsFeCN)

This method is based on that described by Hepler (1980). Pieces, $1mm^3$, were cut under a fixative solution of 2% glutaraldehyde, and 0.05M sodium cacodylate buffer containing 5mM calcium chloride at pH 7.2. The pieces were transferred to fresh fixative solution and left at room temperature for 2-3h. They were then washed in three 20 min changes of 0.05M sodium cacodylate buffer containing 5mM calcium chloride at pH 7.2, and post-fixed in a solution of 1% osmium tetroxide and 0.8% potassium ferricyanide in 0.05M sodium cacodylate buffer. The specimens were subsequently treated with 2% aqueous uranyl acetate for 2 h followed by dehydration, embedding and sectioning for electron microscopy as described in Sections 2.2.1 and 2.2.2.

### 4.2.2 Zinc Iodide - Osmium Tetroxide (ZIO)

This method is based on that described by Harris & Chrispeels (1980). Pieces, $1mm^3$, of infected and uninfected C. hirsuta were excised under a fixative solution of 1.5% formaldehyde and 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer at pH 7.0, transferred to fresh fixative and left for 20h at room temperature. The specimens were then washed in five 20 min changes of double distilled water and treated with a solution containing equal volumes of 2% osmium tetroxide and zinc iodide solution. The zinc iodide was prepared by sonicating 3g zinc powder in 20ml distilled water for 30s, adding 1g resublimed iodine, stirring for 5 min and clarifying the solution by filtration, which was then used immediately. The specimens were treated for either 4, 8 or 16h and washed in distilled water before dehydrating, embedding and sectioning for electron microscopy as described in Sections 2.2.1 and 2.2.2.

### 4.2.3 High Voltage Electron Microscopy (HVEM)

Sections (0.5-1μm thick) of osmium ferricyanide-treated specimens and conventionally fixed specimens were heat-sealed to grids by placing the grid, sections uppermost, on a drop of water on a hotplate at 60°C and evaporating to dryness. Sections of conventionally fixed material were subsequently stained by the PATCHSP procedure as follows: 1h in 10% periodic acid, washed in distilled $H_2O$, 72h in 0.2% thiocarbohydrazide in 20% acetic acid, washed in a series of acetic acid solutions and finally distilled water and placed in the dark in an aqueous solution of 1% silver proteinate for 2h.

Sections were observed on the AEI EM7 HVEM at 500 KV. Micrographs were made at 0° tilt and also ± 6° tilt to obtain stereo pairs.

## 4.3 Results

### 4.3.1 Osmium Ferricyanide

The contents of the nuclear envelope (NE) and endoplasmic reticulum (ER) of about a quarter of the cells within the leaf were stained by this method, but the majority of cells exhibited no selective staining, irrespective of their type or infection by haustoria. In the cells which were selectively stained, the reaction of the contents of the NE and ER varied from medium to intense electron density (Plates 4: 1,3) and a similar variation of staining reaction of ER cisternal contents occurred within a cell (Plate 4: 3). The extrahaustorial matrix was lightly to moderately stained and occasionally contained electron dense striations (Plate 4: 1). Although the extrahaustorial membrane and the host ER were frequently closely associated (Plate 4: 3) no definite connections were observed. About 2/3 to 3/4 of the way along the haustorial neck from the penetration site a region of the host and fungal plasmalemmas and small

extensions of these membranes were intensely stained and the haustorial neck wall between them was of greater electron density than elsewhere within the haustorial wall (Plate 4: 2). The fungal cytoplasm was not selectively stained and other regions of the infected cells exhibited the usual staining characteristics obtained with uranyl acetate (Plates 4: 1-3).

4.3.2 Zinc Iodide - Osmium Tetroxide

Similar results were obtained with all treatment times. The fungal cytoplasm was very electron dense and no clear detail could be discerned within it (Plate 4: 4). In some parts of the host cytoplasm similar dense precipitates were observed even in tissue treated for only 4h. However, in most of the host cells only the contents of the ER and Golgi cisternae were intensely stained (Plates 4: 4,5). The extrahaustorial matrix was only of light electron density. Frequent close associations but no definite connections between the host ER and the extrahaustorial membrane were observed (Plate 4: 4).

4.3.3 High Voltage Electron Microscopy

Thick sections of leaves treated with OsFeCN gave no more information than thin sections. Some possible connections were observed but were not definite.

Thick sections treated with the PATCHSP technique, which usually stains the extrahaustorial matrix more intensely than the contents of the ER, demonstrated that the vesicles and tubules seen within the host cytoplasm adjacent to the matrix in thin sections were intertwining tubular extensions of the extrahaustorial matrix (Plates 4: 6,7). No definite connections between them and the host ER were seen.

## 4.4 Discussion

Investigations have shown few definite connections between the host ER and the extrahaustorial membrane and with the OsFeCN, ZIO and PATCHSP techniques the contents of the ER usually stained differently from the extrahaustorial matrix. Thus it is unlikely that the lumen of the ER and the extrahaustorial matrix are continuous. However, this does not exclude the possibility that the extrahaustorial membrane is formed from the ER. With the OsFeCN technique, although variation between cells is probably due to failure of the reagents to penetrate all cells equally (Hepler, 1980), the variation of ER staining within a particular cell indicates a difference in function and/or a lack of continuity between different regions of the ER. A similar variation has enabled the identification of discontinuous sub-regions of the ER in photoreceptor cells of the leech Hirudo medicinalis (Walz, 1982). Thus, since variations in staining of the different regions of the ER can occur, differential staining of the contents of the ER and the extrahaustorial matrix does not disprove the theory that the extrahaustorial membrane is derived from the ER. Similarly the lack of connections between the ER and the extrahaustorial membrane does not disprove the theory since Golgi cisternae are derived from those of the ER although the two are not continuously connected (Morré & Ovtracht, 1977). Thus a similar developmental process with rare (transient) connections, may account for the formation of the extrahaustorial membrane. Investigations of many rust haustorial infections show a close association between the ER and the haustorium (Hardwick, Greenwood & Wood, 1971; Heath & Heath, 1971; Heath, 1972; Littlefield & Bracker, 1972; Harder, Rohringer, Samborski, Kim & Chong, 1978). The detailed study of P. graminis tritici (Harder et al., 1978) has revealed a complex network of ER tubules and cisternae surrounding the haustorial body. In addition, during the formative stages

of the haustoria, extensive host ER proliferation occurred around the neck between the neck ring region and the haustorial body with frequent points of contact or near contact with the invaginated PM. It is significant that such points of contact occur at and beyond the neck ring where the transition in host PM characteristics is known to occur. In the present study, the intense staining with OsFeCN confined to a specialised part of the haustorial neck wall within region B, the associated host and fungal plasmalemmas and the small extensions from the host PM suggest that connections with the host ER may also occur here. However, connections were not observed and OsFeCN is not specific, having been shown to stain a variety of substances, other than the contents of the ER and NE, which are not visible with conventional staining techniques (Schnepf, Hausmann & Herth, 1982).

Structural similarities observed between the extrahaustorial membrane and the ER in other investigations also support the theory of ER origin. Freeze-fracture studies of Uromyces appendiculatus and P. coronata avenae have shown that sterols, present in the uninvaginated PM, are absent from both the ER and the extrahaustorial membrane (Harder & Mengden, 1982). Other freeze-fracture studies have revealed the absence from the extrahaustorial membrane of a rust (Littlefield & Bracker, 1972) and a powdery mildew (Gil, 1976) of 10nm particles which are present in the normal host PM. Similarly, ER lacks the particles (Morré & Ovtracht, 1977) whereas there is a progressive increase in their number from the forming to mature faces of dictyosomes of the alga Micrasterias denticulata (Staehelin & Kiermayer, 1970). However, it should be noted that newly formed plasmalemmas of nerve fibres (Pfenniger & Bunge, 1974) and regenerating cilia (Satir, Sale & Satir, 1976) also lack particles as do the cytoplasmic vesicles which fuse with them, suggesting that particles are inserted into the PM subsequent to its formation. Thus, the absence of

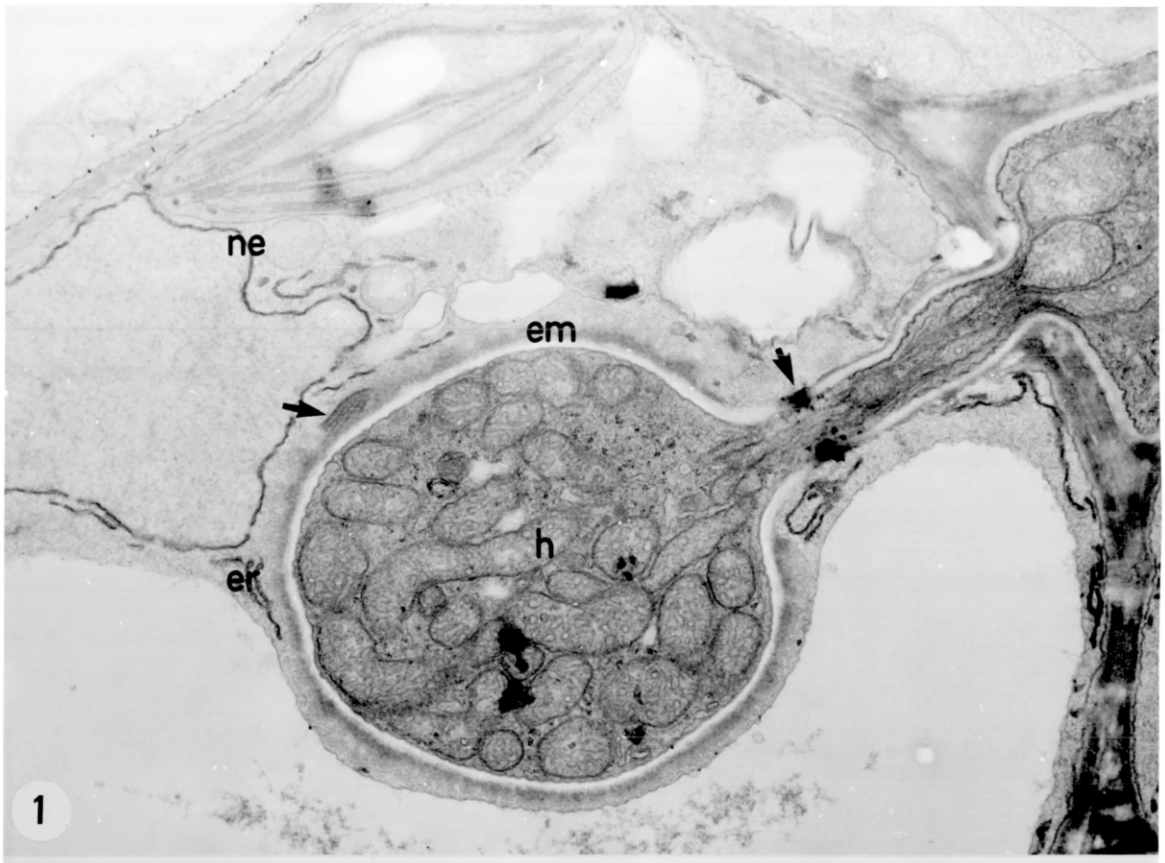
particles within the extrahaustorial membrane may only be a result of its recent formation.

If the invaginated PM is not derived from the ER it presumably originates from the Golgi as does normal host PM, or by extension of existing plasmalemma, and is subsequently modified by the fungus. Although Golgi are frequently seen in association with powdery mildew haustoria (Gil, 1976; Fig. 16 in Bushnell & Gay, 1978) this is not a consistent occurrence in other infections. However, it has been suggested by Ingram, Sargent & Tommerup,(1976) that the PM of lettuce infected by Bremia lactucae proliferates prior to penetration of the cell by the fungus. In these circumstances a close association between the Golgi and the haustoria would not be necessary.

In summary, the evidence for formation of the extrahaustorial membrane from the host ER is inconclusive, but it seems unlikely that the extrahaustorial matrix and the lumen of the ER are a spatial continuum. Irrespective of the source of invaginated PM, there is still the possibility that it may be subsequently altered by the fungus.

## Plates 4:1 and 4:2

Section of an haustorium (h) of A. candida infecting a mesophyll cell of C. hirsuta in material prepared by the OsFeCN technique. Plate 4:1 shows that the contents of the host nuclear envelope (ne) and endoplasmic reticulum (er) are intensely stained, whereas the extrahaustorial matrix (em) is only moderately electron dense apart from the more darkly stained striations (arrowed). The host endoplasmic reticulum and the extrahaustorial matrix are closely associated but no connections can be seen. The fungal cytoplasm is not selectively stained. About 2/3 to 3/4 of the way along the haustorial neck from the penetration site a region of the host and fungal plasmalemmas and small extensions of these membranes are intensely stained, as is the region of haustorial neck wall between them (arrowed). This can be seen most clearly in Plate 4:2 and although this may suggest continuity of this region with the lumen of the host ER, no connections with the ER in this region can be seen. Plate 4:1, x16500; plate 4:2, x40500.


ne
em
h
er
1

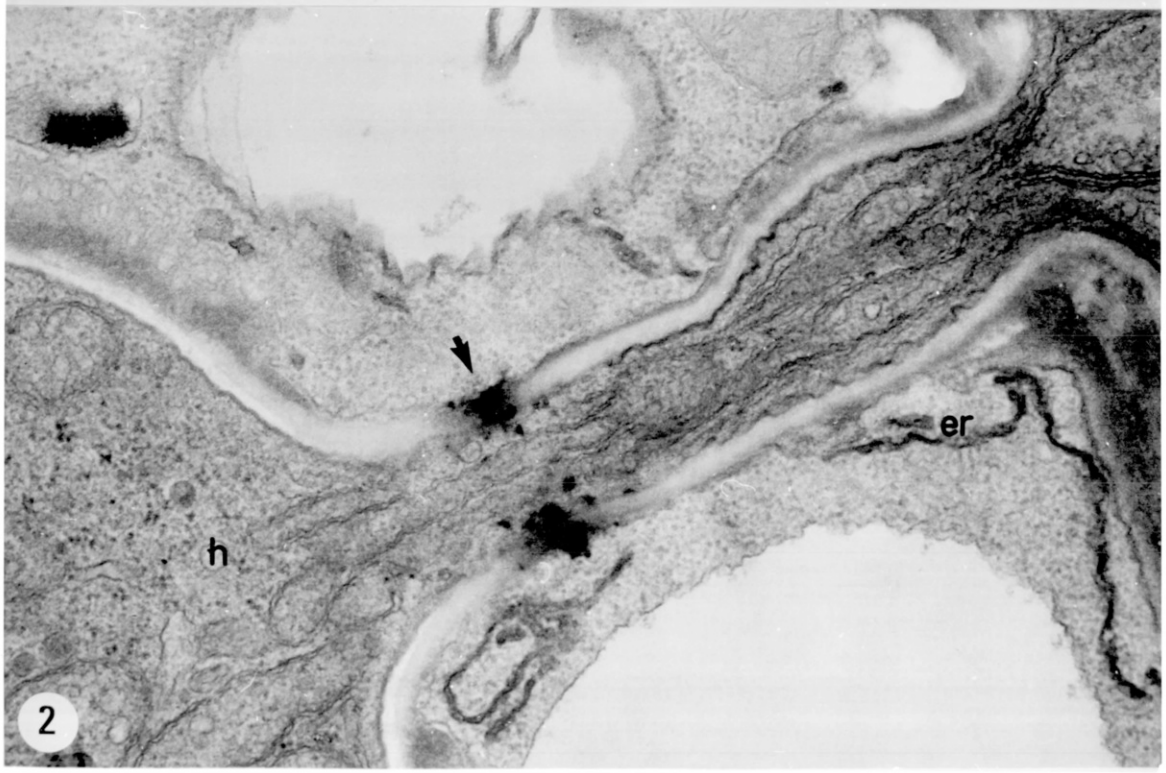
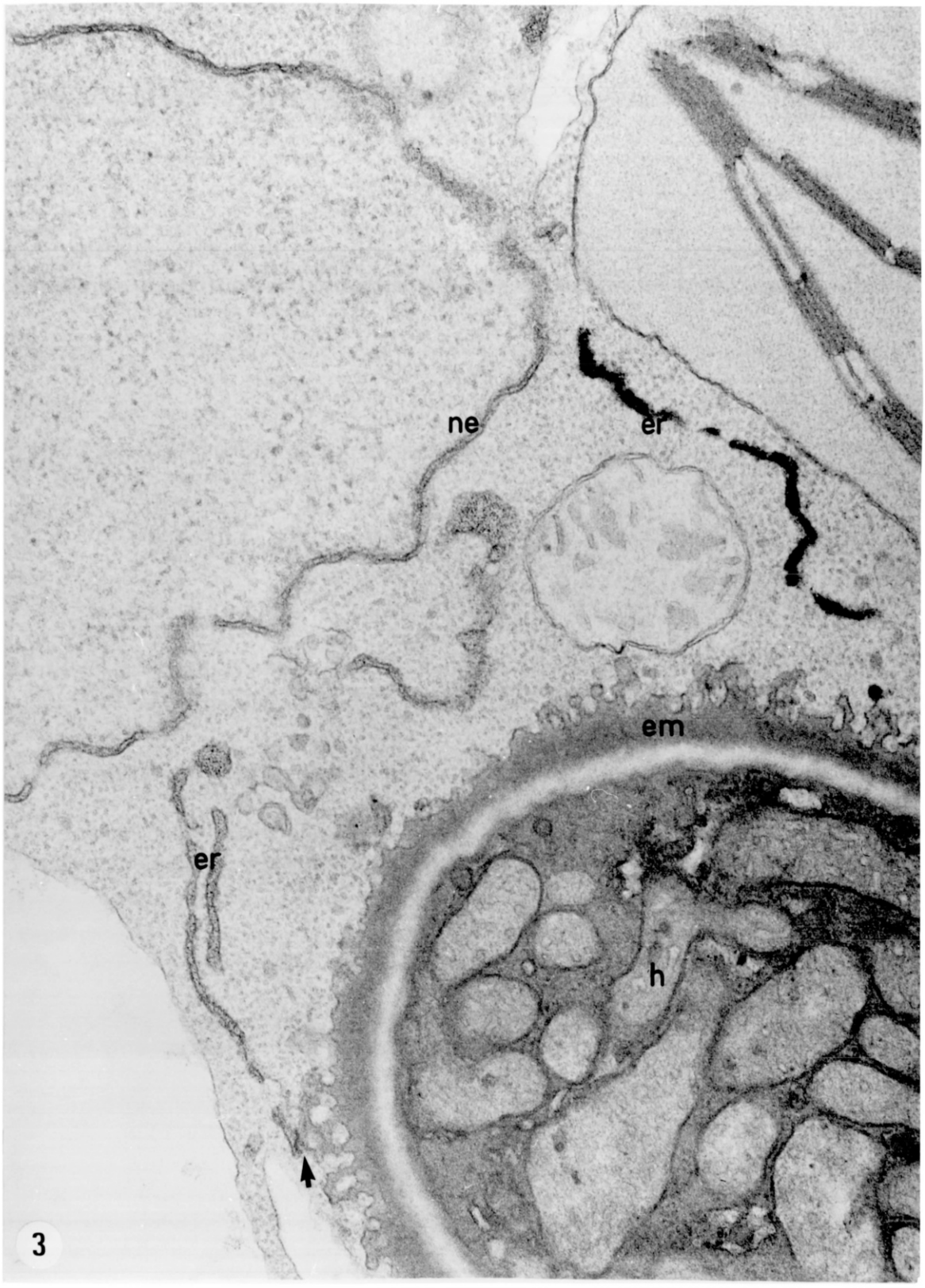

er
h
2

Plate 4:3

Section of an haustorium (h) of A. candida infecting a mesophyll cell of C. hirsuta in material prepared by the OsFeCN technique. The contents of the host nuclear envelope (ne) are moderately stained as is the extrahaustorial matrix (em) whereas the host endoplasmic reticulum (er) varies from moderate to intense electron density. There is a very close association between the host ER and the extrahaustorial matrix (arrowed), but no connections can be seen. X48600.

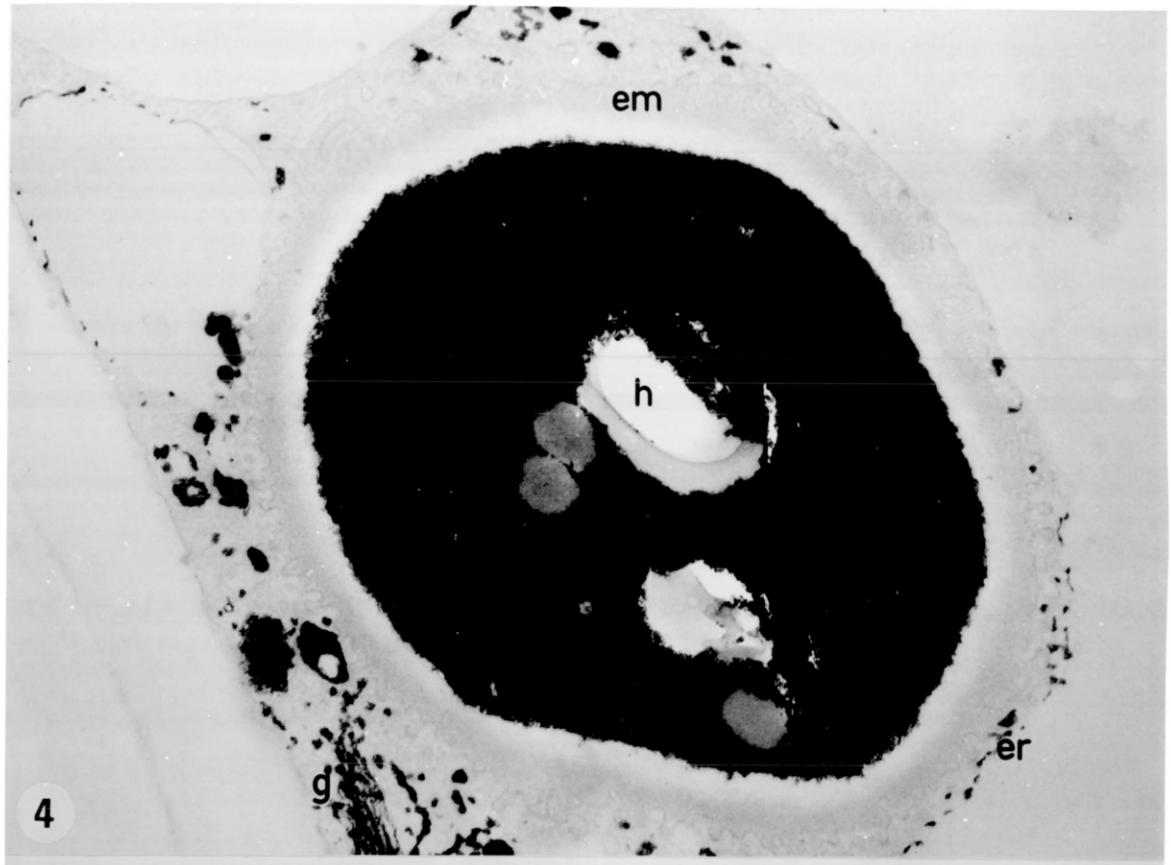

ne
er
em
er
h
3

## Plates 4:4 and 4:5

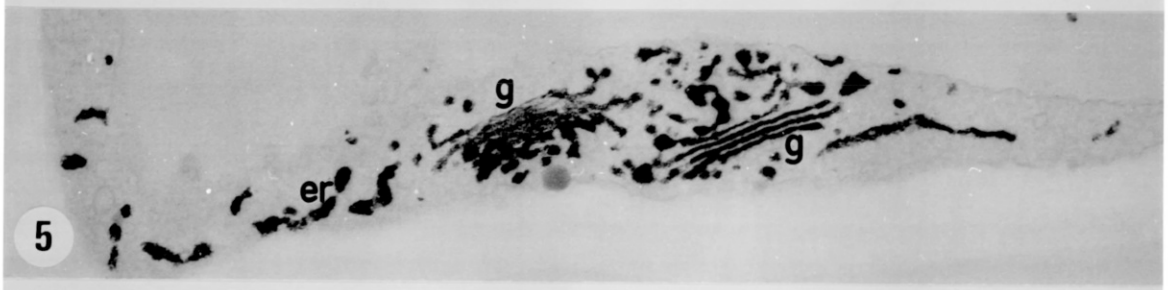
Sections of an haustorium (h) of A. candida infecting C. hirsuta and an uninfected cell of C. hirsuta from material prepared by the ZIO technique. Plate 4:4 shows that the haustorial cytoplasm is very electron dense and no detail can be discerned within it. The contents of the Golgi bodies (g) and endoplasmic reticulum (er) within the host cytoplasm are selectively intensely stained (shown most clearly in Plate 4:5), whereas the extrahaustorial matrix (em) is only of light electron density. This indicates that the extrahaustorial matrix is not continuous with the endomembrane system despite frequent close association. Plate 4:4, x36950; Plate 4:5, x27200.

## Plates 4:6 and 4:7

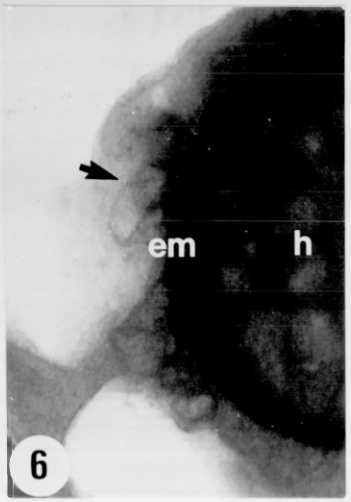
Section, 0.5-1μm thick, of an haustorium (h) of A. candida infecting C. hirsuta, stained by the PATCHSP procedure. The micrographs were made with the specimen at $0^{\circ}$ tilt (Plate 4:6) and $6^{\circ}$ tilt (Plate 4:7) to give a stereo pair. There are intertwining tubular extensions (arrowed) of the extrahaustorial matrix (em), but no connections with the host ER can be seen. Plates 4:6 and 4:7 x15050.




4




5




6



7

# 5. The Permeability of the Haustorial Neck Wall of A. candida

## 5.1 Introduction

The presence of a barrier to apoplastic diffusion of solutes within the haustorial neck wall has been established for both powdery mildew (Gay & Gil, 1975; Gil & Gay, 1977; Gay & Manners, in preparation) and rust (Heath, 1976) infections. In experiments with powdery mildews in which haustorial complexes were isolated, the extrahaustorial matrix was seen to expand in hypotonic solutions, the extrahaustorial membrane remaining tightly adherent to the haustorial neck wall at the neckband (Gil & Gay, 1977). In addition, the permeability of both powdery mildew (Gay & Manners, in preparation) and rust (Heath, 1976) neckbands have been investigated with the use of apoplastic markers. In neither investigation did the markers pass beyond the neckband, thus demonstrating that despite the differing morphologies, a common characteristic exists in rust and powdery mildew neckbands. It has been suggested (Heath, 1976; Spencer-Phillips & Gay, 1981) that this barrier is important for the efficiency of a host/pathogen nutrient transfer mechanism.

Since the specially stained region B of the haustorial neck wall of A. candida exhibits some of the characteristics of the neckbands of powdery mildew and rust haustoria (Woods & Gay, 1983), the permeability of the haustorial neck wall was investigated for evidence of an apoplastic barrier, which may influence the functioning of the haustorium.

## 5.2 Materials and Methods

Three methods were employed.

### 5.2.1 Experiments with a Fluorescent Apoplastic Marker

This method was adapted from that of Peterson, Emanuel & Wilson

(1982). Leaves and stems of Cardamine hirsuta infected with Albugo candida were vacuum infiltrated with Tinopal BOPT. Leaves were prepared by removing their edges with a razor blade to provide a greater area for entry of the reagent and stems were cut into 5-10mm lengths. They were placed in aqueous solutions of Tinopal in a sealed dessicator attached to a vacuum pump. The reagent was employed at concentrations of 0.01% and 0.05%. The pressure was reduced to 250mm of mercury for 20 min and then returned to atmospheric. This procedure was repeated and the specimens were left in the reagent for a further 15-20h after which they were rinsed for at least 1h in flowing cold tap water to remove any unbound dye. The specimens were too soft to section by hand, and therefore 30-60μm sections were cut using a sledge microtome with a freezing stage. These were mounted in water on a slide and observed immediately using a Zetopan fluorescence microscope fitted with a mercury vapour lamp. The exciter filter had peak transmission at 365nm and 50% cut off at 340nm and 385nm and the barrier filter emitted wavelengths at greater than 50% transmission above 465nm.

Controls were prepared by treating infected specimens with distilled water instead of Tinopal.

5.2.2 Experiments with an Electron Dense Apoplastic Marker

Aqueous uranyl acetate (0.5mM) was injected with a fine syringe between the veins at the bases of infected leaves. Alternatively, leaves and stems were trimmed with a razor blade and vacuum infiltrated with 0.5mM uranyl acetate at a pressure of 250 mm Hg as described above. Pieces of tissue successfully infiltrated were identified by their water-soaked appearance. The specimens were either left in 0.5mM uranyl acetate or allowed to partially dry in air for 1h before excising $1mm^3$ cubes from the infiltrated regions under a fixative solution of 2% glutaraldehyde in 0.1M phosphate buffer. Phosphate buffer was used in order to

precipitate uranyl crystals. The pieces were fixed for 2h at room temperature, washed for 1h in 3 changes of 0.1M phosphate buffer, post-fixed for 1h in 1% buffered osmium tetroxide, dehydrated, embedded and sectioned for electron microscopy as described in Sections 2.2.1 and 2.2.2

5.2.3 Experiments with Hypertonic Treatment

Specimens were prepared for electron microscopy as described below by Mr. P. Rennie. Pieces, approximately $1mm^3$, of infected leaves, were excised under a 0.8M sucrose solution and left in 0.8M sucrose for 4h. The specimens were then placed in a solution of 2.5% glutaraldehyde, 0.1M sodium cacodylate (pH 7.2) and 0.8M sucrose and left to fix at room temperature. They were subsequently washed for three 15 min periods in 0.1M sodium cacodylate and 0.8M sucrose, then dehydrated, embedded and sectioned for electron microscopy as described in Sections 2.2.1 and 2.2.2.

Controls were prepared for electron microscopy omitting the sucrose.

To detect any difference in the width of the extrahaustorial matrix after plasmolysis of the host cell, micrograph negatives of plasmolysed and unplasmolysed infected cells were projected onto the screen of a microcomputer. The average distance between the outside of the haustorial wall and the extrahaustorial membrane around each haustorium was then found from 8 measurements made at approximately $45^{\circ}$ intervals by drawing on a graphics tablet, linked to the computer, using the programme 'Stereometric Particle Measurement and Analysis' by VIDS, Micro Measurements Ltd., Saffron Walden. The matrices around 15 haustoria were measured in both plasmolysed and unplasmolysed tissue. The mean and standard deviation of these average widths were calculated for each treatment and are shown in Table 5:1.

Table 5:1

Means and standard deviations of the widths of extrahaustorial matrices around haustoria of A. candida infecting C. hirsuta in plasmolysed and unplasmolysed tissue.

| | Mean Width (nm) | Standard Deviation (nm) |
|---|---|---|
| Plasmolysed Tissue | 94 | 21 |
| Unplasmolysed Tissue | 92 | 16 |

## 5.3 Results

### 5.3.1 Experiments with a Fluorescent Apoplastic Marker

In sections of control leaves and stems, a bright yellow-green autofluorescence was exhibited by the sporangiophores and sporangia and the intercellular hyphae autofluoresced a very faint green, but haustoria were not visible. Vascular tissues gave a bright yellow-green autofluorescence and chloroplasts were bright red.

Leaves impregnated with Tinopal could not be used because the autofluorescence of the chloroplasts was so bright that it would have obscured any fluorescence of haustoria which may have been present. However, stems were not affected in this way and the best results were obtained after impregnation with 0.05% Tinopal when the cell walls fluoresced green, thus demonstrating penetration of the reagent into the specimen (Plates 5: 1-4). Intercellular hyphae also fluoresced bright green as did cross sections of hyphae which could be seen below host cells (Plates 5: 1-4). Haustoria gave various reactions. In 5 experiments the majority fluoresced green (Plates 5: 3,4), but a few haustoria, visible in bright field, did not fluoresce in UV light (Plates 5: 1,2).

### 5.3.2 Experiments with an Electron Dense Apoplastic Marker

Uranyl crystals were present within the cytoplasm of host and fungal cells in leaves into which uranyl acetate had been injected (Plate 5: 5). Specimens which had been vacuum impregnated contained little uranyl acetate. Where crystals could be seen they were also present within the host cytoplasm.

### 5.3.3 Experiments with Hypertonic Treatment

The plasmalemma had withdrawn from the wall in most uninfected and infected mesophyll cells treated with sucrose, but did not in controls.

In contrast the invaginated region was not withdrawn from the haustorium and remained closely adherent to the neck wall from the edge of the collar (Plate 5: 6) and at its normal distance around the extrahaustorial matrix. The latter is demonstrated by the absence of a significant difference between the widths of the extrahaustorial matrices in plasmolysed and unplasmolysed tissue (Table 5: 1).

Where encased haustoria were present in plasmolysed cells the host plasmalemma had retracted from the encasement as well as from the host cell wall (Plate 5: 7).

## 5.4 Discussion

Tinopal is a commercial fabric whitener and has been used by Peterson et al. (1982) to investigate the presence of apoplastic barriers within the hypodermal walls of onion roots. The dye does not pass readily through living cell membranes and is therefore confined to the apoplast and binds to the subunits of the microfibrils of cellulose (Maeda & Ishida, 1967; Rattee & Breur, 1974; Haigler, Brown & Benziman, 1980) and chitin (Herth, 1980).

The absence of staining of a few haustoria suggests that they either lacked components which stain or did not have access to the stain. Since they were well developed the latter explanation seems more likely. Conversely, the observation that many haustorial walls fluoresced indicates that those were accessible to the stain and so lacked a barrier to apoplastic diffusion. The vacuum infiltration may have influenced the result, but any redistribution of reagent which may have occurred following damage to membranes caused by freezing and sectioning should have been avoided by the washing which proceeded the freeze-sectioning.

It is concluded that at least a few haustoria have a barrier to apoplastic diffusion, but in the others it is uncertain whether a barrier is lacking or whether preparative methods allowed redistribution.

The suggestion that the staining of haustoria with Tinopal may have been due to damage by vacuum infiltration is supported by the observation of uranyl crystals within the cytoplasm of cells in leaves and stems vacuum impregnated with uranyl acetate. Like Tinopal, a dilute aqueous solution of uranyl acetate does not normally diffuse across living cell membranes. Thus, it is concluded that vacuum impregnation forced uranyl acetate across plasma membranes into the host cytoplasm. Injection of uranyl acetate had a similar effect.

Although no definite conclusions about an apoplastic barrier can be drawn using the diffusion markers, hypertonic treatments provided additional information. The lack of any change in the width of the extrahaustorial matrix and the absence of retraction of the invaginated host PM from it indicate that the extrahaustorial matrix is rigid and that either there is a barrier within the haustorial wall to diffusion between the extrahaustorial matrix and the general apoplast or the invaginated PM is adherent to the entire surface of the extrahaustorial matrix and the haustorial neck wall.

If an apoplastic barrier exists it is very unlikely to extend around the whole haustorium since it would prevent diffusion of metabolites from host to fungus. It is therefore likely to be located within the haustorial neck wall at region B to which the invaginated PM remains attached in plasmolysed cells. These findings are in agreement with those of Coffey (1983). A similar tight seal and barrier has been demonstrated at the neckbands of Erysiphe pisi (Gil & Gay, 1977; Gay & Manners, in preparation), Melampsora lini (Allen, Coffey & Heath, 1979), Uromyces phaseoli vignae and Puccinia sorghi (Heath, 1976) by use of apoplastic markers or experiments with hypertonic treatments. It is thus a common characteristic of powdery mildew and rust neckbands and the location of such an apoplastic seal within haustorial neck region B of A. candida would strengthen the proposal that this region is equivalent to a neckband

(Woods & Gay, 1983). However, the possibility that the invaginated host PM in A. candida infections may be adherent to both the haustorial neck wall and the extrahaustorial matrix cannot be excluded. The adherence of the invaginated PM to the haustorial surface of Peronospora humuli has been suggested by Pares & Greenwood (1979) and a similar attachment may exist in infections by B. lactucae (Section 8). The significance of membrane adherence in the maintenance of differences between the invaginated and uninvaginated regions of the host PM is discussed further in relation to B. lactucae infections (Section 8).

The existence of an apoplastic seal is essential for efficient haustorial uptake of nutrients by the mechanism proposed in Section 3.4. By sealing together the 2 plasmalemmas with an apoplastic diffusion barrier, the host and pathogen form a single functional unit with the possibility of directing transport towards the pathogen (Manners & Gay, 1983). In the absence of such a barrier, solutes undergoing transport from the host to the haustorium are likely to leak from the extrahaustorial matrix to the general apoplast. In addition, any solute influx into the host cell which is dependent on a proton gradient* may no longer be fully polarised towards the haustorium, since some protons extruded through the wall lining host PM by ATPase driven pumps may re-enter the host cell through the extrahaustorial membrane. This re-entry may, however, be limited by the degree to which the haustorial wall resists proton diffusion and the magnitude of the proton gradient across the wall-lining host PM through which the protons have been extruded. The absence of a seal has been indicated in a study of the monokaryon rust Puccinia poarum (Section 7) where this is discussed further.

*Footnote: It is assumed that a proton gradient is established across the PM by ATPase activity (Baker, 1978) but an electrochemical gradient involving other ions may equally apply to this hypothesis.

## Plates 5:1 - 5:4

Light micrographs of sections of the stem cortex of *C. hirsuta* infected by *A. candida*, prepared from material vacuum impregnated with Tinopal BOPT. The sections are photographed in bright field illumination (plates 5:1 and 5:3) or when illuminated by UV (plates 5:2 and 5:4). Host cell walls (hw) and intercellular hyphae (ih), visible in bright field, fluoresce under UV, as do haustoria (arrowed) shown in plates 5:3 and 5:4. However, plate 5:1 shows an haustorium which is visible in bright field (arrowed) but does not fluoresce under UV (plate 5:2) indicating that the fluorescent dye was not accessible to the haustorium. Plates 5:1 - 5:4, x320.

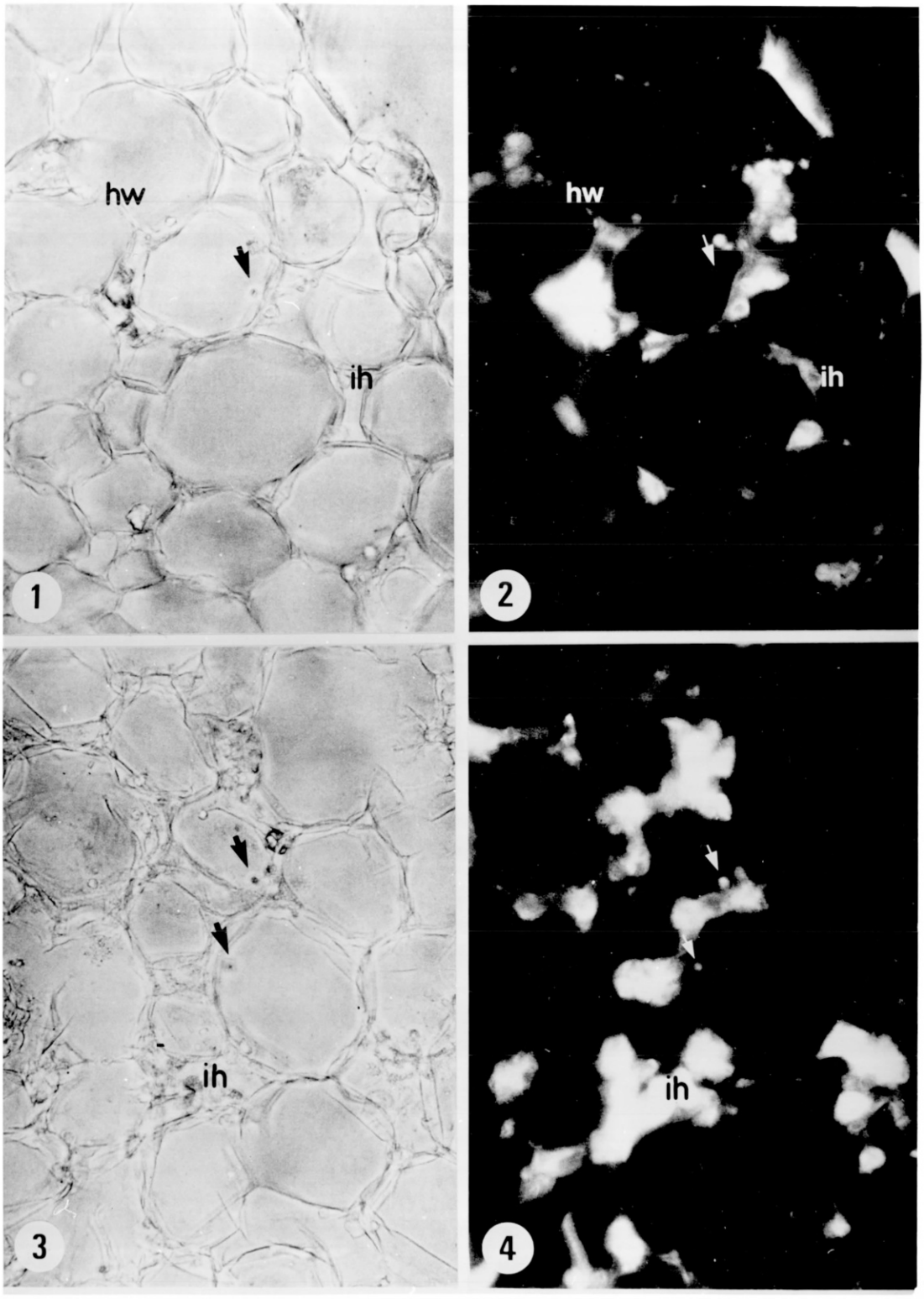

hw
ih
1
hw
ih
2
ih
3
ih
4

Plate 5:5

Section of an haustorium (h) of A. candida infecting a mesophyll cell (m) of C. hirsuta prepared from a leaf injected with a solution of uranyl acetate which was precipitated with $PO^{3-}$ ions in the fixation medium. Uranyl crystals are present within both host and fungal cytoplasms. Since a solution of uranyl acetate does not normally diffuse across living cell membranes it is concluded that vacuum impregnation forced the solution into the cytoplasms. ih, intercellular hypha. X19800.

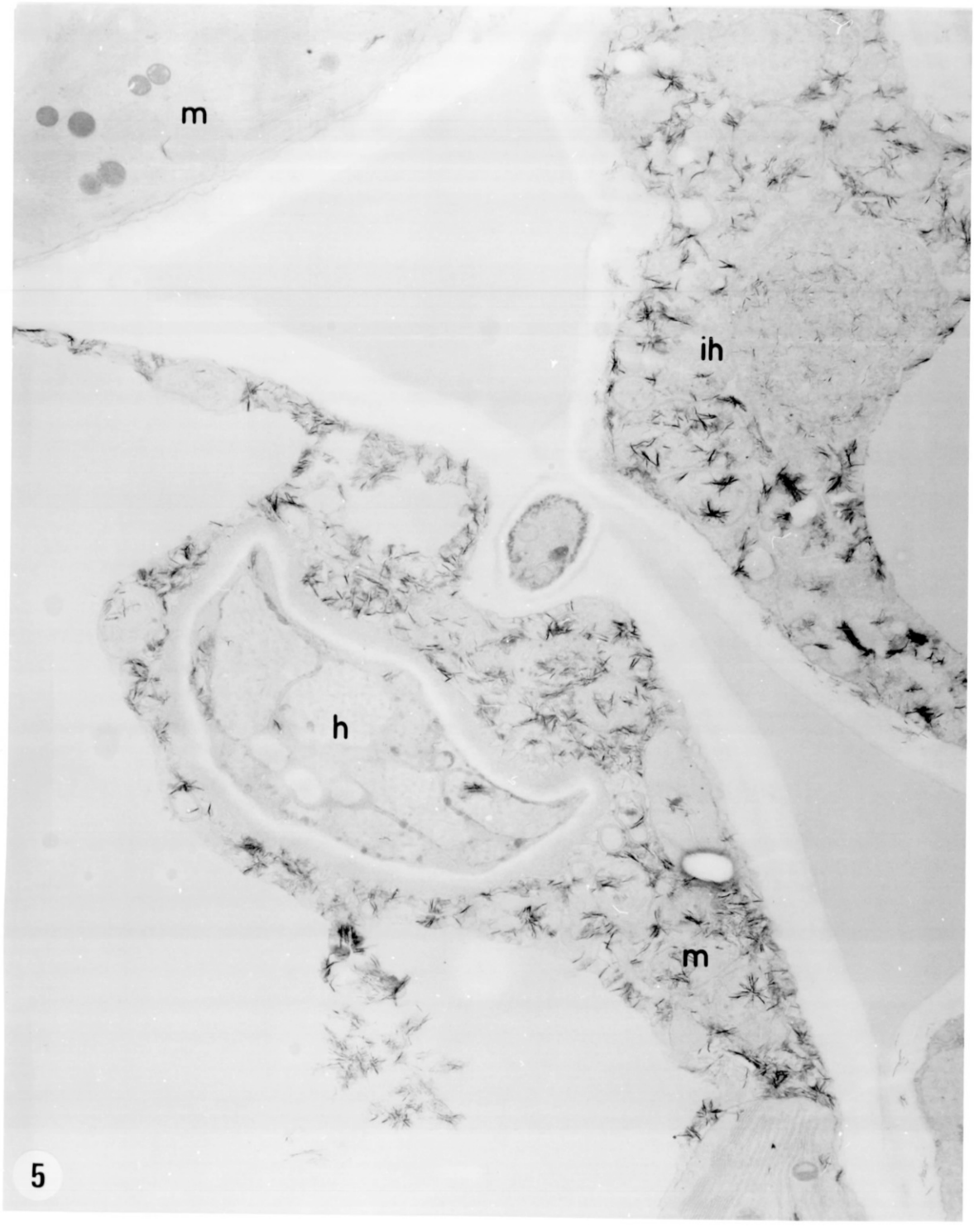
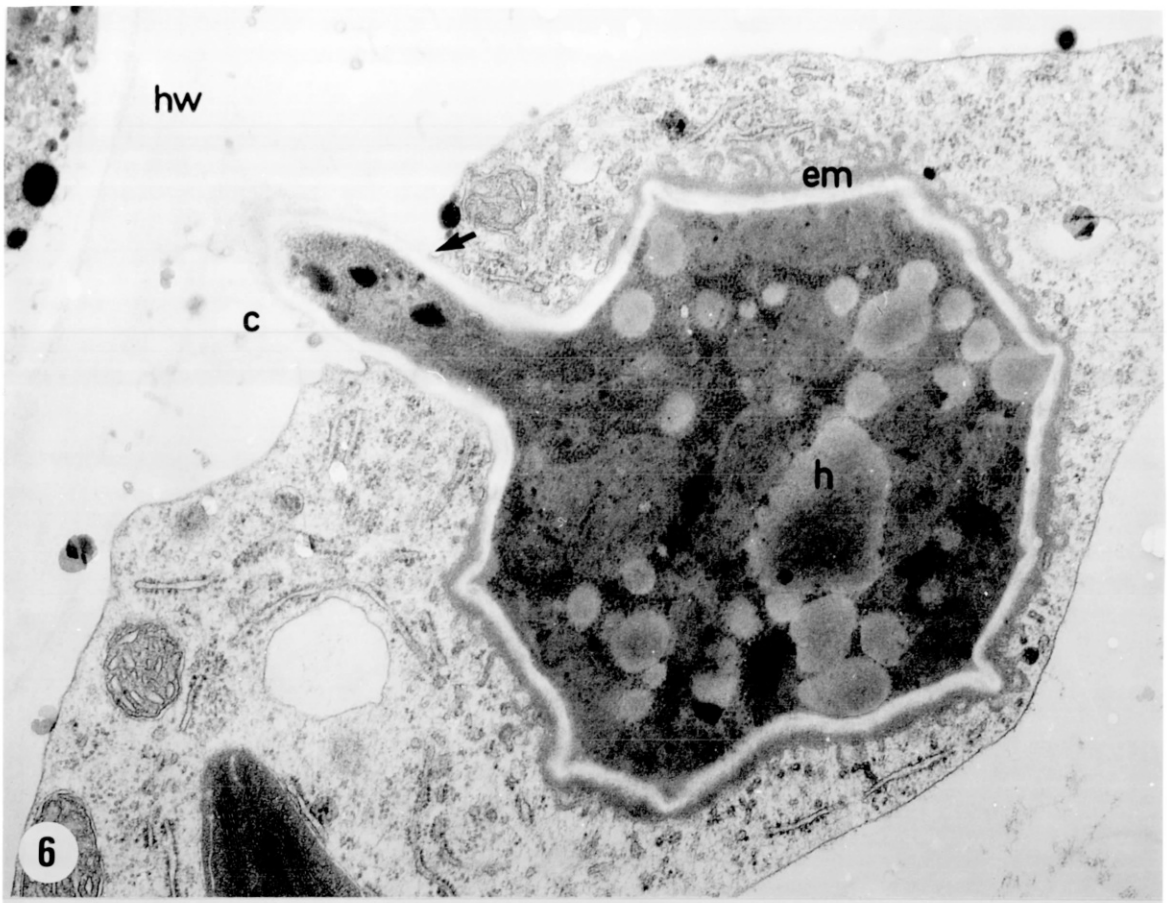

m
ih
h
m
5

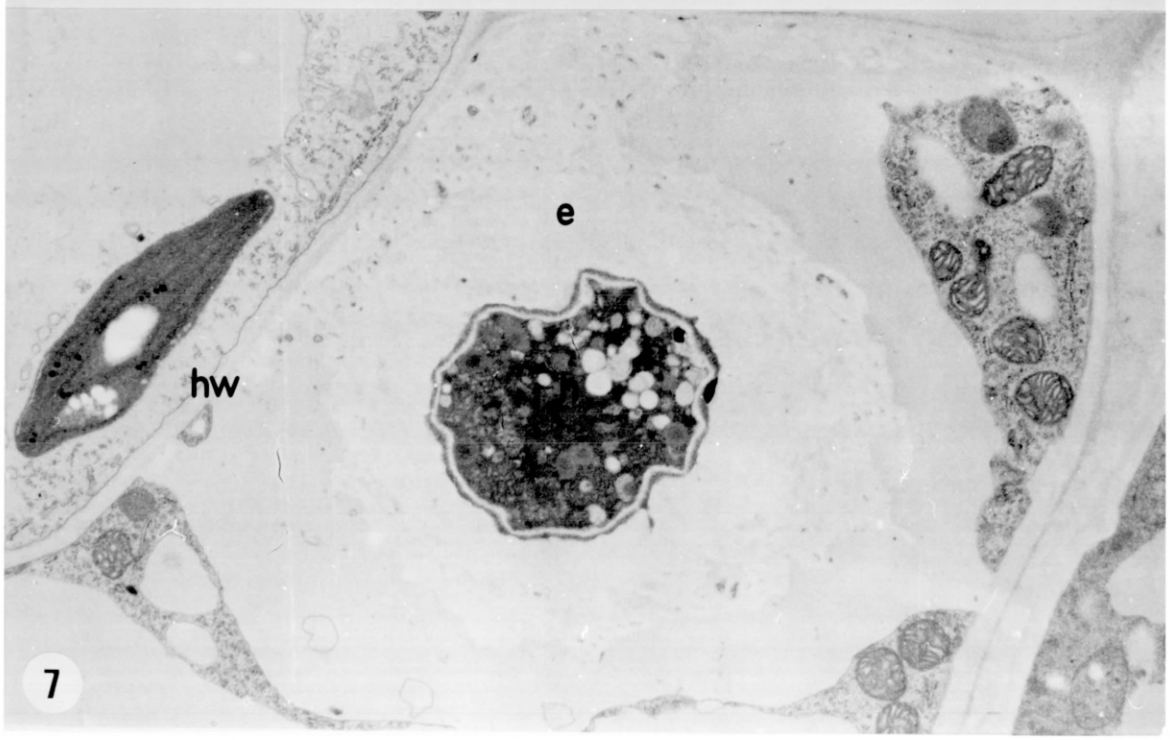
Plate 5:6

Section of an haustorium (h) of A. candida infecting a plasmolysed mesophyll cell of C. hirsuta prepared from material immersed in 0.8M sucrose overnight, stained with uranyl acetate and lead citrate. The host plasmalemma has withdrawn from the host cell wall (hw) but not from the haustorium, remaining closely adherent to the haustorial neck wall (arrowed) from the edge of the collar (c) and at its normal distance around the extrahaustorial matrix (em). X27200.

Plate 5:7

Section of an encased haustorium (h) of A. candida in a plasmolysed mesophyll cell of C. hirsuta prepared from material immersed overnight in 0.8M sucrose. The host plasmalemma has retracted from both the host cell wall (hw) and the encasement (e). X11700.


hw
em
c
h
6


e
hw
7

# 6. X-ray Microanalysis of Haustorial Neckbands and Infected Cells

## 6.1 Introduction

Two of the major characteristics which have been used in the definition of a neckband (Woods & Gay, 1983) are that the fungal wall is of abnormal composition and that it forms an apoplastic seal. Silicon, which can block apoplastic diffusion when deposited between cellulose microfibrils, has been detected within the neckband of the rust Puccinia coronata avenae (Chong & Harder, 1980). Phosphorous, iron and chlorine were also present. Thus, the composition, in particular the silicon content, of the neckband equivalent of Oomycete Albugo candida and the neckband of the powdery mildew Erysiphe pisi was investigated by X-ray microanalysis to determine any consistent components of a neckband which may be responsible for its properties.

There are also numerous reports of silicon in plant cells under fungal attack, particularly in epidermal cells of barley inoculated with E. graminis (Kunoh & Ishizaki, 1975, 1976a, 1976b; Akutsu, Doi & Yora, 1980; Zeyen, Carver & Ahlstrand, 1983). Silicon accumulates within the epidermal wall beneath the appressorium (the halo region) and within deposits including carbohydrate, termed papillae, between the host PM and cell wall. Both haloes and papillae form prior to fungal invasion and subsequently deposition may continue so that the papilla extends as a collar around the haustorial neck. Penetrated papillae have been reported to contain less silicon than unpenetrated papillae and therefore it has been argued that the silicon deposited confers resistance to the host. In one investigation no silicon was detected within a papilla around a young haustorium and around a mature haustorium a silicon content 67 times higher than unaffected regions of the epidermal cell wall was demonstrated, in comparison to a 500-fold increase in silicon content

within unpenetrated papillae (Zeyen et al., 1983).

It has been demonstrated by X-ray microanalysis that silicon may accumulate within papillae of dicotyledonous as well as monocotyledonous hosts (Heath, 1979, 1981a, 1981b) although other investigations using micro-incineration, which is a less sensitive method of detection, indicated that silicon only accumulated within monocotyledonous hosts (Kunoh & Ishizaki, 1975). Thus, the amount of silicon deposited varies between types of plants. The variation may be due to the amount of silicon taken up by the host plant in the transpiration stream, since the proportion of silicon within the Graminaceae is 10-20 times that found in the Leguminaceae (Jones & Handreck, 1965, cited by Lewin & Reimann, 1969) and within the Leguminaceae, bean plants absorb more silicic acid than do pea plants (Barber & Shone, 1966).

Silicon is usually deposited within higher plants in the form of amorphous silica or opal, $SiO_2.nH_2O$ (polymerized silicic acid) derived from monosilicic acid in the transpiration stream (Jones & Milne, 1963; Sangster & Parry, 1976). Deposition presumably takes place from a solution which contains more than 100-140ppm (Scurfield, Anderson & Segnit, 1974), which is the solubility of amorphous silica in water at ordinary temperatures. It has been suggested that such concentrated solutions of monosilicic acid may occur within the transpiration stream through evapotranspiration (Scurfield et al., 1974; Sangster & Parry, 1971; Sargent & Gay, 1977). Thus, the initial deposition of silicon within the halo area of epidermal cells may be due to increased evapotranspiration after the disturbance by the fungus of wax platelets on the epidermal surface. However, as the authors pointed out, this is unlikely to account for subsequent deposition of silica as little water loss can be accounted for by this pathway during the infection and any excess water is lost directly from the fungal mycelium (Ayres, 1976; Hewitt & Ayres, 1975). However, other factors are likely to be important since

it has been demonstrated in experiments with coated and half-coated grass leaves, that silicon may be deposited within epidermal cells from which no evapotranspiration can occur, as long as apoplastic flow is maintained, providing a constant supply of silicic acid (Sangster & Parry, 1971).

Thus, since deposition of elements such as silicon has been related to apoplastic flow, the microanalysis of elements was extended to determine their distribution within the host cells.

## 6.2 Materials and Methods

### 6.2.1 Preparation of Albugo candida infections of Cardamine hirsuta

Pieces of leaves of C. hirsuta infected by A. candida were fixed, without post-fixation in $OsO_4$, and embedded for electron microscopy as described in Section 2.2.1. Sections with a blue interference colour (approximately 200nm thick) were cut as described in Section 2.2.2 and mounted on unfilmed 200 mesh copper grids. These were examined at low magnifications with a Philips 300 transmission electron microscope to locate sections of haustoria with clearly defined necks (Plate 6:1). Their positions on the grids were mapped for ease of subsequent detection for X-ray microanalysis with the Philips 501 scanning electron microscope.

### 6.2.2 Preparation of Erysiphe pisi-infected Pisum sativum

Leaves of Pisum sativum bearing 7-day old infections of Erysiphe pisi were brushed free of their mycelium and then their epidermes were removed under a fixative solution of 2.5% glutaraldehyde and 0.1M sodium cacodylate buffer at pH 7.2 using a pair of forceps. This method tears many of the epidermal cells so that it is possible to view their interior (Chard & Gay, in press). The peeled epidermes were transferred to fresh fixative solution and left for 2h. They were then washed in double distilled water for three 20 min periods and dehydrated in a graded ethanol

series. They were stored in 100% ethanol until required, when they were transferred to 100% acetone via 4 steps: ethanol:acetone (2:1), (1:1), (1:2), 100% acetone. The epidermes were then critical point dried and mounted, with their torn sides uppermost, on copper-tape on an aluminium stub using an adhesive of carbon paint applied at several points around the edge of each epidermal strip. Mounting directly on the aluminium stub was avoided because the λK for aluminium is adjacent to that for silicon.

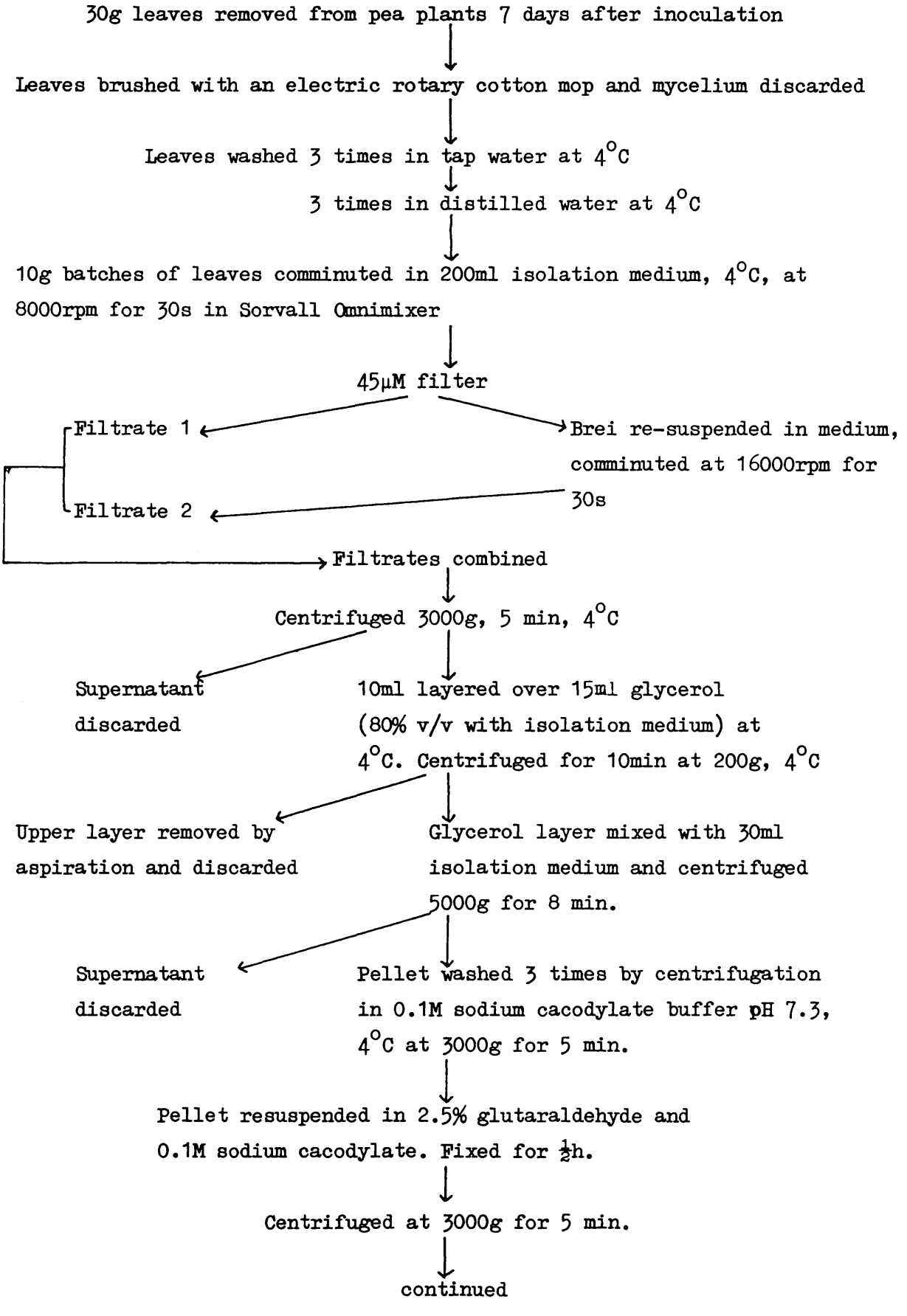
### 6.2.3 Isolation of Haustorial Complexes

The method used was adapted from Gil (1976) and is summarised in Fig. 6: 1. An isolation medium of 0.1M phosphate buffer at pH 6.8 and 0.2M sucrose was used, omitting $MgSO_4$ used in previous investigations (Gil, 1976; Manners, 1979) because the λK for Mg was adjacent to that of silicon. Drops of the haustorial complex suspension were placed on formvar-coated 100 mesh copper grids and allowed to dry in air. The grids were subsequently mounted on a copper stub for analysis.

### 6.2.4 Analytical Procedure and Treatment of Data

In preparation for examination each grid bearing sections was mounted at the end of a carbon tube using a small amount of carbon paint as a conducting adhesive. The sections were then viewed in the reflexion mode with the Philip's SEM 501 at 12KV (a fault in the equipment prevented use of a transmission detector). The beam was focussed on points to be analyzed and the emitted X-rays were collected by a solid state detector coupled to a multichannel analyzer and counter (EDAX). Each analysis was carried out for 100 sec at a beam current previously set to give the total count rate of 150-230 counts/sec on an area of resin surrounding the leaf section. All spectra were recorded on computer discs.

Figure 6:1 Flow diagram for isolation and fixation of haustorial complexes from Pisum sativum infected with Erysiphe pisi



Pellet resuspended in 0.1M sodium cacodylate,
centrifuged, 3000g, 5 min.

↓

Resuspended in distilled $H_2O$

= Lightly fixed haustorial complex suspension

Peeled epidermes were analyzed after location of torn epidermal cells in which haustoria could be seen (Plate 6: 3). The live-time was 100 sec with a beam current previously set to give a total count rate of 22-2300 counts/sec on the aluminium stub. Similarly, when epidermal strips were re-mounted on aluminium stubs for the determination of relative specimen thicknesses of individual structures (described later) the beam current was set so that a count rate of 22-2300 counts/sec on the aluminium stub was obtained.

All analyses of thin sections and epidermal peels were carried out at the University of Minnesota, St. Paul, Minnesota, USA under the supervision of Dr. R.J. Zeyen and Mr. G.G. Ahlstrand.

Isolated haustoria were analyzed with JEOL 733 Superprobe at 12KV for 100 sec live time at a setting which gave a total count rate of 22-2300 counts/sec on an aluminium stub.

When whole structures were analyzed within epidermal peels, it was necessary to obtain an estimate of the relative thickness of the different structures within an infected cell and thus estimate the relative concentrations of elements. Spectral peaks indicated that the electron beam penetrated to the support beneath the specimens and that this penetration was related to the thickness of the structure analysed. Therefore, the proportion of the beam absorbed by the specimen was estimated for each point analyzed. This was first attempted by measuring the net area under the copper peak for each spectrum and also the average net area under the copper peak of a spectrum obtained from the copper tape (termed the Background Copper Count).

$$\text{Thus, Proportion of Beam Absorbed} = \frac{Cu - Cu_x}{Cu}$$

Where $Cu$ = Average net area under copper peak of spectrum obtained from copper tape = Background Copper Count

$Cu_x$ = Net area under copper peak of spectrum obtained from point analyzed

However, although fairly constant within an area the size of an epidermal cell, the background copper count from the copper tape varied from point to point on different parts of the copper tape e.g. at either side of the epidermal strip. Thus the same background copper counts could not be used in the equation above for each cell analyzed. The epidermal strips were therefore re-mounted on aluminium stubs, the aluminium peaks from which did not vary greatly between different areas, and the previously-analyzed cells were re-located. Ideally, each point of the specimen previously analyzed would have been re-analyzed to obtain an estimate of its relative thickness from the formula $\frac{AL - AL_x}{AL}$ where AL = Average net area under aluminium peak of spectrum obtained from aluminium stub and $AL_x$ = Net area under aluminium peak of spectrum obtained from point analyzed. However, due to limited time, each point could not be re-analyzed and therefore 5 of the 10 epidermal wall spectra for each of the 8 infected cells were recorded again in addition to spectra from the aluminium stub. The information from these spectra were then applied to the formula shown below:

$$\text{Mean Proportion of beam absorbed by epidermal wall of particular cell} = \text{mean}\ \frac{(Cu_Z - CU_E)}{Cu_Z} = \text{mean}\ \frac{(AL - AL_E)}{AL} \quad \text{(i)}$$

where $Cu_Z$ = Background copper count for a particular cell, Z;

$Cu_E$ = Copper count for a point on the epidermal wall of cell Z;

AL = Background aluminium count;

$AL_E$ = Aluminium count for a point on the epidermal wall of cell Z.

From (i) above,

$$\overline{Cu_Z} = \frac{\overline{Cu_E}}{1-\frac{(\overline{AL}-\overline{AL_E})}{\overline{AL}}}$$

Since $\overline{Cu_E}$, $\overline{AL}$ and $\overline{AL_E}$ could be determined for each cell examined, an

average background copper count $\overline{Cu_Z}$ for each cell could be calculated and used in the original formula $\frac{Cu-Cu_x}{Cu}$ which is equivalent to $\frac{\overline{Cu_Z}-Cu_x}{\overline{Cu_Z}}$ to find the proportion, P, of the beam absorbed by the specimen at each point analyzed. This gave a measure of the specimen thickness at that point. To obtain an estimate of the relative concentration of an element per unit thickness, the net peak area of the element at each point was divided by P. The distribution of the element in different parts of an infected cell could thus be examined.

In summary:

Relative element concentration at point X in cell Z =

$$\frac{\text{Net area under peak for element}}{\text{Proportion P of beam absorbed by specimen at point X}}$$

where $P = \frac{\overline{Cu_Z} - Cu_x}{\overline{Cu_Z}}$ and $\overline{Cu_Z} = \frac{\overline{Cu_E}}{1-\left(\frac{\overline{AL}-\overline{AL_E}}{\overline{AL}}\right)}$

## 6.3 Results

### 6.3.1 Albugo candida infecting Cardamine hirsuta

Silicon was not present above the level of background counts from the resin surrounding the leaf, within the wall or cytoplasms of the host, intercellular hyphae or haustoria in any of the six cells analyzed. Peaks for phosphorous, sulphur and chlorine were obtained at levels higher than within the resin, but no consistent trends of location were observed. In 2 cells P and Cl were more concentrated within the neck wall region than elsewhere and in 3 cells sulphur was more concentrated within the neck wall, but other cells did not show higher concentrations of these elements in any particular region. Further analysis of data was therefore not carried out. Examples of spectra from the resin and from the haustorial neck wall are shown in Figs. 6:2 and 6:3 respectively.

### 6.3.2 Erysiphe pisi infecting Pisum sativum

Within epidermal peels, the peaks for Si, P, S, Cl and Ca obtained were above levels from background counts on copper tape and aluminium stubs. No consistent location of P, S, Cl or Ca within the infected cells was observed. However, although the peaks were small, Si tended to be located in particular structures. Examples of spectra obtained from different regions of an infected host cell are represented in Figs. 6: 4-8. In order to determine whether there was a significant difference in the quantities of silicon in different parts of the specimen it is usual to examine the results by a two-way analysis of variance. However, as can be seen from Figs. 6: 9-16, although the relative distributions are similar, the absolute amounts of silicon differed greatly between cells. The large variation in the quantities of silicon would thus produce large error values in a two-way analysis of variance. It was therefore inappropriate to apply this test to the results and they are therefore represented as histograms (Figs. 6: 9-16).

The figures show that the greatest concentration of silicon in each infected cell was in the epidermal wall immediately adjacent to the haustorial neck or in the proximal end of the haustorial neck. The papilla could not be clearly identified and thus the epidermal wall immediately surrounding the haustorial neck was termed the papilla region. The silicon concentration in the host wall increased towards the papilla. The concentrations were higher than in uninfected cells and in one case the maximum concentrations were 10 times those in uninfected epidermal walls (Fig. 6: 9). The proximal region of the haustorial neck contained more silicon than its distal part and the haustorial body contained very little silicon, usually no more than the uninfected cell wall.

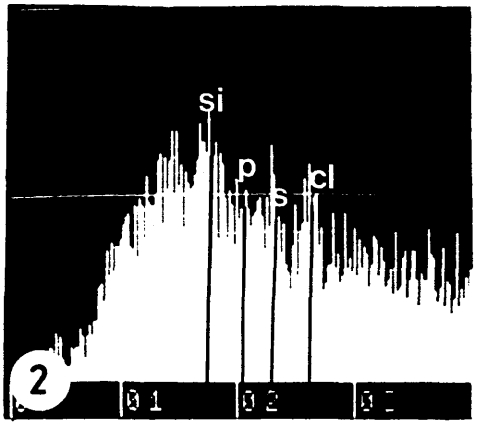
Analysis of isolated haustorial complexes showed that although P and S were present in varying amounts, no silicon above background level was present in either the haustorial neck or the haustorial body.

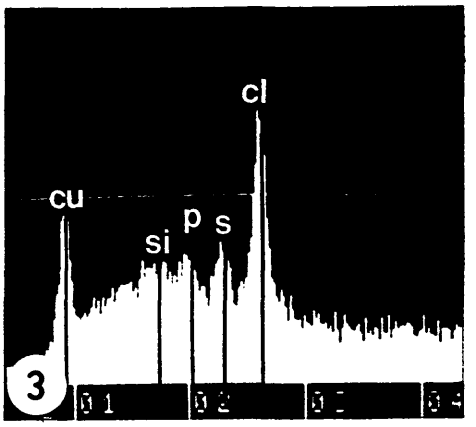
## Figures 6:2 and 6:3

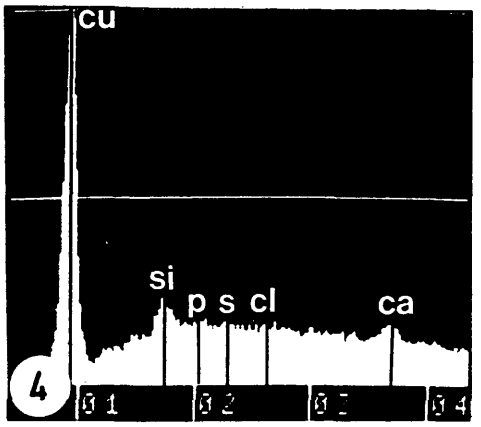
Spectra obtained from analysis of sections of A. candida infecting C. hirsuta. Figure 6:2 shows the spectrum from a region of the resin surrounding the embedded leaf. Figure 6:3 shows the spectrum from part of the haustorial neck wall of A. candida with net peak areas for P, S and Cl but not Si which are greater than those obtained from the resin.

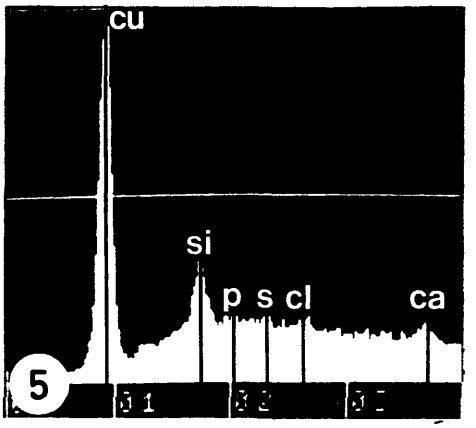
## Figures 6:4 - 6:8

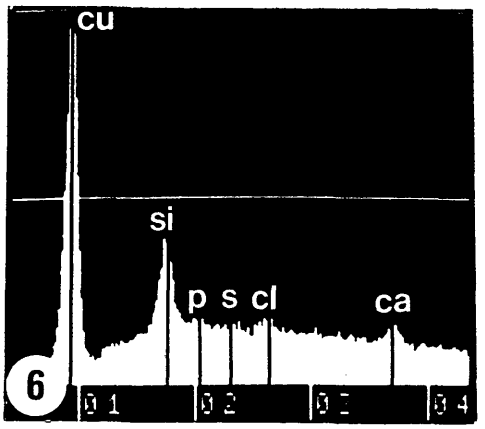
Spectra obtained from analysis of an epidermal cell of P. sativum infected by E. pisi. The regions analysed are as follows: figure 6:4, epidermal wall in unaffected part of cell; figure 6:5, epidermal wall close to infection site; figure 6:6, papilla region; figure 6:7, haustorial neck; figure 6:8, haustorial body. The net peak area for Si is greatest within the papilla region and least within the haustorial body.

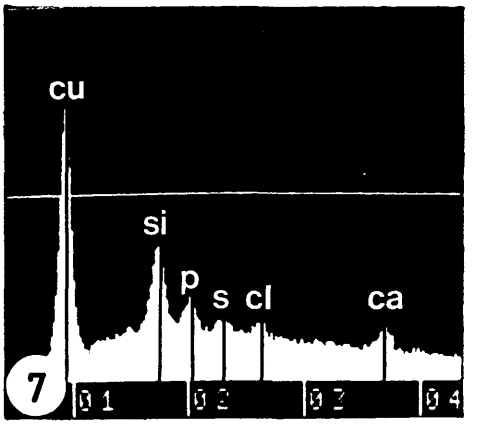

si
p
s
cl
2

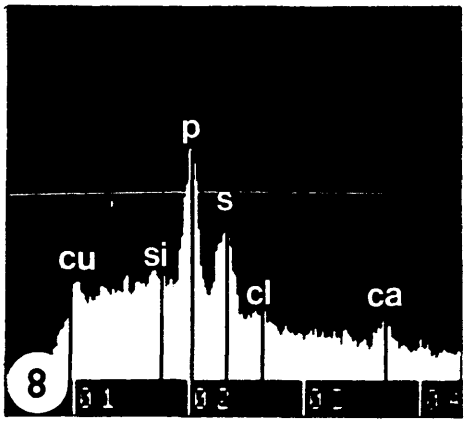

cl
cu
p s
si
3


cu
si
p s cl
ca
4


cu
si
p s cl
ca
5


cu
si
p s cl
ca
6


cu
si
p
s cl
ca
7


p
s
cu
si
cl
ca
8

Figures 6: 9-16

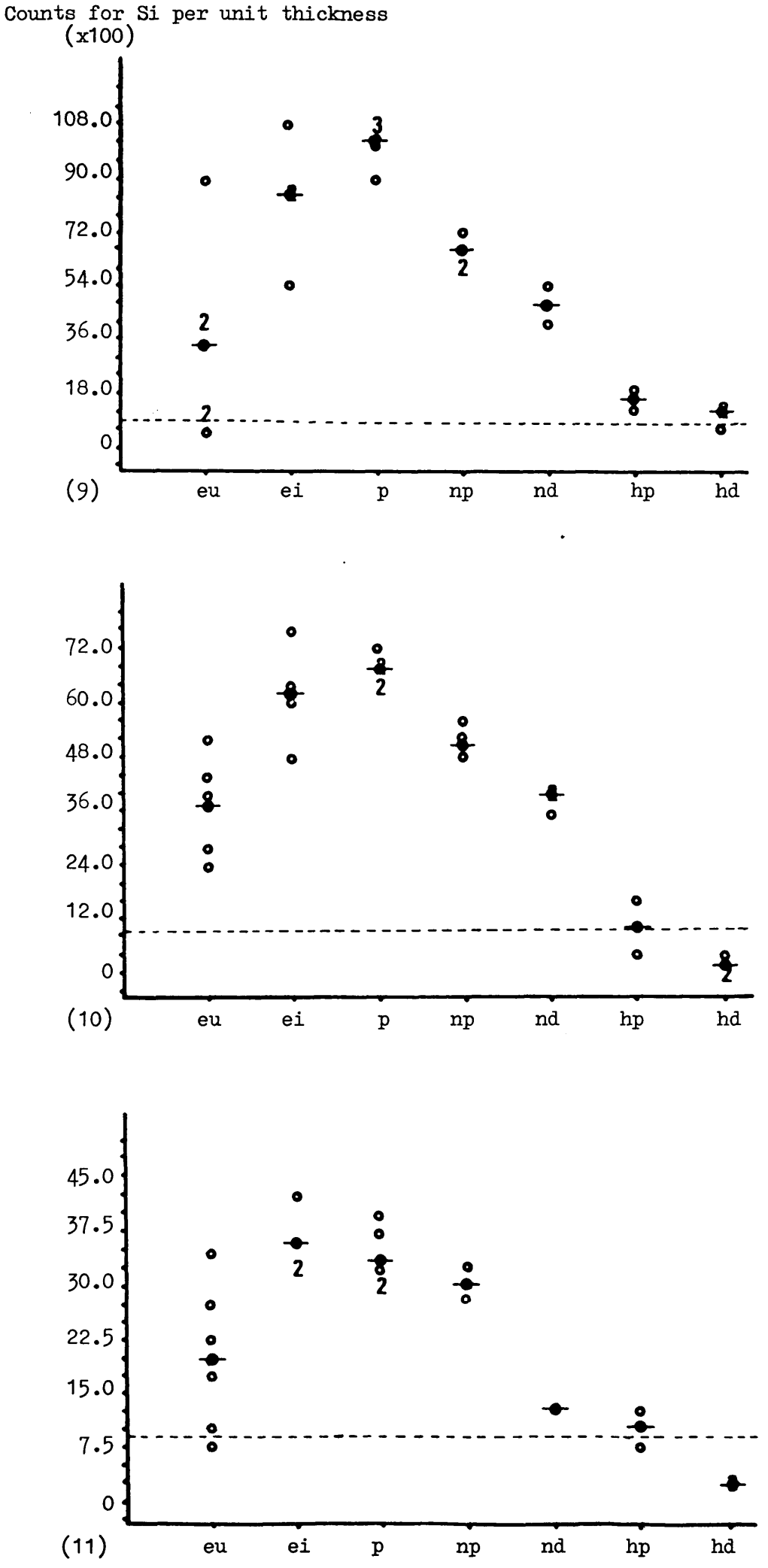
Histograms to show the distribution of Si within cells of peeled epidermes of P. sativum infected by haustoria of E. pisi. Each histogram represents a different cell and the relative concentrations of Si are shown for the epidermal wall in an unaffected part of the cell (eu), epidermal wall close to the infection site (ei), papilla region adjacent to the haustorial neck (p), proximal haustorial neck (hp), distal haustorial neck (nd), proximal haustorial body (hp) and distal haustorial body (hd).

o , Si concentration at a single point

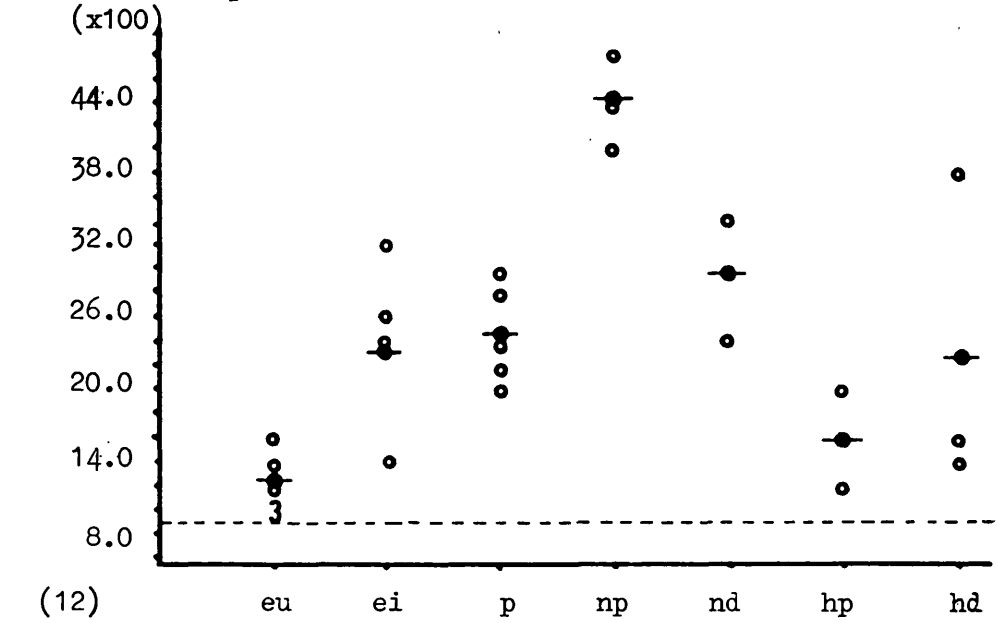
2 or 3, Si concentration at 2 or 3 points analysed

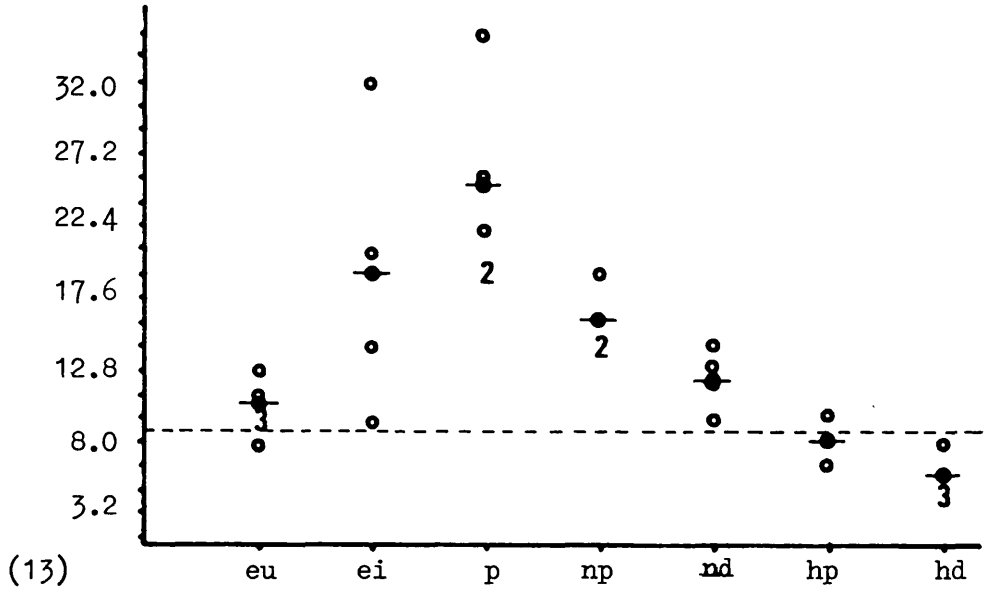
-●- , average Si concentration for region

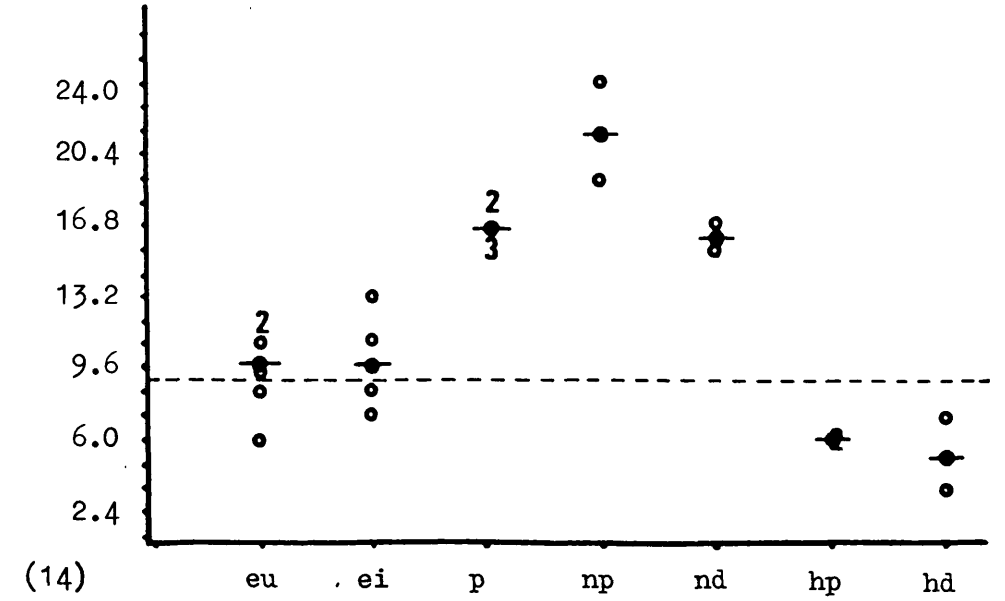
---, average Si concentration in epidermal wall of uninfected cells

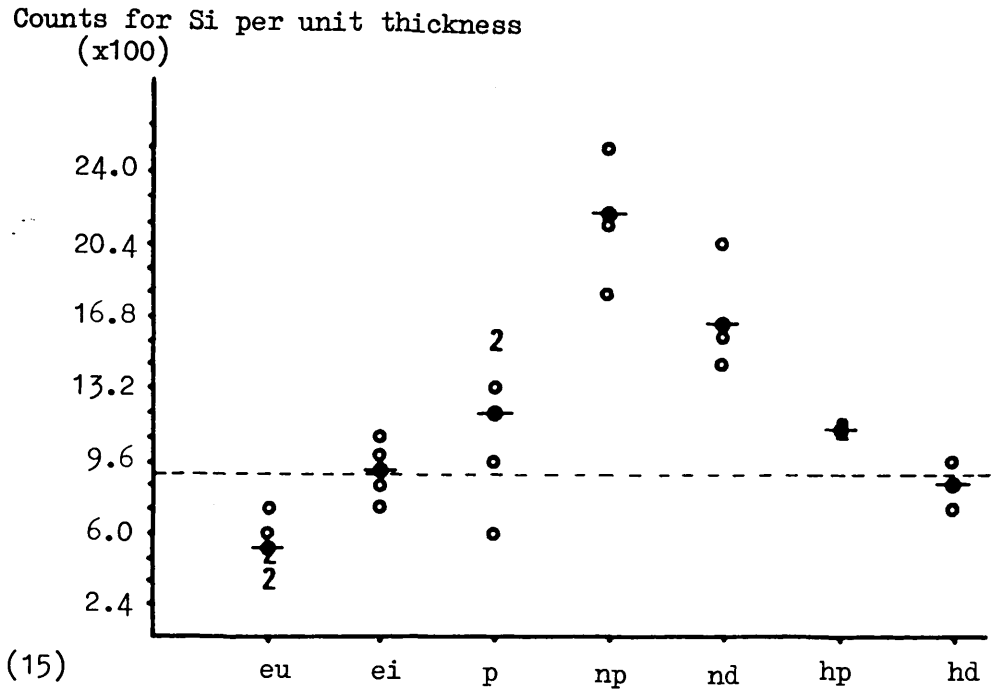

Counts for Si per unit thickness
(x100)
108.0
90.0
72.0
54.0
36.0
18.0
0
(9)
eu
ei
p
np
nd
hp
hd
72.0
60.0
48.0
36.0
24.0
12.0
0
(10)
eu
ei
p
np
nd
hp
hd
45.0
37.5
30.0
22.5
15.0
7.5
0
(11)
eu
ei
p
np
nd
hp
hd

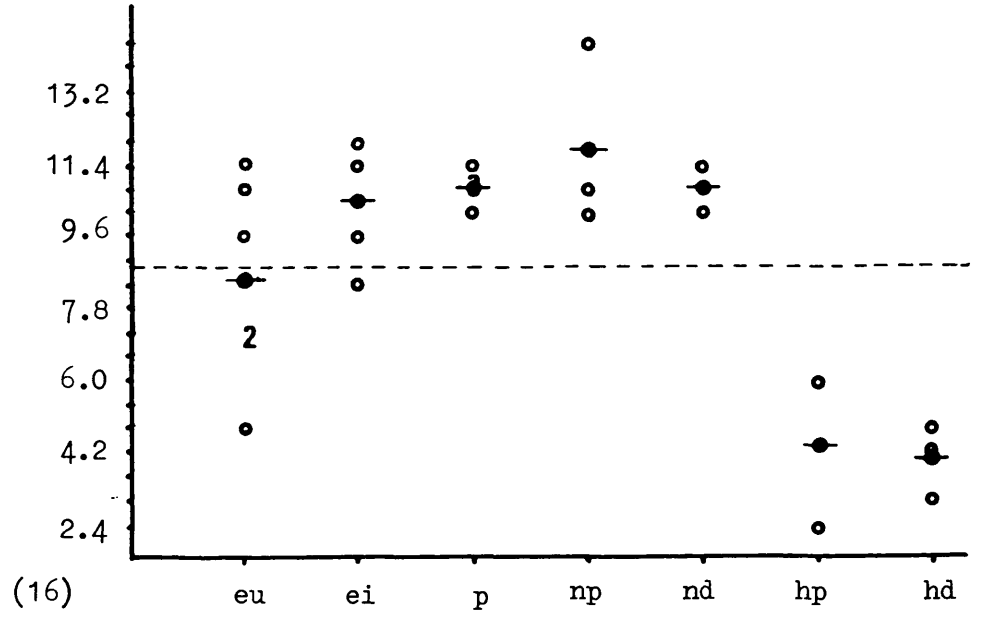
Counts for Si per unit thickness




Counts for Si per unit thickness
(x100)
24.0
20.4
16.8
13.2
9.6
6.0
2.4
2
2
2
(15)
eu
ei
p
np
nd
hp
hd


13.2
11.4
9.6
7.8
6.0
4.2
2.4
2
(16)
eu
ei
p
np
nd
hp
hd

## 6.4 Discussion

Small but variable amounts of silicon have been shown in pea epidermal cells in regions corresponding to haloes and papillae associated with infection sites. Silicon was detected in the proximal regions of haustorial necks of *E. pisi in situ* but it was absent from the necks of isolated haustoria. Thus the silicon detected in *in situ* analyses was presumably due to penetration of the electron beam through the neck to the papilla or collar associated with the haustorium. Only small spectral peaks for silicon were obtained in the investigation and, at most, represented concentrations ten times higher than in uninfected epidermal walls. This contrasts with the 67-fold increase described in mildewed barley (Zeyen et al., 1983).

Accumulation of only small amounts of silicon in penetrated papillae within the epidermal cells of dicotyledonous hosts is consistent with evidence from previous research (Kunoh & Ishizaki, 1975). The low levels may account for some of the variability in the silicon content of papillae in different cells, as small differences in the absolute amount of silicon would have a large proportionate effect. High variation was similarly obtained by Kunoh, Takamatsu & Ishizaki (1978) when low count rates of calcium X-ray signals were used to determine differences between germinated and ungerminated conidia. However, variation in the silicon content of barley papillae has also been shown (Zeyen et al., 1983) and it may be due to penetration, and therefore interruption, at different stages of papilla formation. Silicon accumulation occurs within barley epidermal papillae and, subsequently, in halo regions, only after their formation (Kunoh & Ishizaki, 1976b). Since it has been suggested (Zeyen & Bushnell, 1979) that fungal penetration may arrest or slow the process of papilla formation, it is likely that it also reduces the deposition of silicon. The amount accumulated would therefore

depend on the stage of deposition reached before penetration. Alternatively, variation may be due to different ages of the infection sites, penetration occurring prior to silicon accumulation which, as suggested by Zeyen et al. (1983) continues within the papilla and halo as the haustorium develops.

It was explained in the introduction that silicon may accumulate passively from the transpiration stream where there is a high concentration of monosilicic acid, but it is unlikely that an increase in the concentration within the halo and papilla is due to evapotranspiration (Sargent & Gay, 1977). This suggests that the wall structure in these regions may be altered so that it forms an impedance, but not a barrier, to apoplastic flow and/or binds silicon present in the transpiration stream. Investigations have indicated that lipid or lipidic cuticular precursors from a non-membraneous source within the cytoplasm accumulate within the halo region and papilla (Sargent & Gay, 1977; Zeyen & Bushnell, 1979). It is expected that this would impede apoplastic flow and thus increase the concentration of monosilicic acid until it is sufficient for silicon to be deposited. A sufficiently high concentration of silicic acid would be achieved especially quickly, and thus lead to more silicon deposition, in plants, such as Graminae, which contain a high proportion of silicic acid. The additional presence, within the papilla and halo, of phenols, which are known to bind silicon, has been suggested (Sargent & Gay, 1977), but tests for different phenolics so far applied have proved negative (Sargent & Gay, 1977; Heath, 1979). However, silicon has been shown to bind to many organic materials and so some other host metabolite which binds silicon may be deposited in addition to lipid. If apoplastic flow is blocked by the continued deposition of either a metabolite which impedes the transpiration stream or by the silicon, then no further accumulation of silicon would occur.

Thus, in summary, silicon may accumulate passively from the transpiration stream in the absence of evaporation, indicating sites of impeded apoplastic flow and/or the presence of a substance which will bind silicon.

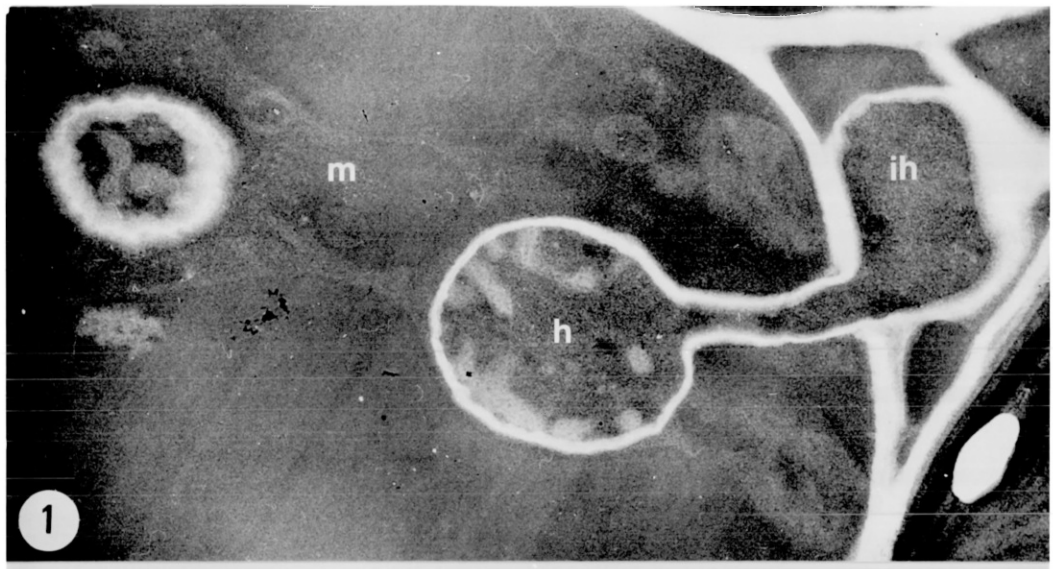
Silicon has been detected within the haustorial neckbands of Puccinia coronata avenae (Chong & Harder, 1980). Neckbands of Puccinia sorghi and E. pisi have been shown to form barriers to apoplastic flow (Heath, 1976; Gil & Gay, 1977; Gay & Manners, in preparation) and it has been suggested by Chong & Harder that the accumulation of silicon may be responsible for the impermeability. However, silicon was not detected within the haustorial neck regions of E. pisi or A. candida in the present investigation, indicating that silicon is not an essential constituent of an impermeable neckband. It was concluded above that silicon accumulation occurs passively at sites where apoplastic flow is impeded and/or substances are present which bind silicon and that deposition would not continue if apoplastic flow, and therefore the supply of silicon, ceased. Thus, the accumulation of silicon at the neckband of P. coronata avenae must occur prior to the formation of a complete barrier. This suggests that within the neck wall a host or fungal metabolite is deposited or the wall structure is altered so that apoplastic flow is impeded and finally ceases. No further deposition of silicon can then occur. This theory is consistent with the absence of an observable neckband in very young haustoria. The high concentration of silicic acid necessary for silicon deposition is less likely to be reached prior to the formation of the impermeable neckband in infections of dicotyledonous hosts and this has been demonstrated by the absence of silicon within E. pisi and A. candida haustorial necks.

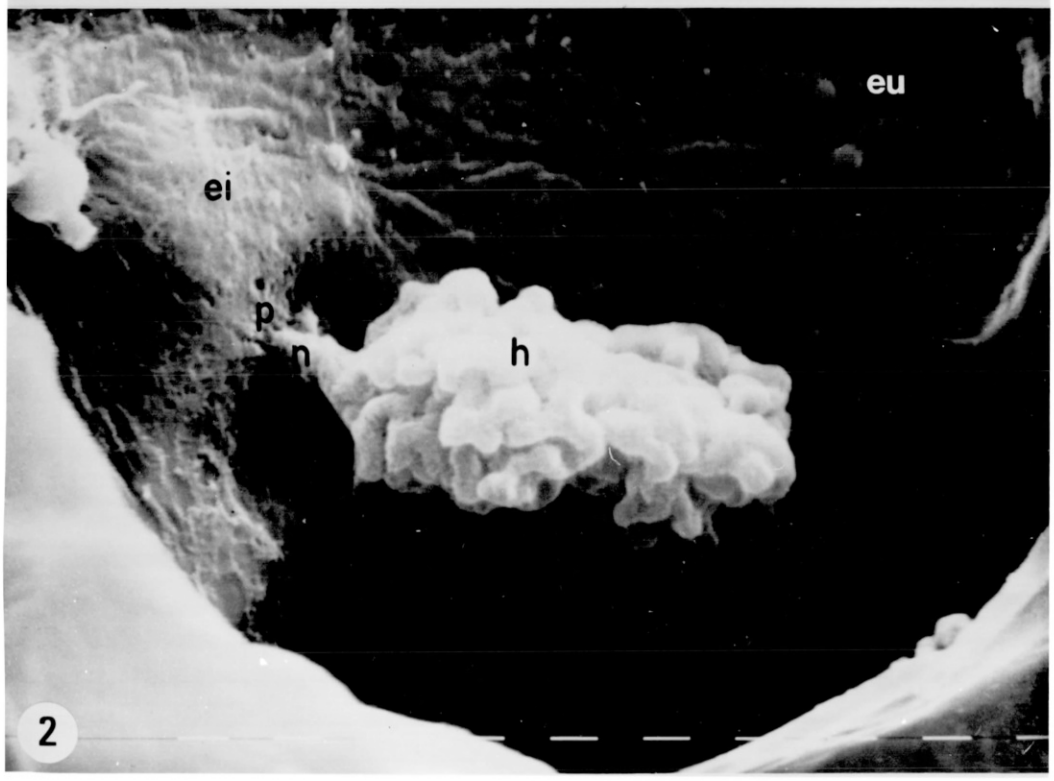
Plate 6:1

Median section of an haustorium (h) of A. candida infecting a mesophyll cell (m) of C. hirsuta prepared from unosmicated material, observed without staining. The walls and cytoplasms of the host, intercellular hyphae (ih) and haustorium were subsequently subjected to x-ray microanalysis. X11200.

Plate 6:2

Scanning electron micrograph of an haustorium of E.pisi infecting an epidermal cell of P. sativum prepared from a leaf stripped of its epidermis under fixative. The haustorial body (h), haustorial neck (n), papilla region (p), epidermal wall close to the infection site (ei) and epidermal wall (eu) in the unaffected part of the cell were subjected to x-ray microanalysis. X6000.


m
ih
h
1


eu
ei
p
n
h
2

## 7. The Host-Pathogen Interface of Tussilago farfara Infected by Puccinia poarum

### 7.1 Introduction

Ultrastructural investigations of monokaryotic rust infections have demonstrated the formation of irregularly shaped filamentous haustoria which terminate within the host cell (Rijkenberg & Truter, 1973; Walles, 1974; Robb, Harvey & Shaw, 1975; Harder, 1978; Al-Khesraji, Lösel & Gay, 1980; Borland & Mims, 1980; Chong, Harder & Rohringer, 1981; Al-Khesraji, 1981; Al-Khesraji & Lösel, 1981). These have been variously termed P-haustoria or M-haustoria. Since most of the fungal mycelium of Puccinia poarum external to the aecium in pycnial-aecial stages is monokaryotic (Al-Khesraji, 1981), the intracellular structures will be referred to as M-haustoria in this account. In a few of the monokaryotic rusts examined, filamentous intracellular structures were observed to exit from the host cell (Gold, Littlefield & Statler, 1978; Gold & Littlefield, 1978), and thus, according to Bushnell's definition (1972) cannot be classed as haustoria. They are therefore referred to as intercellular hyphae.

The investigations cited above have shown the host PM to be invaginated around the M-haustorium, but M-haustoria differ from haustoria of dikaryotic stages (D-haustoria) in many respects: no structure recognizable as a neckband has been observed; they are enclosed to varying extents by host wall-like material; they may be septate and are only slightly constricted at the penetration site. There is no clear evidence that the PM invaginated around M-haustoria is structurally or physiologically different from the uninvaginated PM as is the PM invaginated around D-haustoria, although there has been some indication, using the PACP staining technique, that a difference may exist (Fig. 38 of Chong, Harder & Rohringer, 1981; Heath, cited in Littlefield & Heath,

1979). As specialization of the invaginated region of the host PM and the maintenance of the two regions by a tight junction at the neckband, is thought to be necessary for nutrient transport into haustoria (Spencer-Phillips & Gay, 1981), an investigation of the monokaryotic phase of Puccinia poarum (Niels.) on its pycnial-aecial host Tussilago farfara L. was carried out. These investigations were concentrated on the presence or absence of a neckband and the extent of structural and physiological specializations of the invaginated host PM. Thus, the possible contribution of M-haustoria to the uptake of nutrients by the fungus could be considered.

## 7.2 Materials and Methods

### 7.2.1 Fixation and Embedding of Material for Differential Staining

Pieces of leaves of Tussilago farfara infected by Puccinia poarum were excised under fixative from colonies where the majority of aecidia had not dehisced. Fixation, post-fixation, dehydration and embedding were carried out as described in Section 2.2.1. Sections were cut (Section 2.2.2) either parallel to the surface of the leaf or perpendicular to it, in an attempt to obtain median sections of haustoria.

### 7.2.2 Cytochemical Staining Methods

Sections were mounted on unfilmed 200 mesh gold grids and alternate grids were then subjected to the PACP or PATCHSP staining procedures described in Section 2.2.3. Thus sections of the same haustoria could be located which had been treated by both methods.

### 7.2.3 Localization of ATP-ase Activity

Infected and uninfected leaves were treated by the ATP-ase localization procedure with controls described in Section 2.2.4. Sodium

fluoride (10mM) was used for the ATP + inhibitor control treatment with infected material, whereas sodium orthovanadate (150μM) was used with uninfected material to test both inhibitors. Sections on alternate grids were observed either unstained or after PATCHSP staining to identify with certainty the material between the invaginated host PM and the haustorial wall.

## 7.3 Results

M-haustoria were observed in mesophyll cells but occurred more frequently in xylem and phloem parenchyma and transfer cells, xylem vessels and bundle sheath cells (Plates 7: 1,2,21). They were filamentous without a narrower neck region and were only slightly constricted at the site of penetration of the host cell wall (Plates 7: 11,13-17,19). The wall-lining region of the host plasma membrane was continuous with the membrane surrounding the M-haustoria and between the invaginated membrane and the haustorial wall, several different types of material were identified by the PACP, PATCHSP and uranyl acetate-lead citrate (UA/Pb) staining procedures. These are referred to as host wall-like material, proximal matrix and distal matrix.

Host cell wall-like material extended from the penetration site to varying lengths along the haustorium. Around some haustoria the material tapered to a rim to form a collar (Plates 7: 11-18) whereas other haustoria were completely enclosed (Plates 7: 3-5,11,19-21). The material gave similar staining reactions to the host cell wall with PACP (lightly stained) (Plates 7: 4,6,10,11,13), PATCHSP (moderately stained) (Plates 7: 3,5,7,16,20) and UA/Pb (lightly stained) (Plates 7:14). Frequently, parallel fibrils were observed in this material as in the host cell wall (Plates 7: 3,4,5,6,7). The invaginated PM followed the contours of the wall-like material but was slightly undulate. Haustoria which were

totally enclosed usually had vacuolated cytoplasms (Plates 7: 11,19,21) but enclosed younger haustoria with few vacuoles, were also observed (Plates 7: 3,4). Those which were only partially enclosed contained few or no vacuoles (Plates 7: 6-18).

In haustoria which were only partially enclosed, another substance could be distinguished between the haustorium and the invaginated host PM beyond the region of cell wall-like material. This was discerned by the PACP technique (Plates 7: 6,8,10-13) and, to a lesser degree, with UA/Pb stain (Plate 7: 14). Both procedures resulted in a more electron dense reaction than with host wall-like material and neither showed fibrils. With the PATCHSP technique, however, the region was stained with similar intensity to that of the collar of host wall-like material, but it was distinguishable by its amorphous texture and narrower dimensions (Plates 7: 7,9,16). A similar layer has been described as the proximal matrix by Chong, Harder and Rohringer (1981). The invaginated host PM followed the contours of the matrix and was, in most cases, closely associated with it, although not completely smooth. The matrix continued along the length of the haustorium to the distal portion where it merged with the third type of material between the haustorial wall and the invaginated membrane, similar to that described by Chong et al. (1981) as the distal matrix. This region continued round the tip of the haustorium and could be distinguished from the proximal matrix by both PACP and PATCHSP procedures. With PACP, fibrils of similar electron density to the proximal matrix were observed to radiate from the haustorium within the wider transparent region of the distal matrix (Plates 7: 8.10). With PATCHSP the distal matrix was mainly unstained, but with occasional electron dense inclusions (Plates 7: 9,16). The invaginated host membrane was convoluted around the distal matrix.

Within infected cells, distinct regions of the host PM were distinguished using PACP or ATPase localization procedures although they

could not be discerned with PATCHSP or UA/Pb staining techniques. Sections on alternate grids of specimens which had been incubated with ATP as substrate were observed either unstained or stained by the PATCHSP procedures. This enabled the identification of the material adjacent to each region of the host PM.

Similar distributions of precipitates within cells were obtained with ATP (Plates 7: 15,17-19,21) and βGP (Plate 7: 22) as substrates and also with the ATP + NaF controls. However, the no substrate controls showed no precipitate apart from a fine lead deposit within the endomembrane system of both host and fungus (Plate 7: 23). Precipitates on the PM of cells of uninfected leaves were obtained with both ATP (Plate 7: 24) and βGP substrates and the level of precipitates with ATP was greatly reduced in comparable cell types in the presence of sodium orthovanadate (Plate 7: 25). Since sodium orthovanadate is a more specific inhibitor of plant PM ATP-ase than NaF, the results indicate that precipitates obtained on the PM when ATP was employed as substrate represent ATPase activity rather than non-enzymic hydrolysis. In the absence of an orthovanadate control with βGP it is not possible to distinguish between ATPase and general phosphatase activity.

The wall-lining region of the host PM was intensely stained with the PACP technique (Plates 7: 4,6,8,10,11-13), particularly within transfer cells and parenchyma associated with vascular tissue. Flocculent precipitates obtained by the ATPase localization procedure, indicating enzyme activity, were also formed on the host cell wall-lining region of the PM (Plate 7: 15,17,18,19,21). The level of activity varied across a block of tissue according to penetration of the reagents, but within the same region, activity varied with cell type, low in mesophyll cells but high in xylem and phloem parenchyma and transfer cells.

The invaginated host PM adjacent to the host cell wall-like material either partially or totally surrounding the haustorium was also

intensely stained by the PACP method (Plates 7: 4,6,10-13) and possessed a similar level of ATPase activity to the uninvaginated host PM of the same cell (Plates 7: 15,17-19,21).

Where the invaginated host PM was adjacent to either proximal or distal matrix, it exhibited varying reactions to the PACP technique. In most cases it was only lightly to moderately stained (Plates 7: 6,8, 10-13), although it was occasionally intensely stained like the uninvaginated host PM. However, these regions of the invaginated host PM consistently lacked precipitates with the ATPase localization procedure (Plates 7: 15,17,18).

The transition in characteristics of the host PM between the region adjacent to the host cell wall-like material and the region adjacent to the matrix was seldom abrupt. In most cases a gradual change in the intensity of the PACP staining and the level of ATPase activity extended over distances of approximately 1.5μM (Plates 7: 13,18).

The plasmalemma of both intercellular hyphae and haustoria stained lightly to moderately with the PACP technique (Plates 7: 6,10,12,13), but precipitates were not observed on this membrane when either ATP or βGP was employed as substrate (Plates 7: 15,17-19,21,22). The membrane of the endoplasmic reticulum of both host and fungus was lightly stained by the PACP method (Plates 7: 4,6,10,12) in contrast to the intense or moderate reaction of the uninvaginated or invaginated host PM and, although a fine precipitate similar to that in no substrate controls was observed within the cisternae (Plates 7: 15,17,21,22), no densely granular precipitates were observed in them with the ATPase or βGPase localization technique.

## 7.4 Discussion

The M-haustoria of Puccinia poarum infecting Tussilago farfara have been shown to lack a defined neck or any specially stained region

within the haustorial wall resembling a neckband, both of which are characteristic of most other haustoria which have been described, including those of the dikaryotic phase of this fungus (Al-Khesraji & Lösel, 1981). Two different types of M-haustoria were observed, some being partially enclosed by a collar of host wall-like material of varying extent and others being totally enclosed. These observations are in agreement with a previous investigation of P. poarum on T. farfara (Al-Khesraji & Lösel, 1981). An association of completely enclosed haustoria with bundle sheath and mesophyll cells rather than with vascular tissue was noted by these authors, but this was not observed in the present investigation.

Similar structures have been described in ultrastructural investigations of other monokaryotic rust infections which all form filamentous M-haustoria without neckband-like regions. In studies of Cronartium ribicola, P. coronata avenae, and P. podophylli, the haustoria were partially enclosed by host wall-like material (Robb, Harvey & Shaw, 1975; Harder, 1978; Borland & Mims, 1980; Chong, Harder & Rohringer, 1981) whereas in P. sorghi they were totally enclosed (Rijkenberg & Truter, 1973). In addition, SEM studies of P. recondita (Gold, Littlefield & Statler, 1978), Melampsora lini (Gold & Littlefield, 1978) and P. malvacearum (Gold, Littlefield & Statler, cited in Gold et al., 1978) revealed similar intracellular structures, but these exited from the cells and are therefore classed as intracellular hyphae. Although most dikaryotic rust infections form haustoria with a neckband and sometimes a very small collar, exceptions do exist, such as the telial stage of Kunkelia nitens which forms filamentous haustoria partially enclosed by an extensive wall-like collar (Glidewell & Mims, 1979) and the uredial stage of Physopella zeae which, in addition to unenclosed haustoria, forms filamentous haustoria (termed intracellular hyphae) enclosed by material which extends around the host cell wall of infected cells (Heath

& Bonde, 1983). In both these examples no recognizable neckband was observed

Enclosure of haustoria

Although M-haustoria which are entirely enclosed by host wall-like material resemble in some respects the encasement of dikaryotic rust haustoria by resistant host cells (Heath, 1971; Coffey, 1976), the two are not identical. In the resistant reactions described, the encasement is an extension of the callose-containing collar, formed by continued deposition of material by the host PM proximal to the neckband. This traps a layer of cytoplasm, bounded by the extrahaustorial membrane and the newly invaginated host PM, between the extrahaustorial matrix and the encasement. In contrast, the host wall-like material enclosing the monokaryotic haustoria of P. poarum is immediately adjacent to the haustorial wall without an intervening layer of host cytoplasm or plasma membrane. This host wall-like material probably includes callose. This deduction follows from the work of Al-Khesraji and Lösel (1981) who viewed aniline blue-stained sections in ultra-violet light and found that M-haustoria fluoresced a yellow/green colour, characteristic of callose. The authors pointed out that the recent demonstration of the role of callose in cellulose biosynthesis in cotton fibres (Meier, Buchs, Buchala & Homewood, 1981) justifies the hypothesis that the presence of callose indicates sites of synthesis of host wall material (Al-Khesraji & Lösel, 1981). This is also supported by the present finding of intense PACP staining of the invaginated host PM adjacent to the host cell wall-like material since it has been suggested by Van der Woude (1973) that chromic acid-phosphotungstic acid reacts with glycolipids involved in the synthesis of cell wall polysaccharides.

Specialization and origin of the invaginated host PM

The invaginated host PM around totally enclosed M-haustoria is indistinguishable from the normal host PM lining the cell wall, in possessing

ATPase and βGPase activity, an intense PACP staining reaction and, presumably, the ability to synthesize new host wall. However the host PM invaginated around partially enclosed M-haustoria consists of two regions: one, adjacent to the host wall-like collar is indistinguishable from the uninvaginated part of the membrane and the other region, adjacent to the proximal and distal matrices, lacks the plasmalemma characteristics listed above. A similar specialization of the extrahaustorial membrane has been indicated by the PACP technique in M-haustoria of P. coronata avenae (Fig. 38 of Chong et al., 1981) and Uromyces phaseoli vignae (Heath cited in Littlefield & Heath, 1979). The origin of the specialised region of host membrane is uncertain, but, as suggested for A. candida infections (Woods & Gay, 1983), there are two equally plausible explanations: either the fungus modifies the host PM to which it is adjacent by means of a metabolite, or the invaginated region of the host PM may be formed from an abnormal source, e.g. directly from the ER rather than from Golgi vesicles. The absence of ATPase activity observed in the specialised region of PM would follow from each hypothesis. The close association of ER and the extrahaustorial membrane supports the theory of ER origin, but the PACP staining reaction of the region of host PM adjacent to the matrices is different from that of the ER as well as from the uninvaginated PM, suggesting that some source of membrane other than ER, or a modification of normal PM by a fungal metabolite are more likely.

Maintenance of 2PM domains

Both the gradual nature and the varied position of the transition in characteristics of the two regions of host PM are in contrast to the abrupt transition which has been described at neckbands of haustoria (Spencer-Phillips & Gay, 1981; Woods & Gay, 1983). This and the enclosure of haustoria (discussed later) suggest the absence of an extrinsic

barrier to membrane fluidity, as effected by a neckband, around M-haustoria of P. poarum, which would prevent the intermixing of two regions of a fluid membrane. However, rates of lateral diffusion of proteins and lipids within membranes are normally very rapid and it is unlikely that a completely fluid membrane would exist as two distinct regions, even with a gradual transition in characteristics between them. The lateral diffusion coefficient of proteins within membranes is approximately $10^{-9}$ - $10^{-10}$ $cm^2$ $s^{-1}$ and of lipids approximately $10^{-8}$ $cm^2$ $s^{-1}$, giving a lateral diffusion rate in any one direction of 0.2-0.6 $\mu ms^{-1}$ and 2 $\mu ms^{-1}$ respectively. Assuming the alternative source theory for origin of the invaginated region, synthesis of new specialised membrane would be required at a rate corresponding to the growth rate of the M-haustorium. Even with a rapid growth rate, like that of Saprolegnia, of 0.3 $\mu m$ $s^{-1}$ the rate of specialised membrane synthesis would not exceed the rate of diffusion of its components and thus a specialised region would not be maintained. Alternatively, if the modification theory were correct then in order to form a specialised region the fungal metabolite must diffuse and alter membrane proteins such as the ATPase complex at a rate exceeding or equal to the protein lateral diffusion rate. However if this were the case, altered proteins diffusing at the rate given above, would migrate the whole length of an M-haustorium, 20 $\mu m$ long, within 30-100s and thus modification of both the uninvaginated and the invaginated PM would occur. This was not observed. Thus, the existence of a specialised region of invaginated PM in the absence of a neckband which may serve as an extrinsic barrier to membrane fluidity, and the gradual transition in characteristics at varying locations suggest a decrease, but not absence of fluidity of whole or part of the invaginated PM.

Decreased fluidity of biological membranes commonly results from an increase in the content of sterols or saturated, as opposed to unsaturated, fatty acids. Such changes have been reported in senescing or damaged biological membranes (Houslay & Stanley, 1982). Furthermore, they may be responsible for the absence of ATPase activity in the specialised region of the host PM. It has been established that ATPases require a fluid lipid environment (Kimelberg & Paphadjopoulos, 1972) and they may also be sensitive to small changes in the level of unsaturation of fatty acids. The latter has been demonstrated in investigations of a chloroplast $Mg^{2+}$ ATPase which requires unsaturated rather than saturated monogalactosyldiacylglycerol (Pick, Gounaris, Admon & Barber, 1984). However, lowered concentrations of unsaturated fatty acids within the invaginated PM would decrease diffusion rates from host to fungus since high levels of unsaturated fatty acids have been shown to increase passive diffusion rates by occupying a greater molecular area (Demel, Geurts van Kessel & van Deenen, 1972). The consequent decreased rates of diffusion may be compensated for by carrier-mediated transport. If, alternatively, a decreased fluidity of the invaginated PM results from an increased sterol content, this is not a mechanism common to all haustorial infections since a recent freeze-fracture examination of the dikaryotic rusts Uromyces appendiculatus and Puccinia coronata avenae (which both possess neckbands) has shown that although sterols are present in the wall-lining host PM, they are generally absent from the extrahaustorial membrane and the host ER (Harder & Mengden, 1982).

Thus, the existence of a specialised region of the invaginated PM, a gradual transition in membrane characteristics and the absence of ATPase activity may all result from a decrease in fluidity of the invaginated PM brought about by alteration of the fatty acid or sterol content. These differences could be accounted for by either the alternative source or modification theories, but if, for example, host ER is the alternative

membrane source, subsequent modification would be required since ER contains less lipid (Morré & Mollenhauer, 1974) and sterols (Harder & Mengden, 1982) than the host PM and is therefore more fluid.

Although a decrease in membrane fluidity seems most likely to be brought about intrinsically, a mechanism extrinsic to the membrane, such as transient binding to the proximal matrix, cannot be completely discounted.

Development and enclosure of haustoria

The two different types of M-haustoria may represent either independent lines of development or different stages during the formation and ageing of the haustoria. Al-Khesraji and Lösel (1981) examined sections taken from the centre and the edge of colonies of P. poarum on T. farfara. They noted the complete enclosure by host wall-like material of penetration pegs and mature, but not young, M-haustoria in infected bundle sheath cells. Contrary to the findings of this present study, they did not observe enclosed haustoria within other cells of the vascular tissue. Results of the present investigation showed that haustoria with highly vacuolated cytoplasms were always completely enclosed by host wall-like material and a normal host PM whereas those with only slight or no vacuolation were either partially or totally enclosed. Since vacuolation of the cytoplasm indicates ageing of the fungus, these observations and those of Al-Khesraji & Lösel (1981) suggest that the two different types of haustoria represent different stages of maturity and make it possible to suggest a course of events. The penetration peg is completely enclosed by host wall-like material, as observed by Al-Khesraji and Lösel (1981), and subsequently the two types of haustoria may arise either independently or sequentially. Independent origin alone is not consistent with the observation that highly vacuolated (older) haustoria are always completely enclosed. Sequential development following penetration, the young haustorium being partially enclosed

and becoming totally enclosed as it ages, does not account for some haustoria which are unvacuolated (young) and completely enclosed. Thus, a combination of these two patterns of events is suggested, as follows; after penetration of the host wall the peg is surrounded by host wall-like material which is then either ruptured or extends to keep pace with haustorium development. Subsequently, as the partially enclosed haustoria age they become completely surrounded by host wall-like material.

The different courses of events may be due to the relative rates of fungal growth and consequent plasmalemma development, and the intermixing of the new and existing components of the host PM. For example, if extension of the M-haustorium is rapid, the rate at which components are formed or modified within the extrahaustorial membrane may exceed their lateral diffusion rates. Thus, complete intermixing will not occur. Alternatively, if, at an initial or subsequent stage, invasion is slow, diffusion of constituents may keep pace with the formation or modification of the membrane so that even with reduced membrane fluidity the segregation of constituents does not occur. Therefore, distinct regions within the membrane are not differentiated and the haustorium becomes completely enclosed by host wall-like material.

Thus, it seems that in the absence of a neckband which extrinsically prevents diffusion of membrane components, a specialised membrane region is less likely to be formed and, if formed, would be less permanent. This hypothesis may be applied to the enclosure of young dikaryotic haustoria prior to neckband formation in the rusts Uromyces appendiculatus (Hardwick, Greenwood & Wood, 1972) and Melampsora pinitorqua (Coffey, cited in Littlefield & Heath, 1979). Subsequent to neckband formation haustoria may be encased (as described earlier) but there are no reports of enclosures of the type described in P. poarum.

Except within infections by Peridermium pini (Walles, 1974) both enclosed and partially enclosed M-haustoria have not been observed within the same infection in ultrastructural studies of monokaryotic rusts, although none has shown the presence of a neckband. It seems possible that the particular type of M-haustorium observed may depend on the age of material examined, as indicated by research with the monokaryon of Cronartium ribicola (Robb et al., 1975; Colley, 1918). The ultrastructural study by Robb et al. revealed a filamentous haustorium surrounded by a collar of host wall-like material at the proximal end and an extrahaustorial matrix (termed sheath) at the distal. However, in an examination by light microscopy, Colley (1918) noted that young unvacuolated haustoria were unenclosed, but, as the haustorium matured, wall-like material developed around it until the whole haustorium was enclosed. At that stage the cytoplasm was vacuolate. Thus, further detailed ultrastructural studies of monokaryotic rust haustoria are required to determine the exact nature of the enclosing material and whether the absence of a neckband is always associated with the eventual enclosure of haustoria.

Functional implications

It has been suggested that the presence of a modified invaginated PM, lacking ATPase activity, is essential for nutrient transport into haustoria so that all host ATPase pumps are polarised towards the fungus (Spencer-Phillips & Gay, 1981; Woods & Gay, 1983). A neckband maintains this specialised region and forms a seal so that, as discussed in Section 5, the host and haustorial cytoplasms are coupled to form a functional unit (Manners & Gay, 1983). The enclosure of haustoria of P. poarum indicates that there is no such barrier to apoplastic diffusion. The distribution of ATPase activity in cells of T. farfara containing partially enclosed haustoria is similar to that described for A. candida (Woods & Gay, 1983) and U. appendiculatus (Spencer-Phillips

& Gay, 1981) infections, although the present result is more equivocal because both ATPase and βGPase activity were similarly distributed. (A control with βGP and Na orthovanadate (a specific ATPase inhibitor) would distinguish with certainty between ATPase and general phosphatase activity.) Nevertheless, it seems likely that a transport mechanism similar to that proposed by Woods & Gay (1983) may apply. However, it is expected that in the absence of an apoplastic seal and an extrinsic barrier to membrane fluidity, transport would be less efficient since the host and fungus are not coupled. Additionally it would be less permanent, since when an haustorium is completely enclosed by host wall-like material and a normal host PM, it would be equivalent to an intercellular hyphae with regard to nutrient absorption. Therefore it is concluded that the formation, efficiency and permanence of a nutrient uptake mechanism by the filamentous M-haustoria of P. poarum may be adversely influenced by the absence of a neckband.

Despite the above argument for decreased efficiency of transport, examination of the results obtained by Al-Khesraji (1981) from studies of both the monokaryotic and dikaryotic infections by P. poarum reveals no evidence that the monokaryon is any less successful than the dikaryon in spore production, but differences in the details of colonisation have been noted: the intercellular mycelium of the monokaryon was more extensive than that of the dikaryon, but the dikaryon penetrated a greater percentage of host cells. The latter may not indicate greater contact of the dikaryon with the host membrane as each monokaryotic haustorium has a larger surface area. The invasion of the vascular tissue, particularly the phloem, which occurred in the monokaryotic but not the dikaryotic infection, may be a key factor responsible for the success of the monokaryon. The high concentration of nutrients within the vascular tissue would provide a large diffusion gradient between host and fungus which may compensate for the loss of solutes to the general apoplast

and/or re-entry of solutes into the host from the extrahaustorial matrix. In addition, the plentiful supply of nutrients from the vascular tissue may also compensate for the frequent enclosure of the haustoria. The importance of the vascular invasion by the monokaryon is supported by autoradiographic studies by Al-Khesraji (1981), who fed the bases of infected leaf segments with tritiated glycerol. Haustoria in cells of the vascular tissues were found to contain isotope whereas non-vascular haustoria were only labelled at their surfaces. Al-Khesraji concluded that the vascular tissue, particularly the phloem, was the direct source of label incorporated into spores and sporing structures of P. poarum on T. farfara. However, it must be noted that the vascular M-haustoria in material examined by Al-Khesraji (1981) were not enclosed as were mature haustoria in mesophyll and bundle sheath cells. Although this absence of enclosure has not been found in the present study, there is a possibility that the position of autoradiographic label may correspond to the presence or absence of a host wall-like enclosure, rather than to different diffusion gradients.

Invasion of vascular tissues by monokaryotic rust haustoria also occurs in infections by P. coronata avenae (Harder, 1978), C. ribicola (Colley, 1918) and Peridermium (Cronartium) pini (Van der Kamp, 1969; Walles, 1974). Vascular tissues are not infected by either the intracellular hyphae of monokaryotic stages of P. recondita (Gold, Littlefield & Statler, 1978) or P. malvacearum (Allen, 1935; Gold et al., cited in Gold et al., 1978) nor the haustoria of any dikaryotic rust so far reported. Thus, vascular invasion is limited to M-haustoria, but may not be universally characteristic, some investigators having omitted to state the distribution within the host.

Plate 7:1

Section showing distribution of intercellular hyphae (ih) and M-haustoria (h) of P. poarum infecting xylem and phloem parenchyma (p) of T. farfara. Sectioned parallel to the leaf epidermis and stained by the PATCHSP procedure. X4700.

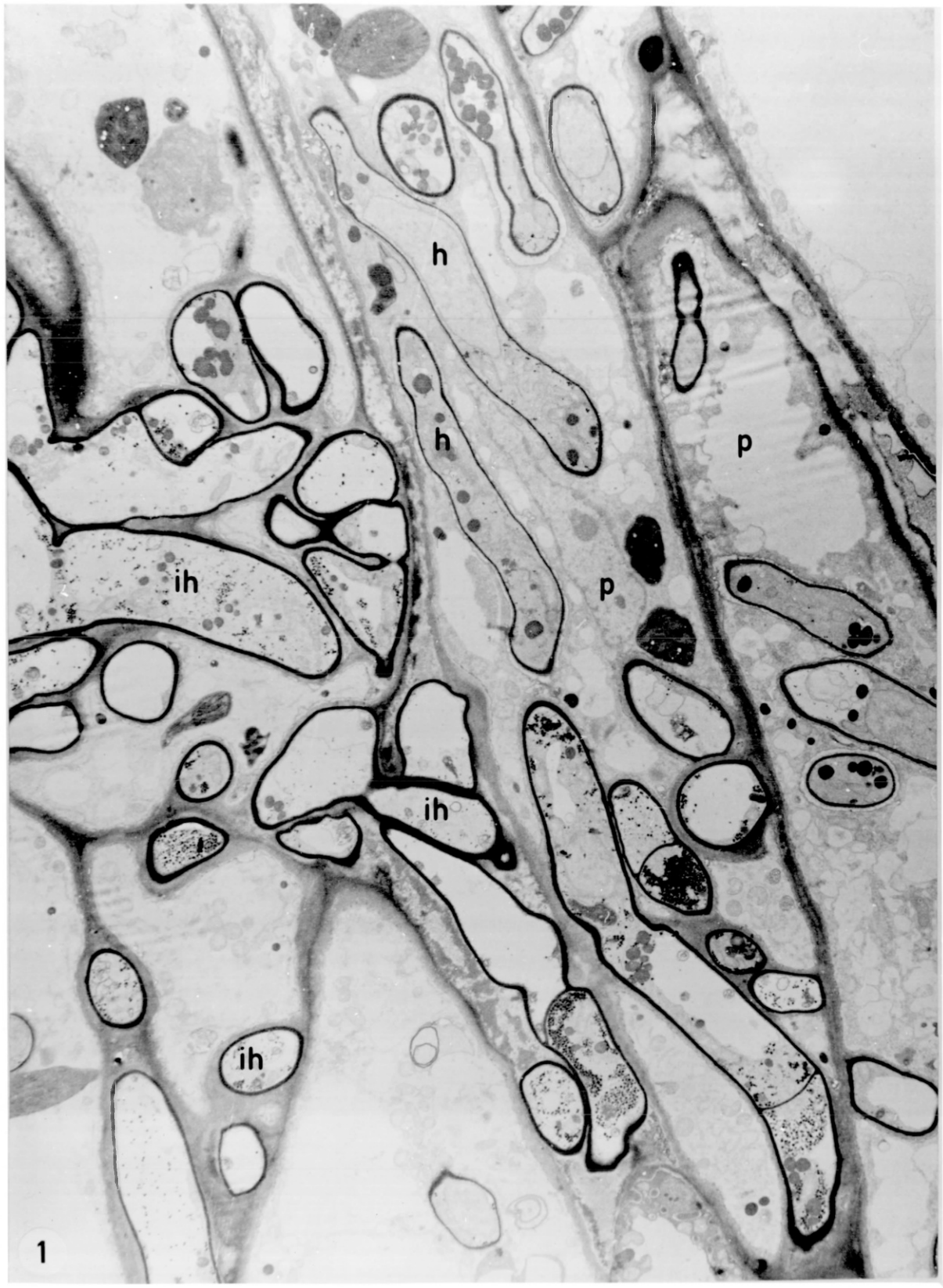
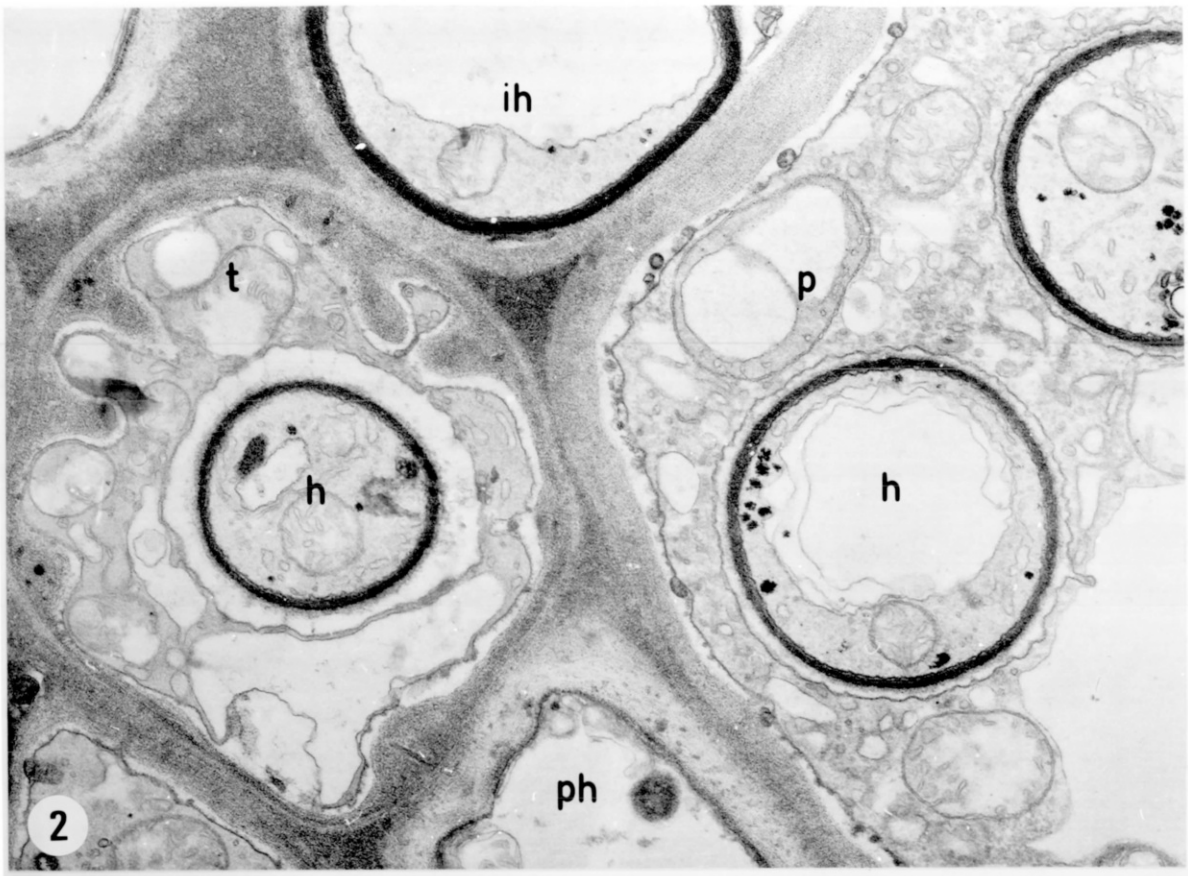

h
h
p
p
ih
ih
ih
1

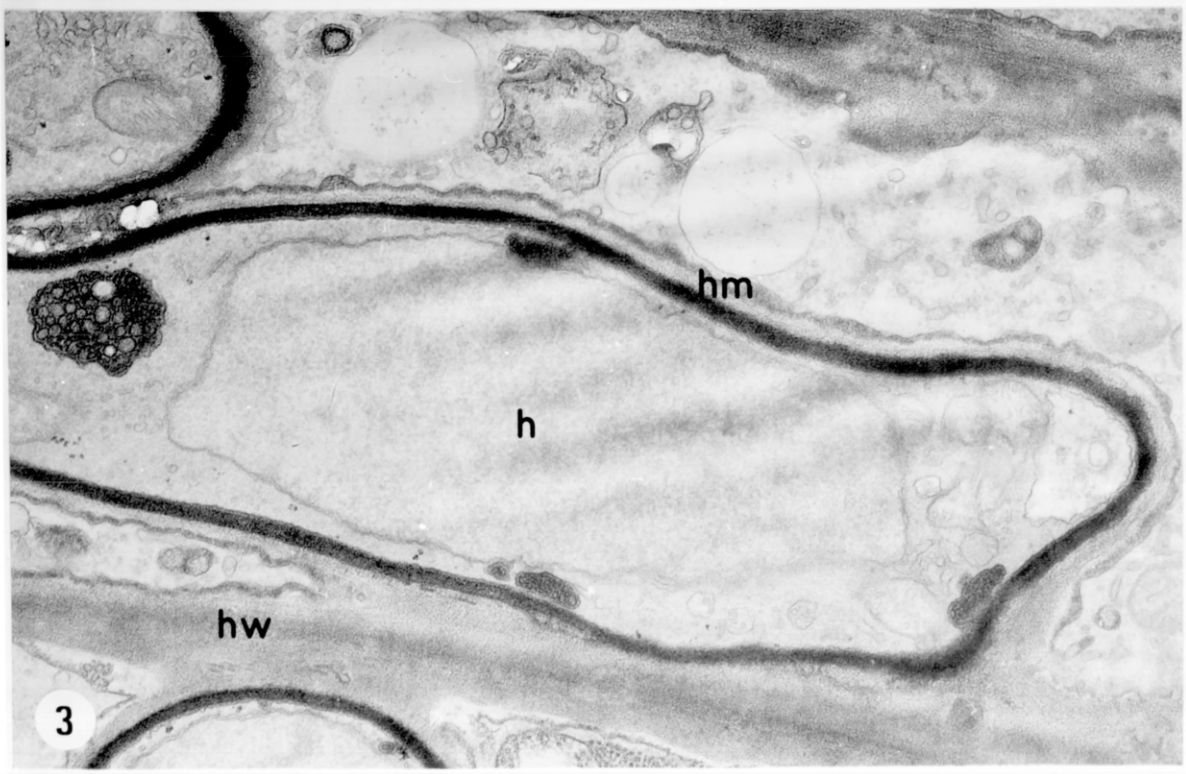
Plate 7:2

Section of M. haustoria (h) of P. poarum infecting phloem parenchyma (p) and transfer cells (t) of T. farfara. Sectioned perpendicular to the leaf epidermis and stained by the PATCHSP procedure. ih, intercellular hypha ; ph, phloem sieve tube. X19800.

Plate 7:3

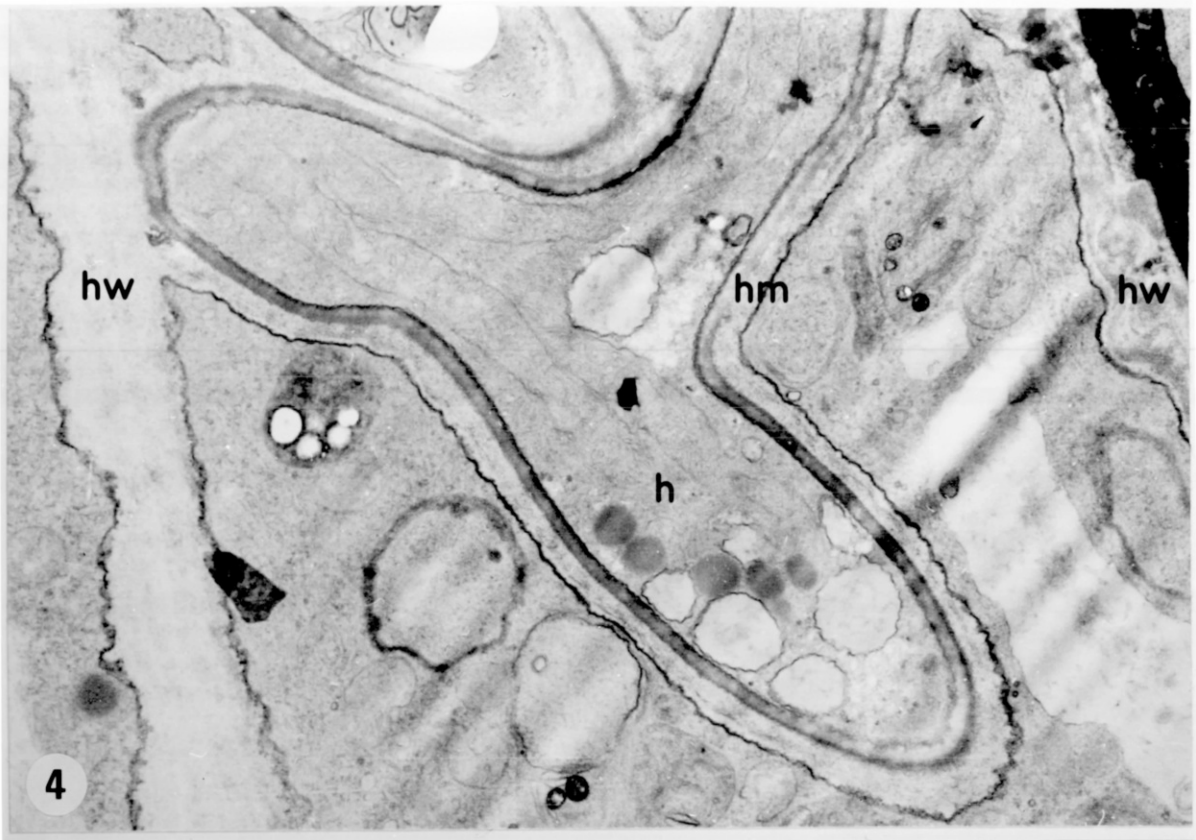
Section of an M-haustorium (h) of P. poarum infecting a parenchyma cell within the vascular tissue of T. farfara, stained by the PATCHSP procedure. The haustorium is completely enclosed by material (hm) which gives a staining reaction similar to the host cell wall (hw). The haustorial cytoplasm is almost unvacuolated indicating that the haustorium is quite young. X19800.


ih
t
p
h
h
ph
2


hm
h
hw
3

## Plates 7:4 and 7:5

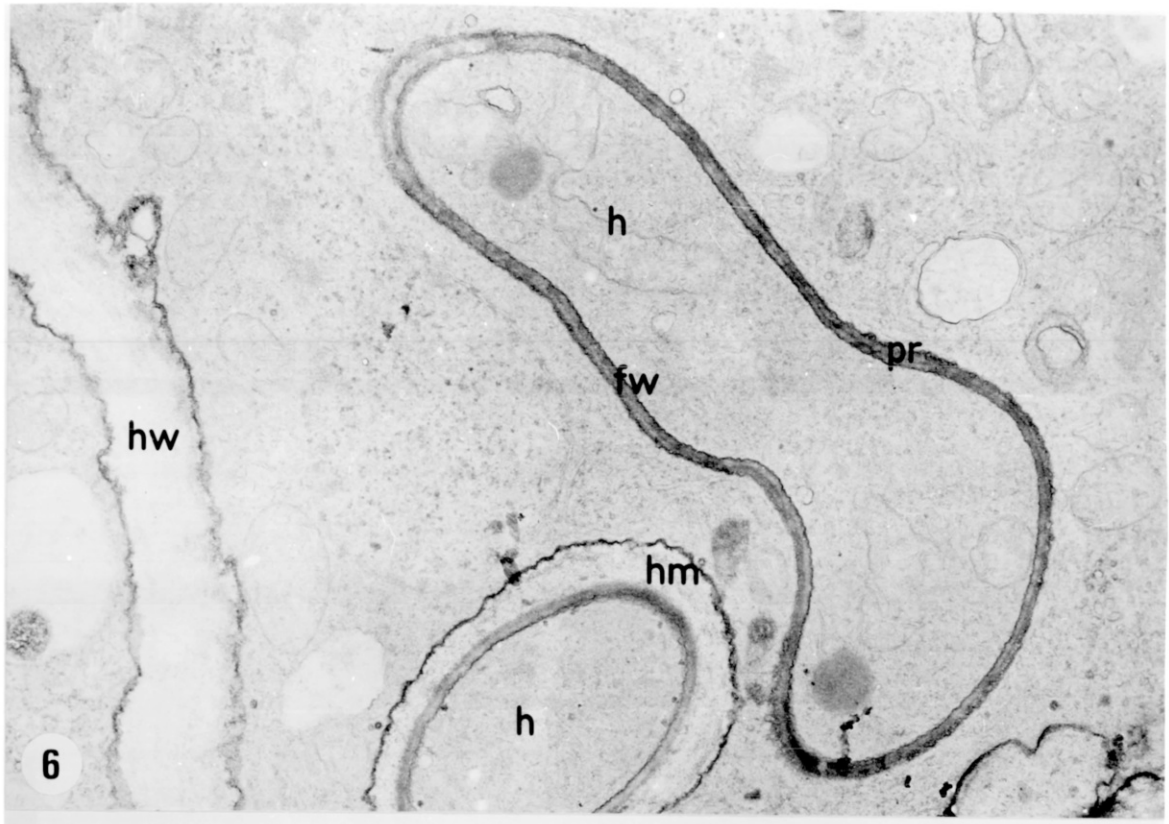
Sections of an M-haustorium (h) of P. poarum infecting a xylem parenchyma cell of T. farfara, stained by the PACP (Plate 7:4) or PATCHSP (Plate 7:5) procedure. The haustorium is completely surrounded by material (hm) which reacts similarly to the host cell wall (hw) when treated by either staining technique. The haustorial cytoplasm is only slightly vacuolated indicating that the haustorium is not old. Plate 7:4, x13600; Plate 7:5, x14900.

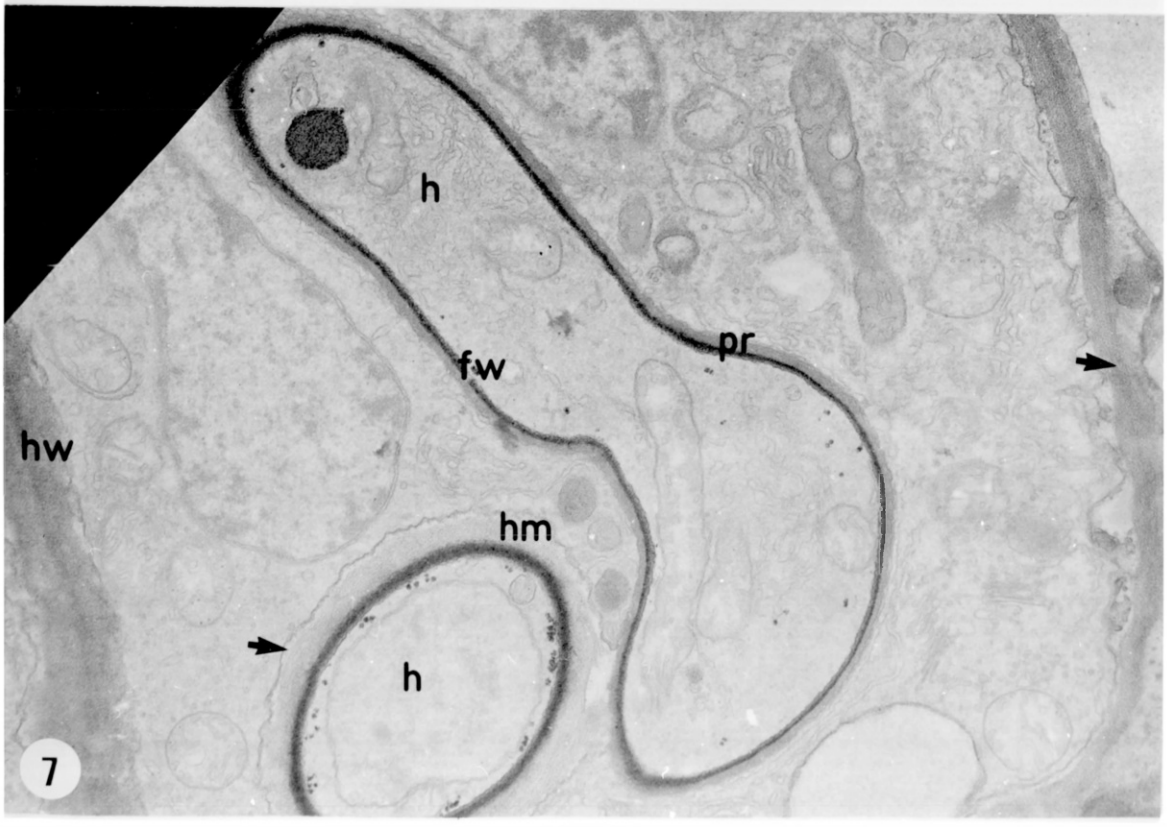

hw
hm
hw
h
4


hw
hm
hw
h
5

Plates 7:6 and 7:7

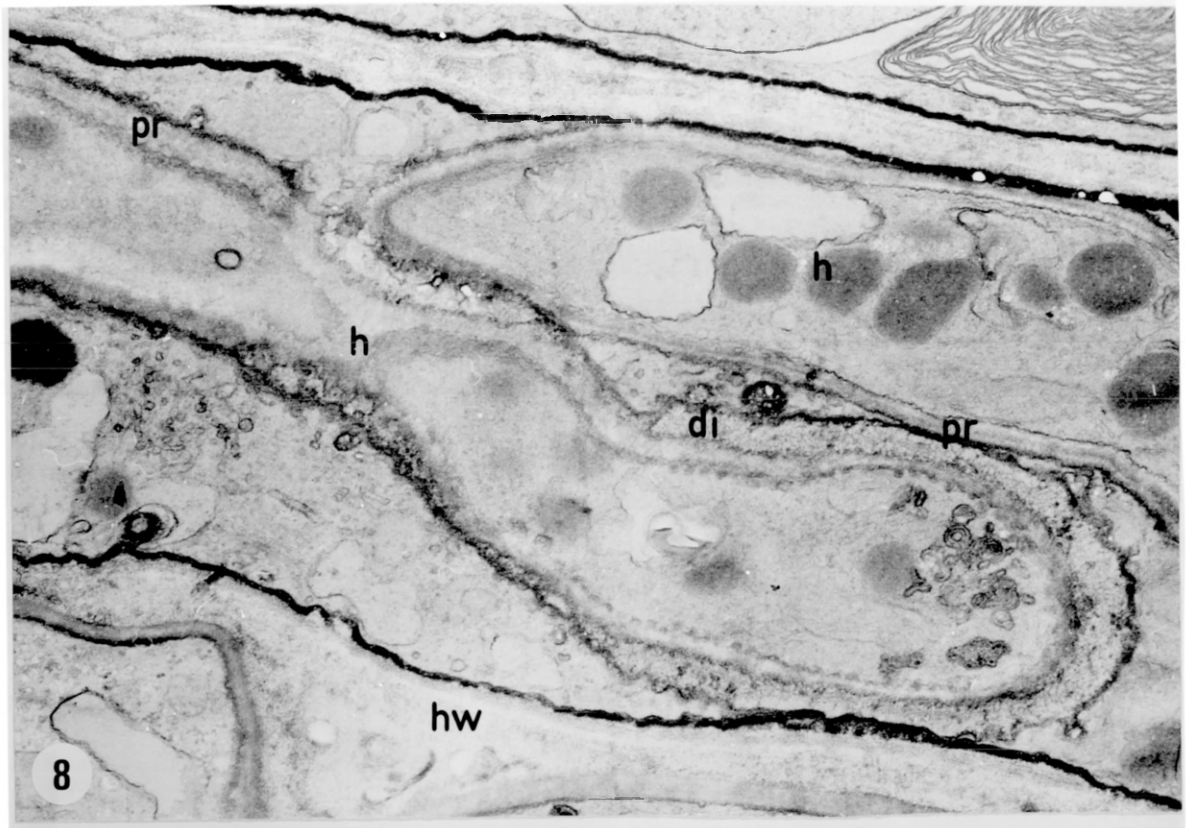
Sections of M-haustoria (h) of P. poarum stained by the PACP (Plate 7:6) or PATCHSP (Plate 7:7) procedure. The haustoria are surrounded by either host wall-like material (hm) or the proximal matrix (pr). Plate 7:6 shows that the proximal matrix is of moderate electron density, similar to the fungal wall (fw) when stained by the PACP technique, but is easily distinguished from the lightly stained host wall-like material. In Plate 7:7 the proximal matrix can be clearly discerned adjacent to the haustorial wall when treated by the PATCHSP procedure, but is stained similarly to the host wall-like material. However, the proximal matrix is amorphous in texture and of more narrow dimensions than the host wall-like material which contains parallel fibrils (arrowed) similar to those in the host cell wall (hw) (arrowed). Plates 7:6 and 7:7 x14900.


h
pr
fw
hw
hm
h
6


h
pr
fw
hw
hm
h
7

Plates 7:8 and 7:9

Sections of M-haustoria (h) of P. poarum in a xylem parenchyma cell of T. farfara, stained by the PACP (Plate 7:8) or PATCHSP (Plate 7:9) procedure. The haustoria are surrounded by proximal (pr) and distal (di) matrices. Plate 7:8 shows that the distal matrix is only lightly stained by the PACP technique and contains fibrils of similar electron density to the proximal matrix. In Plate 7:9 the distal matrix is mostly unstained by the PATCHSP procedure, in contrast to the moderately electron dense proximal matrix. Plate 7:8, x19300; Plate 7:9, x19800.


pr
h
h
di
pr
hw
8

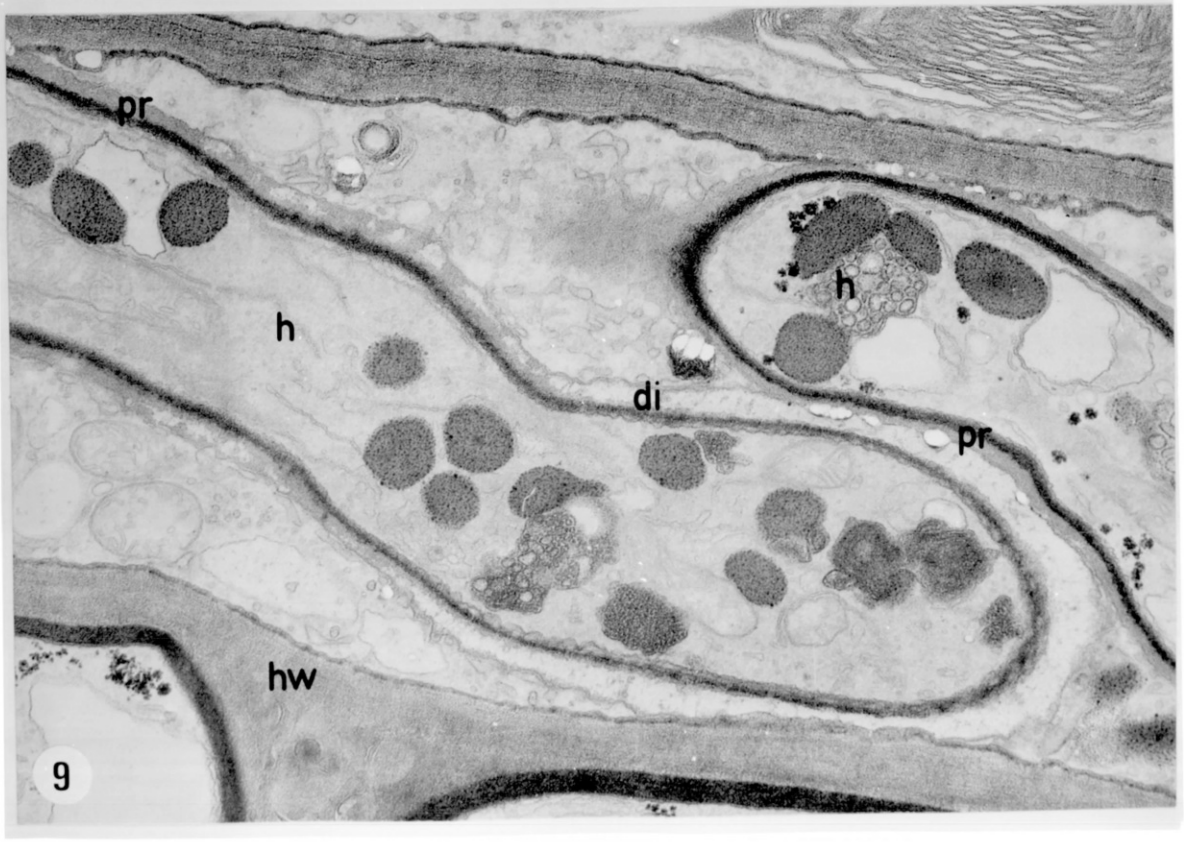
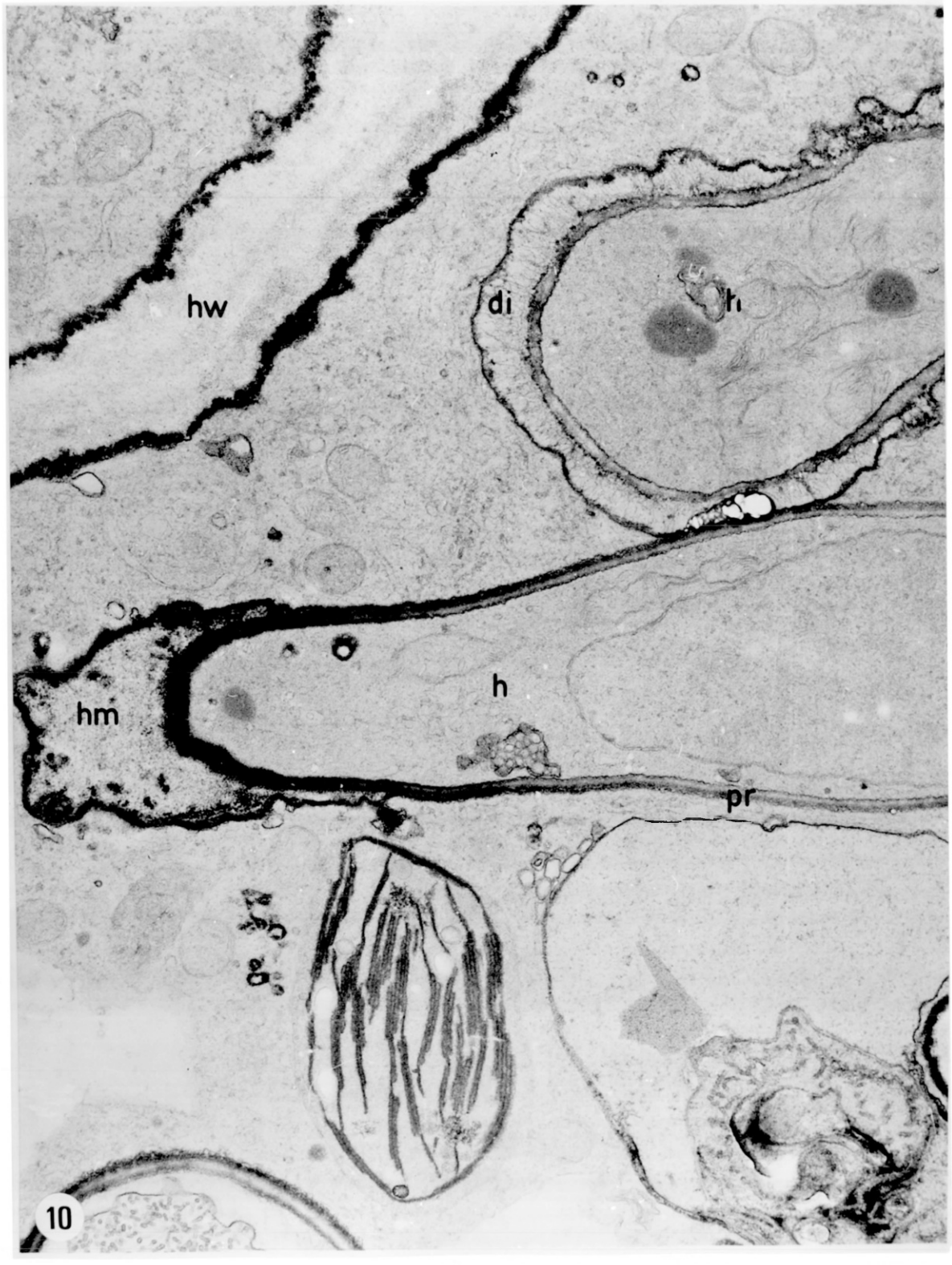

pr
h
h
di
pr
hw
9

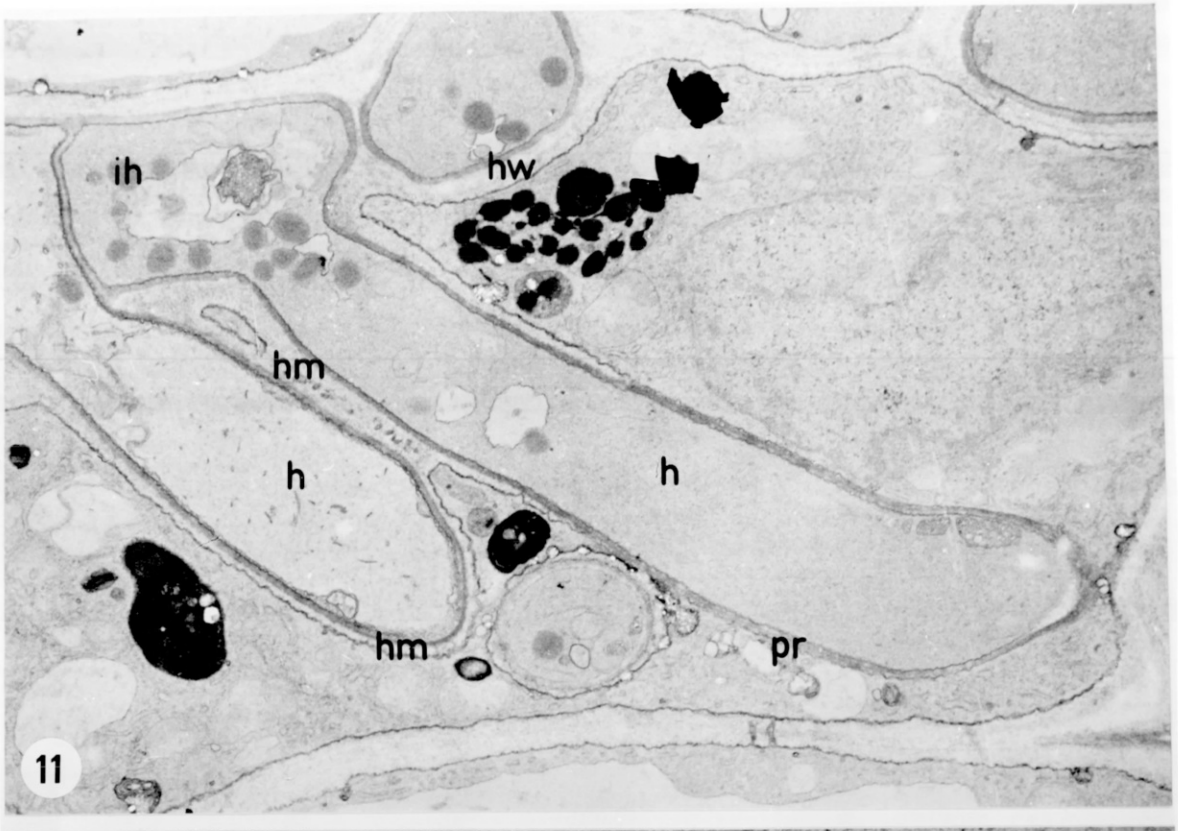
Plate 7:10

Section of M-haustoria (h) of P. poarum, stained by the PACP procedure. The haustoria are surrounded by host wall material (hm), proximal matrix (pr) or distal matrix (di). Moderately electron dense fibrils can be clearly observed to radiate from the haustorium within the electron-lucent distal matrix. The regions of the host plasmalemma adjacent to the host cell wall (hw) and the host wall-like material are intensely stained whereas the regions adjacent to the proximal and distal matrices are only light to moderately stained. X19800.


hw
di
h
hm
h
pr
10

Plates 7:11 and 7:12

Median section of M-haustoria (h) of P. poarum stained by the PACP procedure. Both haustoria are slightly constricted at the site of penetration of the host cell wall (hw). One haustorium has highly vacuolated cytoplasm and is completely enclosed by host wall-like material (hm) and a normal intensely stained host plasmalemma. The other haustorium has only slightly vacuolated cytoplasm, indicating that it is the younger, and is only partially enclosed by host wall-like material which tapers to a rim approximately 4μM along the haustorium from the penetration site. Beyond this region the proximal matrix (pr) can be distinguished and a gradual transition in staining characteristics of the host PM occurs from intense to moderate electron density. This can be seen most clearly in Plate 7:12. Plate 7:11, x9350; Plate 7:12, x15400.


ih
hw
hm
h
h
hm
pr
11

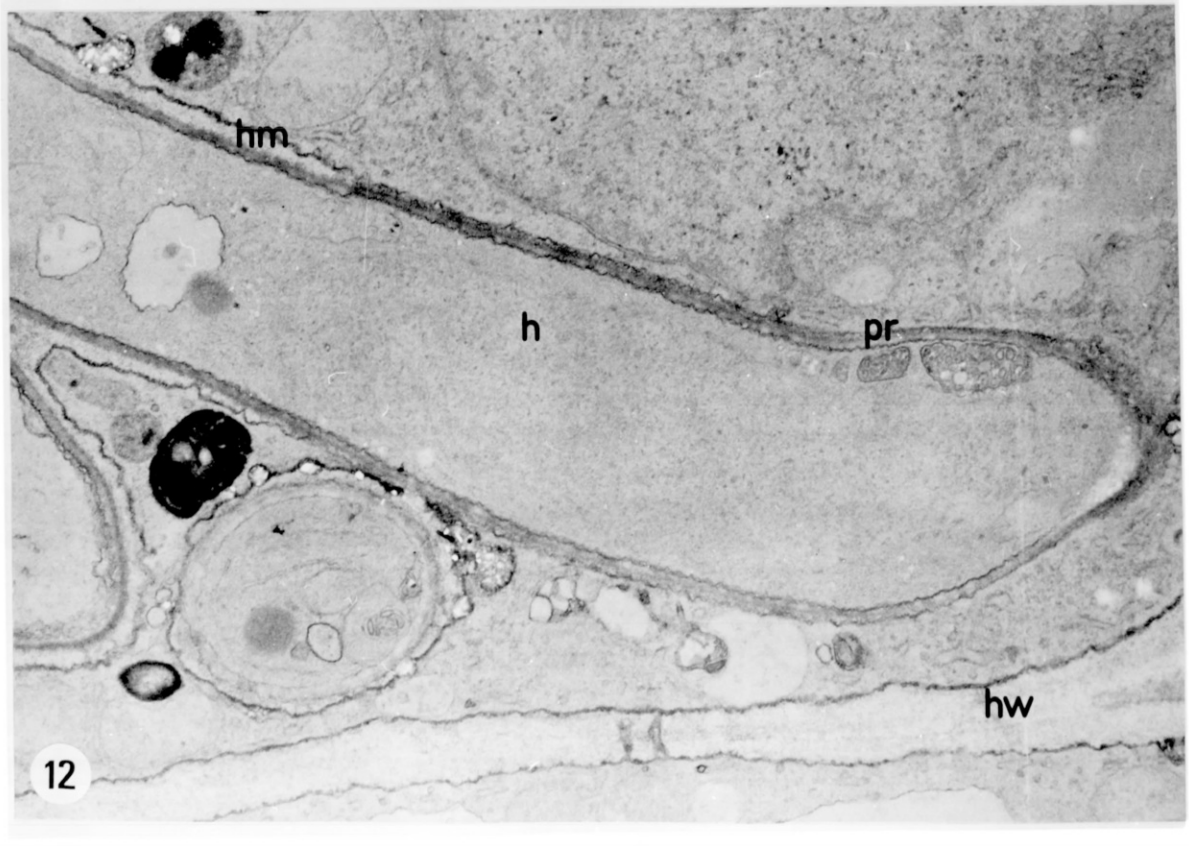
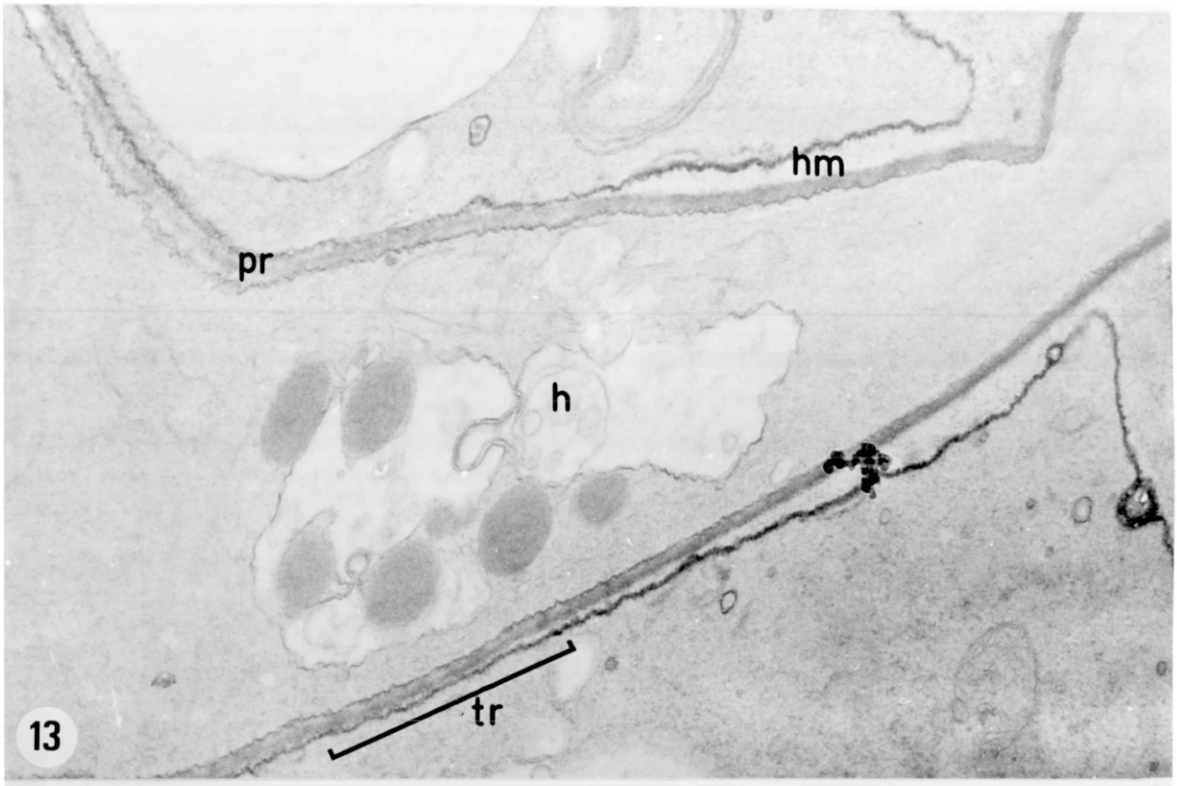

hm
h
pr
hw
12

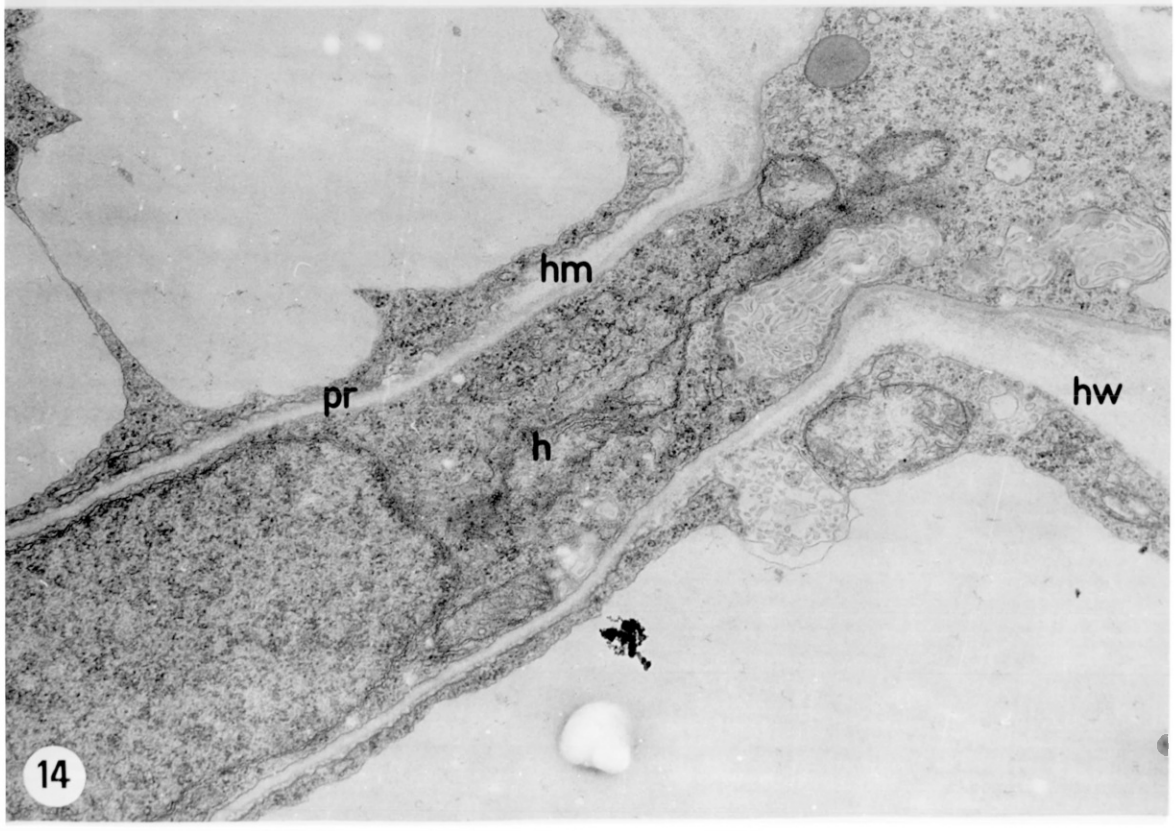
Plate 7:13

Median section of an haustorium of P. poarum infecting a mesophyll cell of T. farfara, stained by the PACP procedure. The collar of host wall-like material around the haustorium tapers to a rim approximately 2μM from the penetration site. A gradual transition in staining intensity of the host plasmalemma occurs over a distance of approximately 1.6μM (tr), between the region adjacent to the host wall-like material and the region adjacent to the proximal matrix (pr). X27200.

Plate 7:14

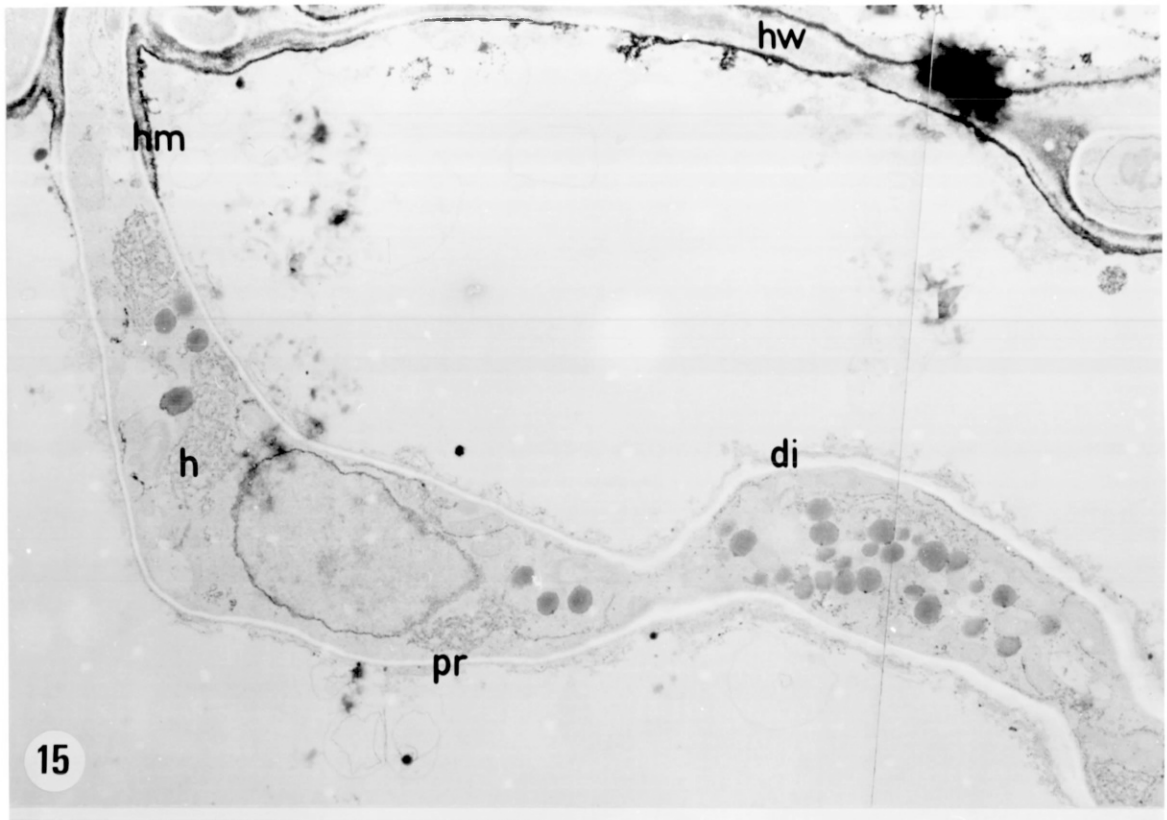
Median section of an M-haustorium (h) of P. poarum, stained with uranyl acetate and lead citrate. The proximal matrix (pr) can be distinguished from the more electronlucent host wall-like material (hm) but no change in staining characteristics of the host plasmalemma can be discerned. X19800.


hm
pr
h
tr
13

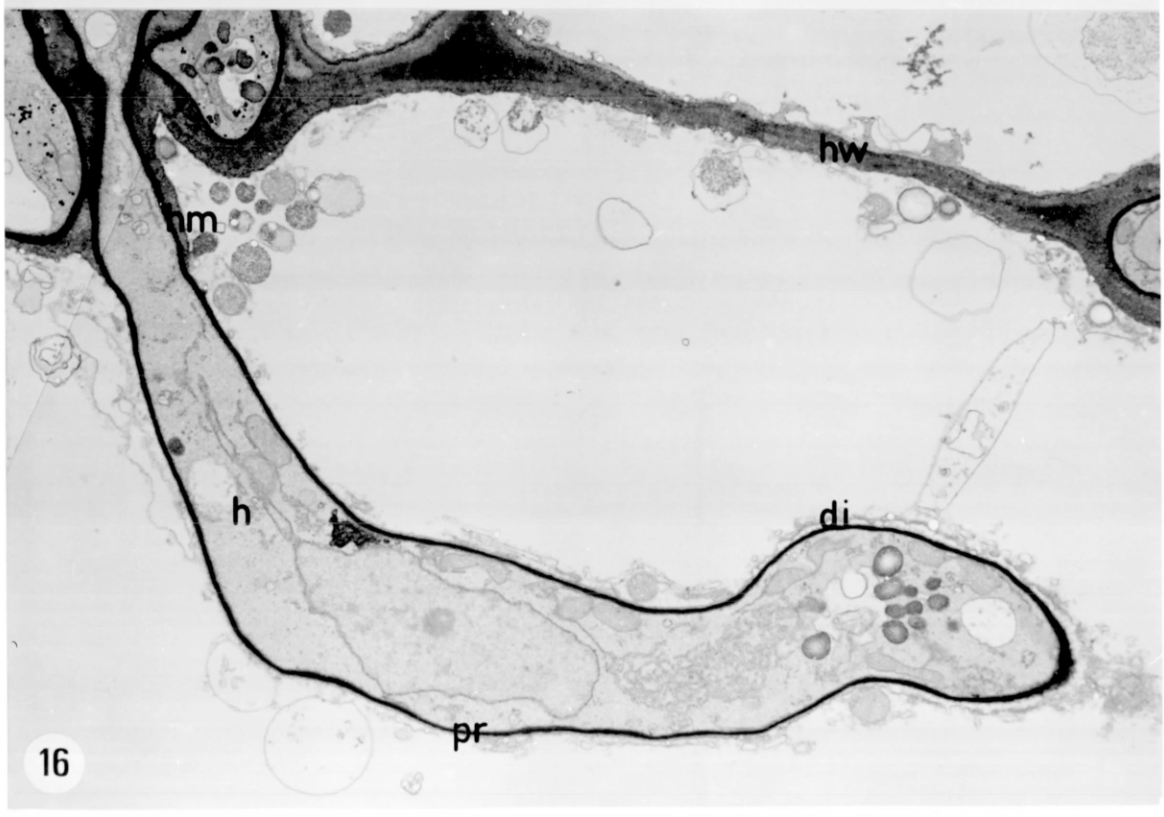

hm
pr
h
hw
14

Plates 7:15 and 7:16

Median sections of an M-haustorium (h) of P. poarum prepared from infected leaves of T. farfara incubated with ATP and magnesium ions at pH 7.2 and observed without staining (Plate 7:15) or after treatment by PATCHSP procedure (7:16). Plate 7:15 shows flocculent precipitates, indicating ATPase activity, on the host PM adjacent to both the host wall (hw) and the host wall-like material (hm) which forms a collar around the haustorium. Reaction product is absent from the host plasmalemma surrounding regions identified by the PATCHSP procedure as the proximal (pr) and distal (di) matrices and is also absent from the fungal plasmalemma. Plates 7:15 and 7:16 x7000.



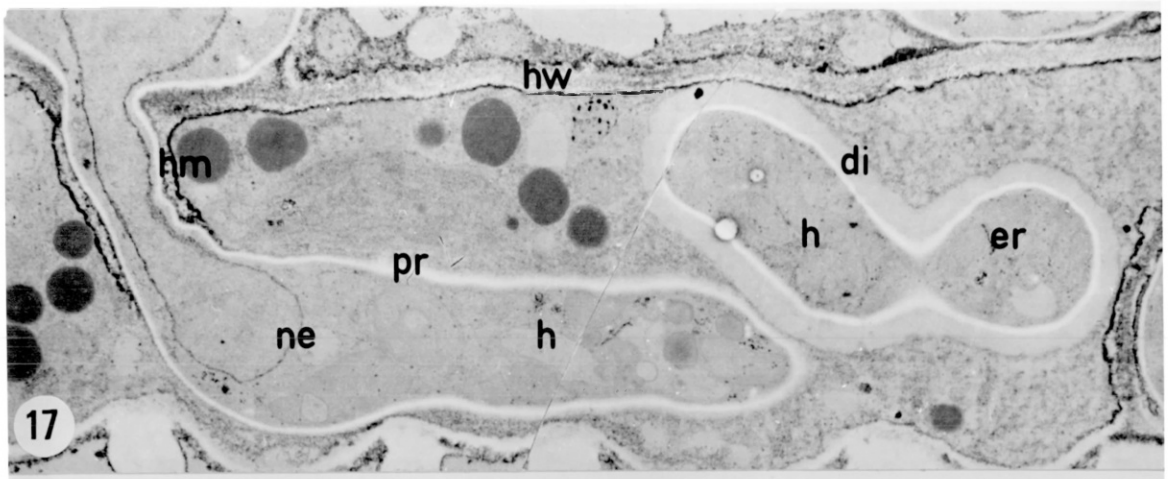

15




16

Plates 7:17 and 7:18

Median sections of M-haustoria (h) of P. poarum infecting xylem parenchyma cells of T. farfara prepared from material incubated with ATP and magnesium ions at pH 7.2. As shown in Plate 7:15, reaction product is localized to the host plasmalemma adjacent to the host cell wall and host wall-like material which partially encloses the haustoria. Precipitates are absent from the fungal plasmalemma and the host plasmalemma adjacent to the proximal (pr) and distal (di) matrices. Plate 7:18 shows most clearly the gradual decrease of precipitate between the ATPase-active and non-active regions of the invaginated host plasmalemma over a distance of approximately 1.3μM (tr). A fine lead precipitate, also observed in no substrate controls, is present in the host and fungal cytoplasms, particularly within the nuclear envelope (ne) and endoplasmic reticulum (er). Plate 7:17, x9350; Plate 7:18, x19800.


hw
hm
di
h
er
pr
ne
h
17

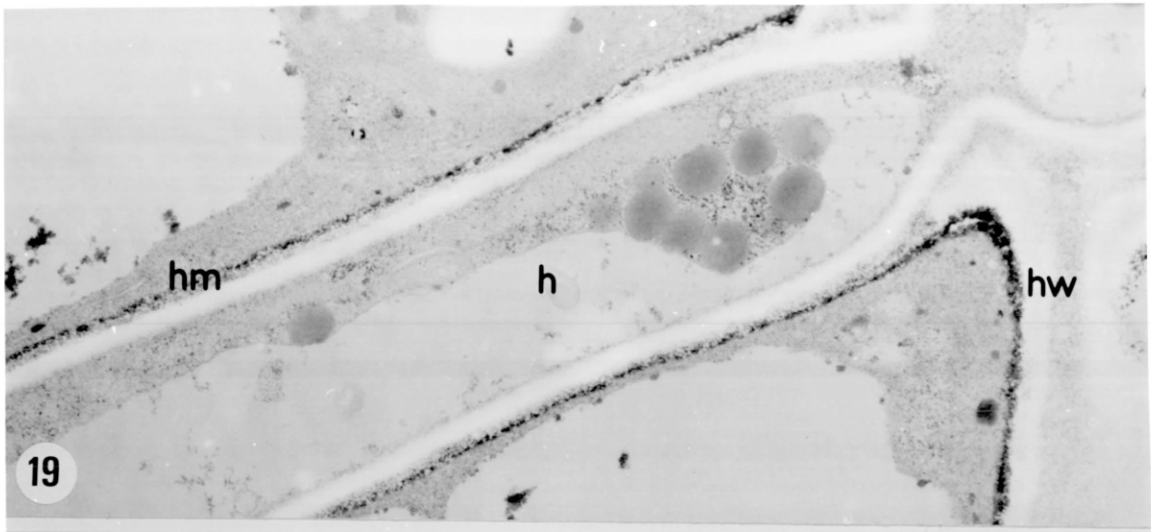

hw
tr
hm
h
pr
18

## Plates 7:19 and 7:20

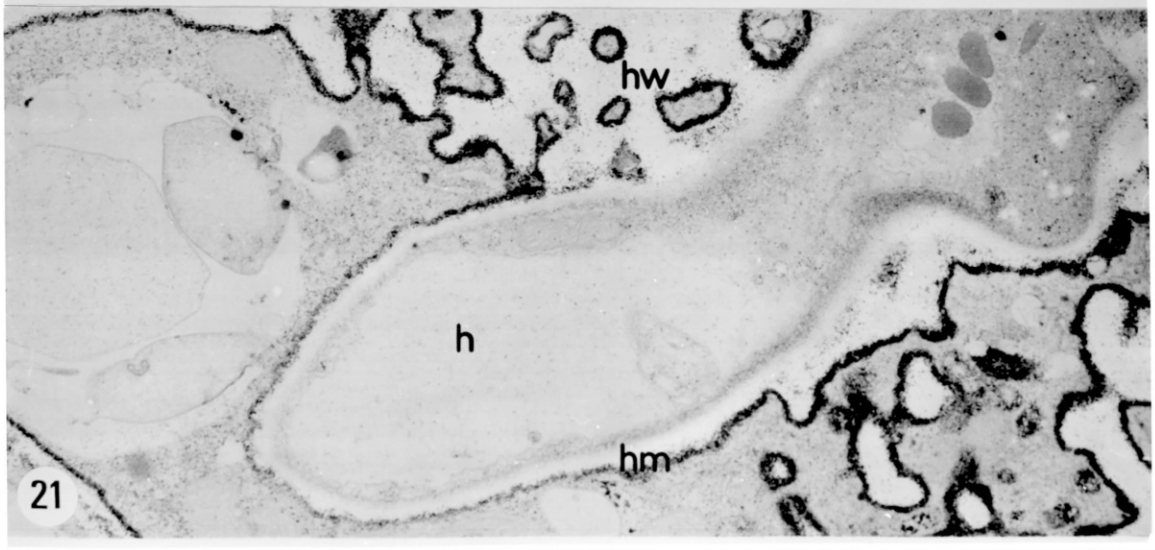
Sections of an M-haustorium (h) of P. poarum prepared from infected leaves of T. farfara incubated with ATP and magnesium ions at pH 7.2 and observed without further staining (Plate 7:19) or after treatment by the PATCHSP procedure (Plate 7:20). The haustorium is completely enclosed by host wall-like material (hm), identified by the PATCHSP staining. Precipitates, indicating ATPase activity, are present on the host plasmalemma adjacent to the host cell wall (hw) and the host wall-like material along the whole length of the haustorium. Plates 7:19 and 7:20 x14850.

## Plate 7:21

Section of an M-haustorium (h) of P. poarum infecting a transfer cell of T. farfara prepared from material incubated with ATP and magnesium ions at pH 7.2 and observed without staining. There are numerous host-wall (hw) ingrowths on one side of the transfer cell through which the haustorium has penetrated. The haustorium is completely enclosed by host wall-like material (hm). Precipitates, indicating ATPase activity, are present on the whole host PM which lines the host wall and continues around the haustorium. X14850.


hm
h
hw
19


hw
h
hm
20

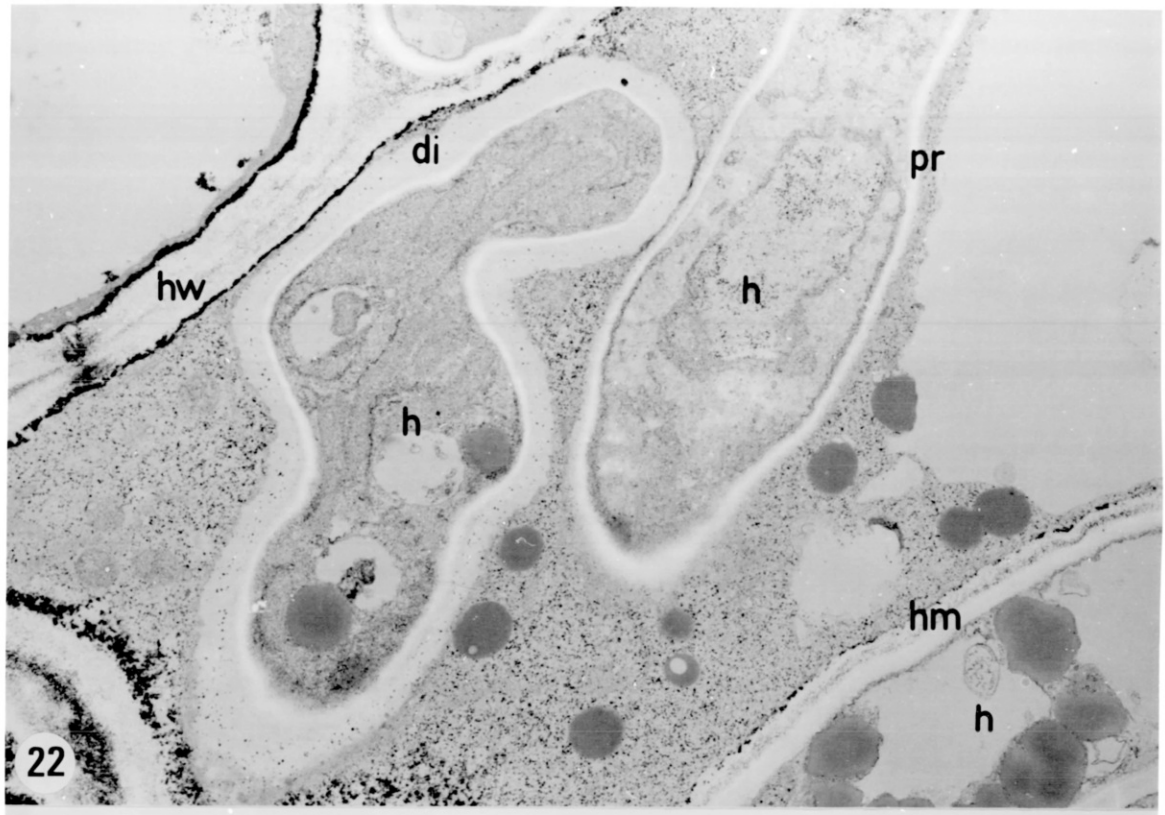

hw
h
hm
21

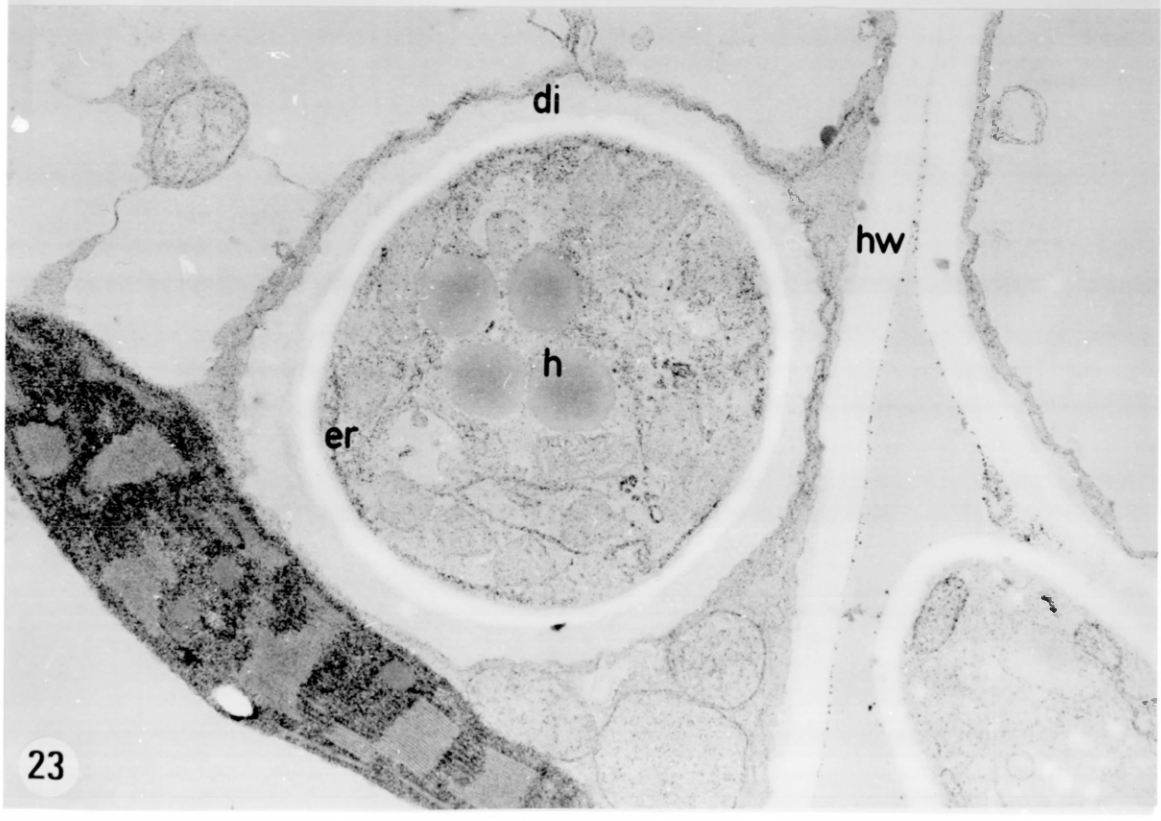
## Plate 7:22

Section of M-haustoria (h) of *P. poarum* infecting a xylem parenchyma cell of *T. farfara* prepared from material incubated with βGP and magnesium ions at pH 7.2. Parts of haustoria surrounded by either proximal matrix (pr), distal matrix (di) or host wall-like material (hm) can be observed. The distribution of reaction product, indicating general phosphatase activity, is similar to the distribution obtained when ATP is employed as substrate. Precipitates are present on the host plasmalemma adjacent to both the host cell wall and the host wall-like material surrounding an haustorium, but are absent from the regions adjacent to the proximal and distal matrices. X11700.

## Plate 7:23

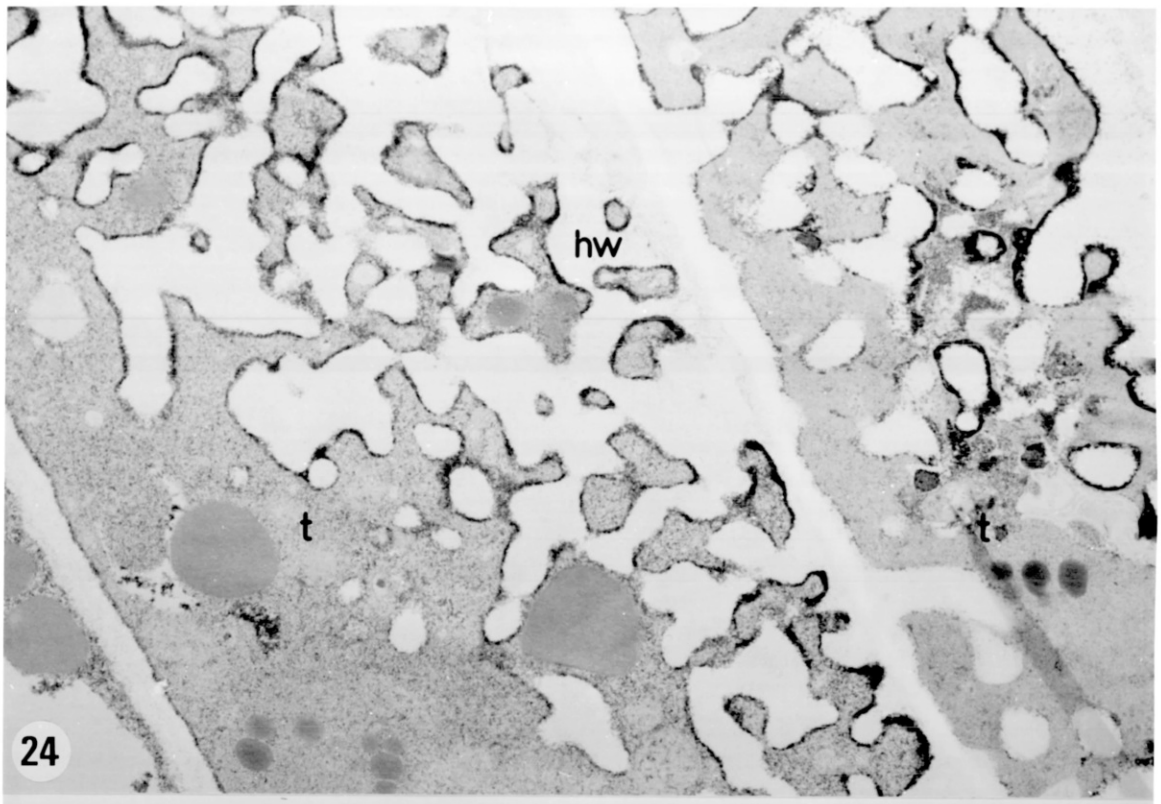
Section of an M-haustorium (h) of *P. poarum* infecting *T. farfara* prepared from material incubated without substrate in the ATPase localization reaction medium at pH 7.2. A fine lead precipitate is present within the host and fungal cytoplasms, particularly within the cisternae of the endoplasmic reticulum (er), but no flocculent precipitates are present on the host or fungal plasmalemmas. hw, host wall; di, distal matrix. X19800.


di
pr
hw
h
h
hm
h
22


di
hw
h
er
23

Plates 7:24 and 7:25

Sections of transfer cells (t) in uninfected leaves of T. farfara incubated either with ATP and magnesium ions at pH 7.2 (Plate 7:24) or in the same medium with the addition of sodium orthovanadate as an ATPase inhibitor (Plate 7:25). Plate 7:24 shows flocculent precipitates, indicating ATPase activity, on the plasmalemma lining the cell wall (hw). Plate 7:25 shows that when an inhibitor is present in the reaction medium precipitates on the plasmalemma of the same cell type are greatly reduced. Plate 7:24, x15400; Plate 7:25, x14850.




24




25

# 8. Modification of the Host PM of Lactuca sativa Infected by Bremia lactucae

## 8.1 Introduction

The more biotrophic of the Peronosporales grow intercellularly within the host and form intracellular haustoria comparable to those of rusts and powdery mildews. As described in Section 3.1, the invaginated region of the host PM in infections by Peronospora pisi (Hickey & Coffey, 1978) and Pseudoperonospora humuli (Pares & Greenwood, 1979) is structurally distinct from the uninvaginated region, despite the absence of an observable neckband. However, a neckband-equivalent, which had been undetected in previous work, was observed in an investigation of an infection by A. candida in which the invaginated host PM is also specialised. Therefore, the haustorial infection by Bremia lactucae of Lactuca sativa was examined to investigate the possibility of a previously undetected neckband (Ingram, Sargent & Tommerup, 1976) and to determine the degree of specialisation of the invaginated region of the host PM.

## 8.2 Materials and Methods

### 8.2.1 Fixation and Embedding of Material for Differential Staining

Pieces of cotyledons of Lactuca sativa either uninfected or with a 4-day infection by Bremia lactucae were supplied by Dr. J. Mansfield and had been fixed in glutaraldehyde, post-fixed in $OsO_4$, dehydrated and embedded in araldite. Sections were cut according to the procedure described in Section 2.2.2.

### 8.2.2 Cytochemical Staining Methods

Sections mounted on 200 mesh gold grids were stained using the PACP procedure described in Section 2.2.3.1.

## 8.3 Results

The haustorium and collar within infected cells were as described by Ingram, Sargent & Tommerup (1976) and Sargent (1981). The host PM followed the contours of the collar and invaginated at the penetration site to surround the haustorium (Plates 8: 1,2). It was in extremely close contact (Plate 8: 4) with an osmiophilic layer which extended around the haustorial wall from the penetration site (Plates 8: 1,2). Ingram et al. were unsure whether this layer corresponded to the extrahaustorial matrix around haustoria of powdery mildew and rust infections. In the present investigation this layer was observed to be similar in appearance to material which formed a meniscus between the intercellular hypha and the host wall and which continued around the wall of the intercellular hypha (Plates 8: 1,2). It will therefore be referred to as the outer haustorial wall. The invaginated host PM was so closely adherent to it that even at high magnifications the trilamellar structure of the membrane could not be clearly discerned (Plate 8: 4).

Within the host cytoplasm immediately adjacent to the extrahaustorial membrane, cisternae of endoplasmic reticulum following the contours of the haustorium were interspersed with vesicles (or cross sections of tubules) with contents of similar electron density to the ER (Plate 8: 3). Occasional haustoria observed within necrotic host cells were completely encased by collar-like material which trapped a layer of host cytoplasm, bounded by the host plasmalemma adjacent to both the haustorium (extrahaustorial membrane) and the encasement (Plate 8: 5).

After treatment by PACP, the fungal PM, both of the haustorium and the intercellular hyphae, was intensely stained (Plates 8: 2,3). Where the host PM surrounded the haustorium it was consistently very lightly stained (Plates 8: 2,3,6), but the staining reaction of the host wall-lining region of the PM varied from cell to cell within infected leaves.

In some cells the whole of this region was intensely stained and thus easily distinguished from the invaginated PM (Plates 8: 2,3). The transition in staining characteristics occurred approximately where the invagination began, although its precise location was obscured by oblique sectioning of the penetration site (Plate 8: 2). Alternatively, the whole wall-lining PM was only lightly stained and in this respect similar to the invaginated region (Plate 8: 6). This variation in staining reaction of the uninvaginated host PM was irrespective of the maturity of the haustorium within the cell and also occurred in cells of infected leaves in which haustorial profiles were not seen, although haustoria may have been present outside the plane of section.

About 1/3 of the cells of infected leaves had plasmalemmas which were only lightly stained, whereas, the host PM of uninfected control material was consistently intensely stained (Plate 8: 7).

The membranes of both host and fungal ER were almost unstained with PACP (Plate 8: 3) and the vesicular membranes within the host cytoplasm immediately adjacent to the extrahaustorial membrane, which was lightly stained, were either moderately or intensely electron dense (Plate 8: 3).

## 8.4 Discussion

The results of PACP staining have distinguished 2 types of infected cell; those with only the invaginated PM exhibiting abnormal staining chracteristics and those in which both the invaginated and uninvaginated host PM stained abnormally.

It was suggested in previous sections 3, 4 and 7 that an invaginated host PM may arise either from normal host PM which is subsequently altered by a fungal metabolite or from an abnormal source such as the host ER. Studies of *Bremia lactucae* (Ingram et al., 1976) have demonstrated a proliferation of the host PM prior to fungal penetration and it is thought that the new membrane invaginates as the haustorium enlarges.

Thus, it seems likely that in Bremia infections, the different characteristics of the invaginated part of the PM are due to a fungal metabolite which modifies the already formed membrane which the haustorium is near.

Cells in which the invaginated and uninvaginated regions of the host PM are distinct indicate either a general decrease in fluidity of the invaginated PM as proposed for cells infected by the monokaryotic stage of P. poarum (Section 7.4) or the prevention of membrane fluidity by, for example, the presence of an extrinsic stabilising structure, equivalent to the neckbands of powdery mildew and dikaryon rust haustoria, to which the invaginated host PM is sealed. It is expected that a general decrease in fluidity of the invaginated PM would result in a gradual transition in the characteristics of the two plasmalemma regions and that the transition would occur at varying positions along the haustorial surface. However, in Bremia -infected cells, the transition was consistently observed near the point of inflexion of the plasma membrane. This indicates that the two PM domains are permanent. This conclusion is further supported by the encasement of some necrotic haustoria in an extension of the collar which traps a layer of host cytoplasm. This cytoplasm is bounded around the haustorium by the extrahaustorial membrane and the host PM adjacent to the encasement. Thus, the vesicles of dictyosomes thought to be responsible for collar deposition (Sargent, 1981) fuse with the unmodified PM but not the extrahaustorial membrane. Similar encasements of necrotic haustoria occur in other haustorial infections where neckbands form a tight seal between the extrahaustorial membrane and the haustorial neck wall (Heath, 1971; Heath & Heath, 1971; Littlefield & Bracker, 1972; Perera & Gay, 1976).

Thus, although a neckband has not been detected directly, its existence around haustoria of B. lactucae is suggested by the single site of transition of the host PM and by the encasement of necrotic haustoria

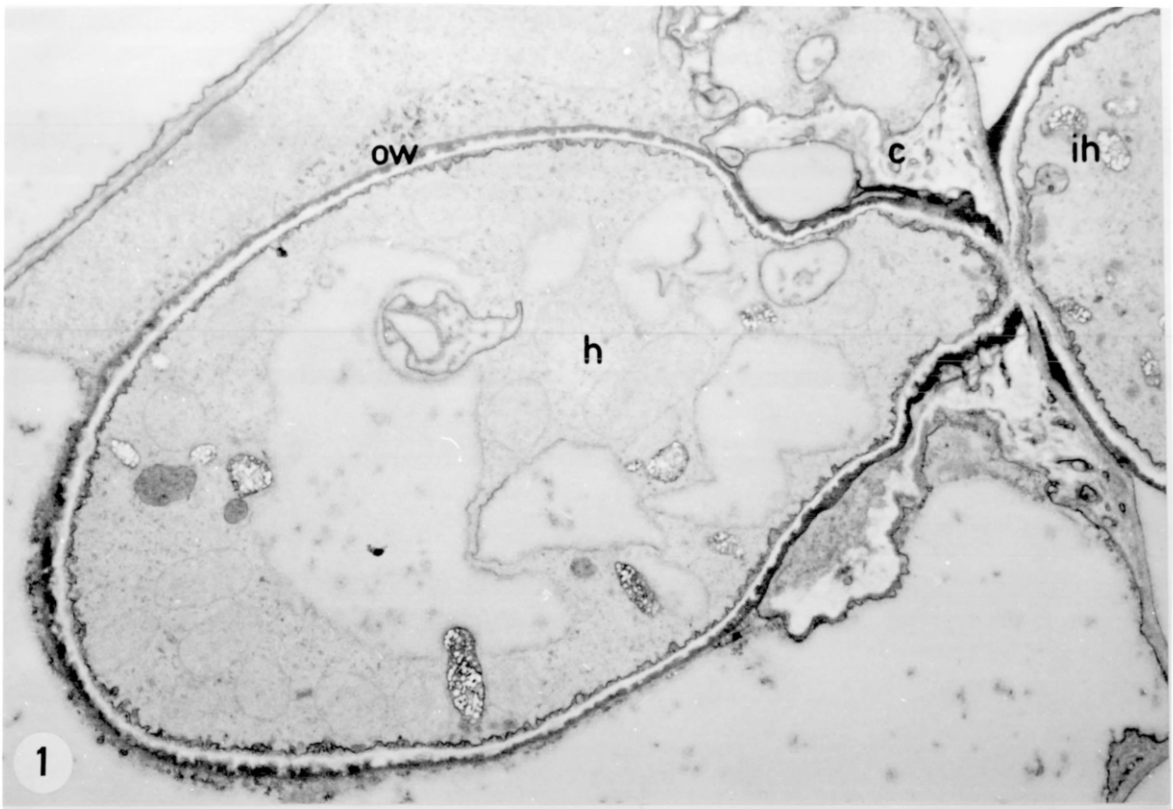
in an extension of the collar. Alternatively, fluidity of the whole extrahaustorial membrane may be prevented by, for example, adherence to the outer haustorial wall as indicated by their very close association. Adhesion has previously been suggested for infections by another member of the Peronosporales, Pseudoperonospora humuli (Pares & Greenwood, 1979) in which a similar specialisation of the extrahaustorial membrane was observed. Thus, the outer haustorial wall may in this respect be analogous to the haustorial neck wall of rusts and powdery mildews. However, although it may be an extrinsic barrier to lateral diffusion within the plasma membrane it is unlikely to be a barrier to apoplastic diffusion if the region allows passage of metabolites between host and parasite. Therefore, the similarity of function between a neckband and the outer haustorial wall is limited to their possible influence on the diffusion of membrane components. However, neither the concept of adherence of the extrahaustorial membrane to the outer haustorial wall, nor the presence of an undetectable neckband explains the existence of cells in which the host plasmalemma is modified throughout. These may arise in one or more of several ways. Since wholly modified cells contain haustoria of different ages it is unlikely that they all develop from cells in which two domains have been previously established. This suggests that at, or prior to, infection the whole membrane of certain cells became modified by the fungus, possibly due to the absence of a barrier preventing domain mixing. It is likely that the modifying process would continue as the extrahaustorial membrane extends around an enlarging haustorium. This pattern of development may also be envisaged in cells in which there is a barrier to domain mixing, but where modification of constituents occurs on both sides of the barrier. However, when considering the above suggested origins of a wholly modified host PM, it must be noted that the structures of the host/haustorial interfaces

of such cells were indistinguishable from those in cells with 2 PM domains. In addition, not all cells with modified wall-lining plasmalemmas were observed to be infected. Thus, there is a third possibility that the wall-lining PM of certain cells may be altered by a mechanism unrelated to invasion of the cell. A fungal metabolite may be released into the apoplast from the intercellular hyphae which causes alteration of the wall-lining PM. However, cells with wholly modified plasmalemmas were not grouped together as would be expected following the action of a freely diffusable metabolite and therefore it seems more likely that the plasmalemmas are modified indirectly by fungal activity. For example, the modification may follow the onset of senescence of individual cells in response to leaf damage caused by fungal invasion of the tissue.

In previous investigations differences in PACP staining between regions of the host PM have been correlated with differences in ATPase activity (Sections 3 and 7). The results of the present study therefore indicate that in the majority of cells containing haustoria of B. lactucae the mechanism of nutrient uptake is similar to that proposed for A. candida (Woods & Gay, 1983), although if there is no apoplastic diffusion barrier, uptake may be less efficient (see Sections 5 and 7). However, experiments designed to locate the activity of ATPase are necessary to confirm this. Previous investigations of electrolyte leakage only encompass the first 12h after inoculation (Maclean & Tommerup, 1979). Thus, similar studies including later stages of infection, in conjunction with ATPase localization and cytochemical investigations, may indicate at what stage alteration of the staining properties of the host wall-lining PM occurs and whether this corresponds to physiological damage. In addition, examination of the host PM in young infections by B. lactucae may provide evidence for the role of the electron dense outer wall layer which is present around haustoria and secondary vesicles, but absent from the primary vesicle.

Plates 8:1 and 8:2

Near median section of an haustorium (h) of B. lactucae infecting a mesophyll cell of L. sativa, stained by the PACP technique. An osmiophilic layer, termed the outer wall (ow) extends around both the haustorium and the intercellular hyphae (ih). The haustorium is partially surrounded by an electron lucent collar (c) with electron dense inclusions. The host plasmalemma lines the host wall (hw) and continues around the collar and the haustorium. The host wall-lining region of the plasmalemma is intensely stained whereas it is only lightly stained adjacent to the outer haustorial wall. This can be seen most clearly in Plate 8:2. The transition in staining characteristics occurs near the penetration site (arrowed), but the precise location is obscured by the oblique section. Plate 8:1, x11700; Plate 8:2, x19800.


ow
c
ih
h
1

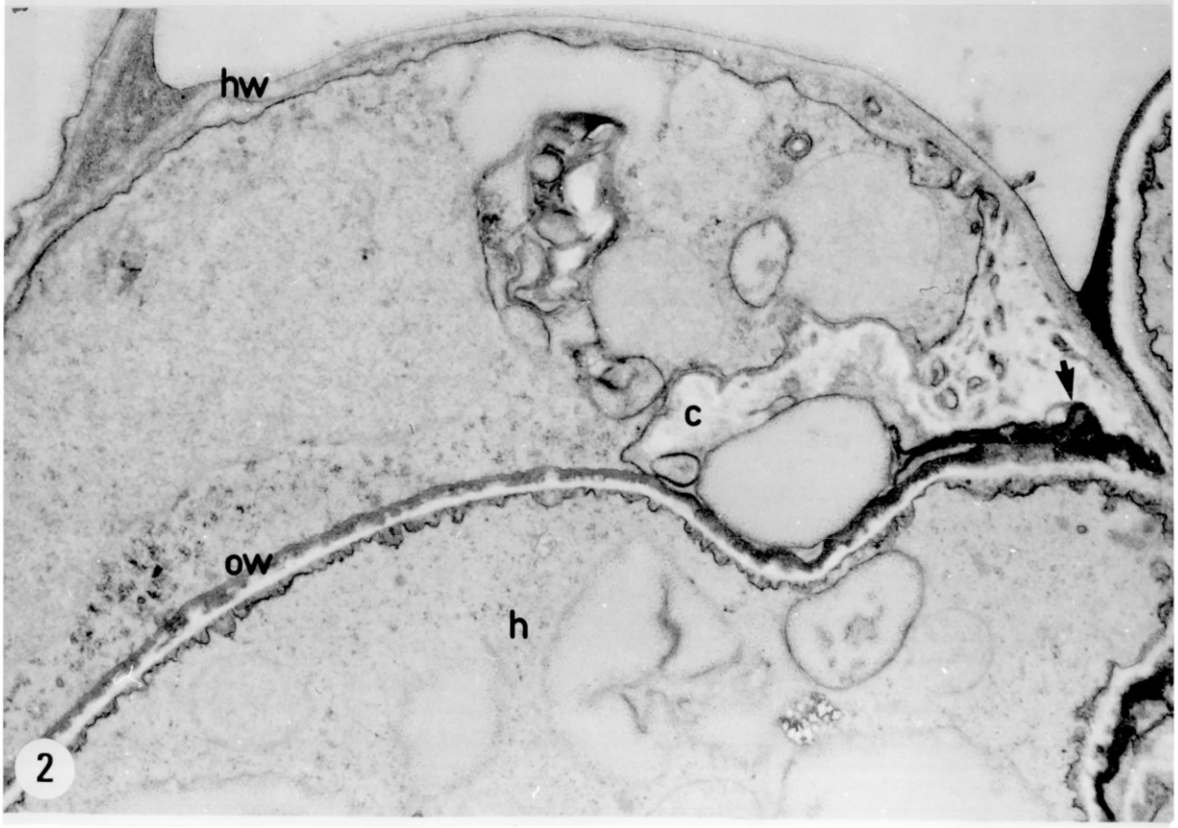

hw
c
ow
h
2

Plate 8:3

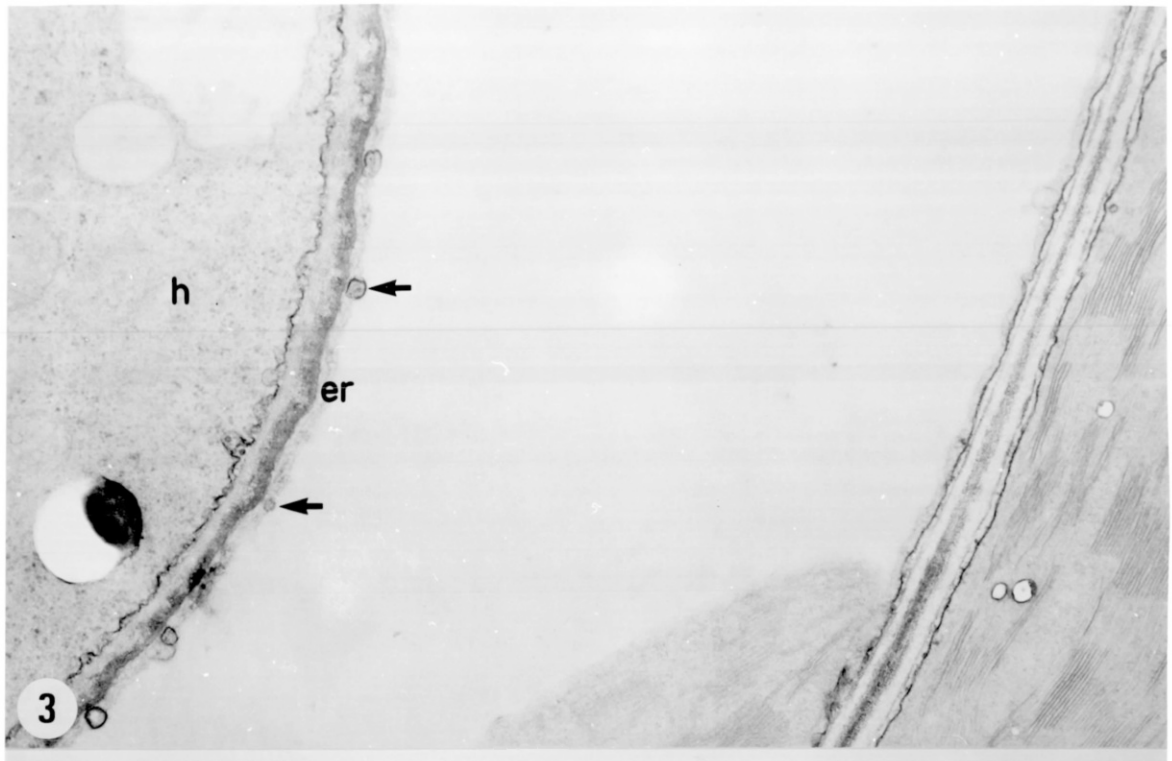
Section of an haustorium (h) of B. lactucae infecting a mesophyll cell of L. sativa, stained by the PACP technique. Within the host cytoplasm immediately adjacent to the outer haustorial wall (ow), very lightly stained cisternae of endoplasmic reticulum (er) follow the contours of the haustorium, interspersed with moderately or intensely stained vesicles (or cross-sections of tubules) (arrowed). X27200.

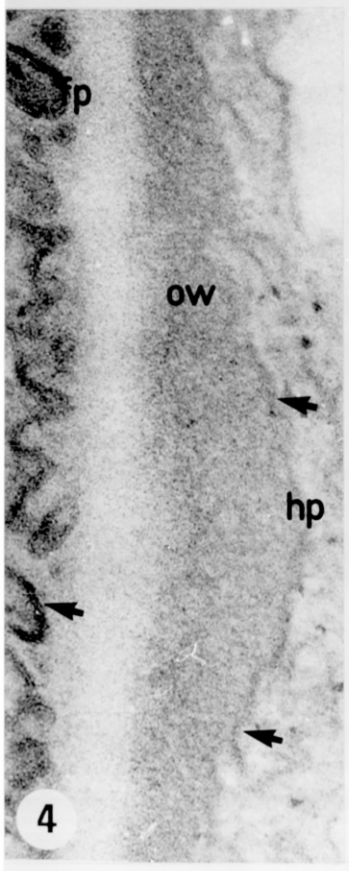
Plate 8:4

Section of an haustorium of B. lactucae stained with uranyl acetate and lead citrate. The tri-lamellar structure (arrowed) of the fungal plasmalemma (fp) can be clearly seen, but the invaginated host plasmalemma (hp) is very closely appressed to the outer haustorial wall (ow) and its tri-lamellar structure can only just be discerned in a few regions (arrowed). X138250.

Plate 8:5

Section of an haustorium (h) of B. lactucae completely encased by an extension of the collar, in a necrotic mesophyll cell of L. sativa, stained by the PACP procedure. A layer of host cytoplasm is trapped between the encasement (e) and the haustorium, bounded by the extrahaustorial membrane and the host plasmalemma adjacent to the encasement (arrowed). X19800.


h
er
3


p
ow
hp
4

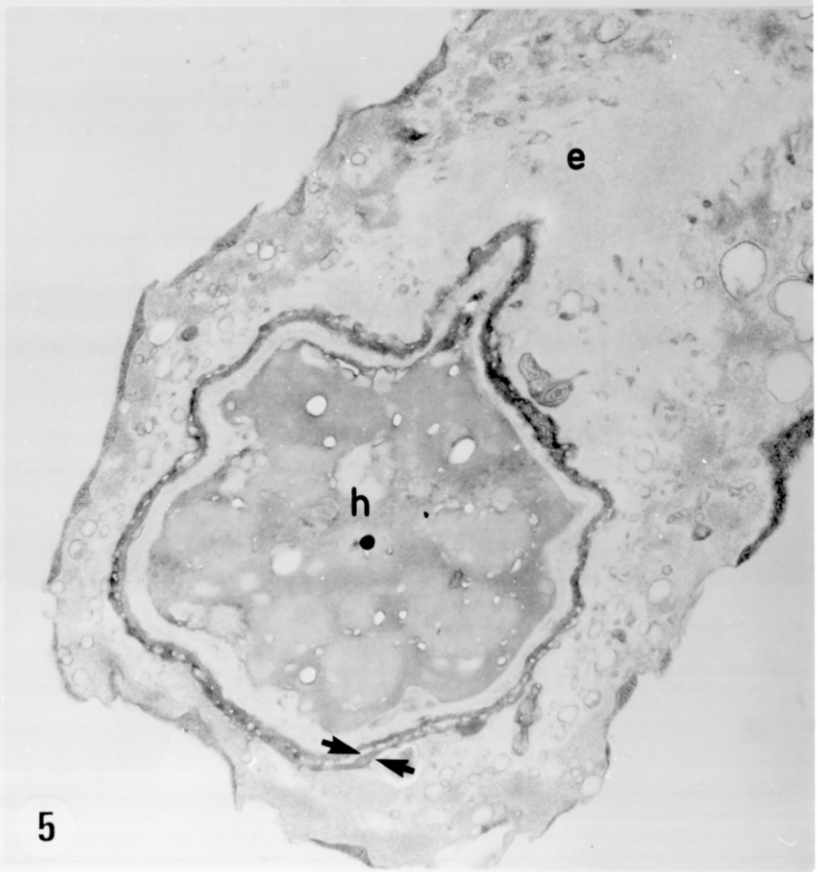

e
h
5

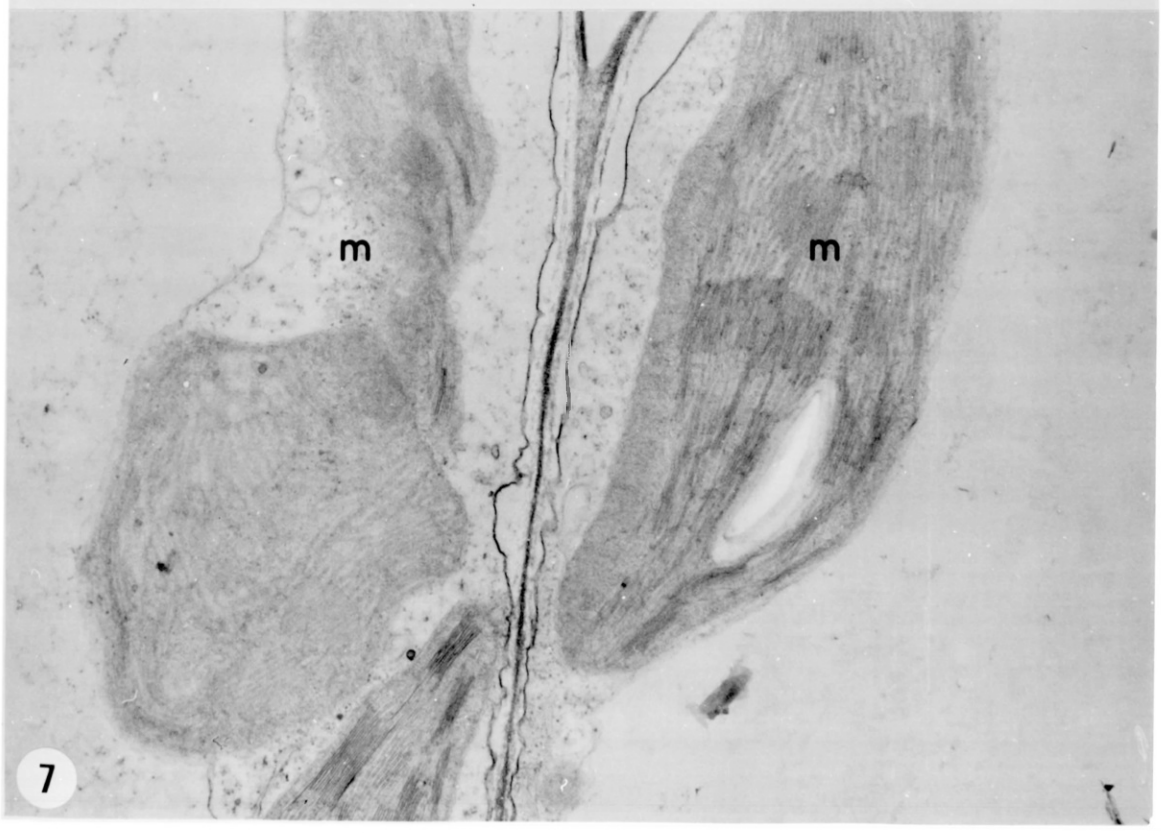
Plate 8:6

Section of an haustorium (h) of B. lactucae infecting a mesophyll cell of L. sativa stained by the PACP procedure. The region of the host plasmalemma adjacent to the outer haustorial wall (ow) is only lightly stained as is the region lining the host wall (hw), in contrast to the normally intense staining reaction of this membrane. The fungal plasmalemma is intensely stained as normal. X19800.

Plate 8:7

Section of an uninfected mesophyll cell (m) of L. sativa stained by the PACP procedure. The wall-lining plasmalemmas exhibit an intense staining reaction, characteristic of this membrane. X19800.


ow
hw
h
6


m
m
7

## 9. Investigations of the Physiology of Haustoria in vivo

### 9.1 Introduction

It has been proposed that nutrient uptake by haustoria is coupled to import of solutes by the host cell. This is thought to be due to the restriction to the wall-lining region of the host PM of an electrochemical gradient generated by an ATPase activated pump (Spencer-Phillips & Gay, 1981). This hypothesis applies particularly to haustoria infecting epidermal cells which do not manufacture photosynthates and therefore must scavenge the apoplast for solutes. However, the evidence for this hypothesis comes from cytochemical studies of the distribution of ATPase activity in infected cells and not from living systems. The generation of electrochemical gradients by ATPase pumps on plant membranes has been examined by monitoring the changes in fluorescence of pH-sensitive or membrane-potential-sensitive probes in sealed vesicles. For example, Dupont, Giorgi and Spanswick (1982) partially characterised an ATPase in microsomal vesicles isolated from corn roots by monitoring the quenching of quinacrine fluorescence which was caused by ATPase-dependent acidification of the intravesicular volume. In addition, changes which occurred after the application of various ATPase inhibitors were measured. Fluorescent probes have also been employed in in vivo investigations of electrogenic pumps and associated potential gradients (Waggoner, 1976). Thus, this approach may be applied to barley coleoptiles infected by E. graminis f.sp.hordei since they can be mounted (Bushnell, Dueck & Rowell, 1967) to permit observation by fluorescence microscopy. Thus any fluorescence changes in the 3 sealed compartments, host cell, extrahaustorial matrix and haustorium, may be monitored after external addition of inhibitors. In order to determine suitable fluorescent probes, inhibitors and experimental conditions the following preliminary investigations were carried out.

## 9.2 Materials and Methods

### 9.2.1 Preparation of Coleoptiles

The method used was adapted from that of Bushnell, Dueck & Rowell (1967). The apical 10-15nm segments of coleoptiles of barley (cv. Atlas) were removed from 7 or 8 day old plants grown as described in Section 2.1.3. On the side opposite the rounded apex, lengthwise cuts were made beside the two vascular bundles. The smaller portion was discarded, leaving a segment with adaxial and abaxial tissue layers with a central cavity, and attached at each edge to a vascular bundle (Figure 9:2). This was subsequently mounted with the adaxial epidermis uppermost between a pair of support wafers as illustrated in Figure 9:1. The wafers were made from plastic for studies by fluorescence microscopy and from epoxy-resin (TAAB) for investigations by electron microscopy. The slot in the lower support wafer provided access for removal of the unwanted abaxial-tissues with a scalpel and forceps, leaving the adaxial epidermis as a single layer in the region of the observation hole (Figure 9:2). The wafers were then immediately sealed with paraffin wax to a special plastic chamber (Figure 9:1) which was filled with 0.01M $Ca(NO_3)_2$ solution. Movement of the solution between the wafers and onto the upper surface of the inner epidermis was prevented by coating the inside of the wafers with a thin layer of an oil spread retardent compound, trade name Nye Bar (William F. Nye Inc., New Bedford, Mass., USA) or PTFE (Teflon), 24h before use. The upper epidermal surface was subsequently inoculated with conidia of *Erysiphe graminis* f.sp. *hordei* so that there were 20-40 spores within the observation region. The prepared coleoptiles were then incubated in the dark at 20°C in a box containing a saturated solution of zinc sulphate. This has a RH of 90% which prevented moisture from condensing on the inoculated surface. After 2 days the majority of spores had germinated

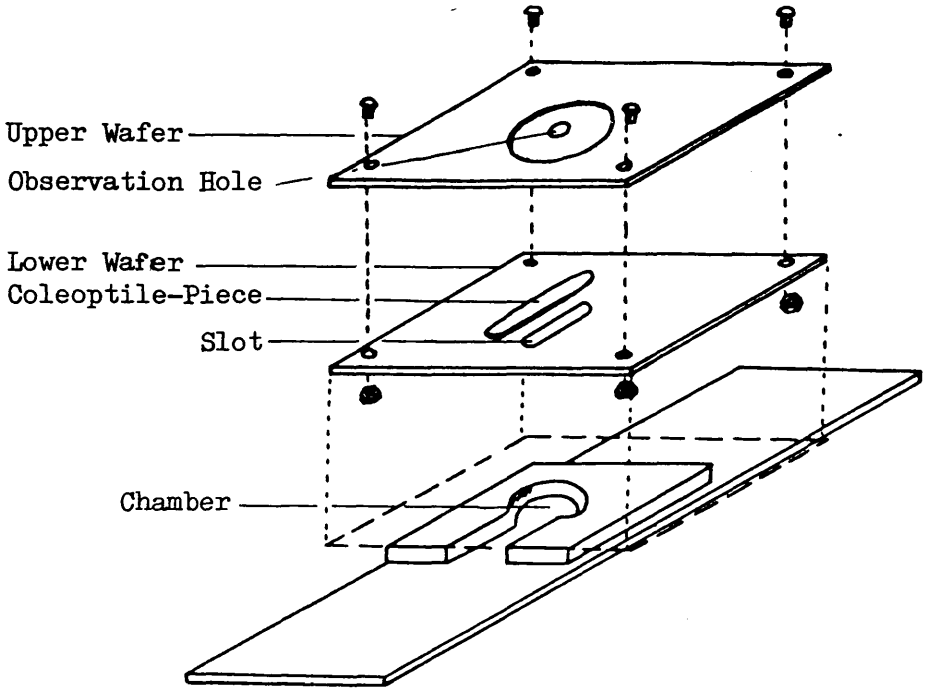



Figure 9:1 Diagram of wafers and chamber used to support and incubate epidermal tissues from barley coleoptiles infected by E. graminis (after Bushnell et al., 1967).

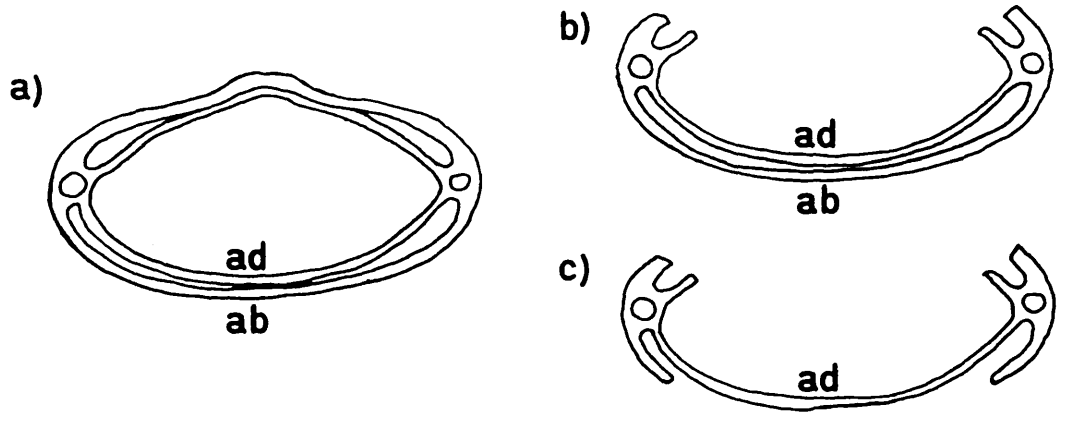



Figure 9:2 Diagrams of cross sections of coleoptiles of H. vulgare and their preparation for infection and observation. a) Intact coleoptile with two vascular bundles. b) Side of coleoptile opposite to rounded apex has been removed leaving adaxial and abaxial epidermal layers with a central cavity separating them. The coleoptile is subsequently mounted in the support wafers. c) The abaxial epidermal layer has been removed from the coleoptile to leave a single layer of epidermal cells. The adaxial side is subsequently infected with spores of E. graminis hordei.

and primary haustoria had formed within host cells.

9.2.2 ATP-ase Localization

Prepared coleoptiles in resin wafers which had been incubated for 2 days were observed by light microscopy to check that spores had germinated and primary haustoria had formed. Subsequently the ATP-ase localization procedure with control treatments was followed as described in Section 2.2.4, except that fixation was for 60-70 min instead of 90 min and sodium orthovanadate (150μM) was used as an inhibitor of ATPase activity in place of NaF. The prepared coleoptiles were held in the wafers throughout the procedure and were fully immersed in solutions. After embedding and polymerisation of the coleoptiles, haustoria were located by light microscopy before trimming and sectioning for TEM as described in Section 2.2.2.

9.2.3 Experiments with Acridine Orange

After 2 days incubation, haustoria within infected coleoptiles were located by light microscopy, their positions were mapped and cytoplasmic streaming of the host cells was checked. The $Ca(NO_3)_2$ solution was then removed from the plastic chamber and replaced with a 1 in $10^6$ or 1 in $10^7$ solution of acridine orange in 0.01M $PO_4$ buffer at pH 7.2. Cells were observed immediately by incident fluorescence microscopy with a filter assembly passing exciting wavelengths of 410-465nm and emission wavelengths above 515nm (Microstar (American Optical Co.) Module no. 2072). Each cell was examined for a maximum of 10s and to further minimize exposure to UV the diameter of the beam was reduced so that only one haustorium was irradiated at each observation. After 1-3h the acridine orange solution was replaced by a solution of an inhibitor in 0.01M $PO_4$ buffer at pH 7 either with or without acridine orange. The inhibitors used were carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1μm and 10μm), dinitrophenol (DNP) (300μm), di-ethylstilbesterol

(DES) (150μM) and sodium orthovanadate (600μM). Cytoplasmic streaming and fluorescence within infected cells were checked immediately and approximately every 5 min for 30 min or until streaming stopped. This limitation of observations minimised damage caused by UV. Subsequent to cessation of streaming the solution containing the inhibitor was replaced with acridine orange in phosphate buffer and cells were again checked for cytoplasmic streaming and fluorescence.

## 9.3 Results

### 9.3.1 ATPase Localization

The coleoptile walls were frequently distorted but the host cytoplasm usually remained intact. Plasmadesmata between adjacent cells were not observed. Haustoria were contained in an extrahaustorial membrane, which is presumed to be an invagination of the host plasmalemma, and had the characteristic lobes and collars of wall-like material around their necks a described in leaves by Bracker, 1968.

In experiments where ATP was employed as substrate, precipitates, indicating ATPase activity, were observed on the PM of the host where it lined the cell walls. It was always unevenly distributed in both infected and uninfected cells, varying from flocculent deposits on the PM adjacent to the anticlinal walls (Plate 9:1) to finely granular on the PM adjacent to the periclinal walls (Plate 9:2). Moderately granular precipitates were also observed on the host tonoplast, but no reaction product was present over either the region of the host PM invaginated around haustoria or the fungal PM (Plate 9:1). When βGP was employed as substrate, precipitates on the wall-lining region of the host PM were similarly distributed in smaller amounts (Plate 9:3). In contrast, deposits on the host tonoplast were heavier than when ATP was used and some reaction product was present on the haustorial PM

(Plate 9:3) although it was absent from the PM of the intercellular hyphae. Precipitates on the host wall-lining PM were much reduced in ATP + sodium orthovanadate controls and absent from the tonoplast (Plate 9:5). In the absence of substrate, no precipitates were observed except for a fine precipitate within the host and fungal ER (Plate 5). A similarly distributed fine precipitate was found with ATP and βGP (Plates 9: 1,3).

9.3.2 Experiments with Acridine Orange

In preliminary experiments no fluorescence was observed when acridine orange at concentrations of less than $10^{-6}$ (w/v) were used and therefore this concentration was used in all subsequent experiments.

When coleoptiles were subjected to UV within a period of 1-2 min after treatment with acridine orange, the host vacuole fluoresced diffusely green and haustorial vacuoles exhibited a bright green fluorescence. The cytoplasms and walls of host and fungus and the extrahaustorial matrix, which was difficult to distinguish, did not fluoresce and no fluorescence was observed before or after addition of an inhibitor with coleoptiles which remained in $Ca(NO_3)_2$ solution. Subsequent to the addition of inhibitors, the effects were confined to the host vacuole and haustorial cytoplasm (Table 9:1). No changes were observed in the host cytoplasm, extrahaustorial matrix or haustorial vacuole and therefore they are not represented in the table. Similar results were obtained after the addition of a solution containing a particular inhibitor whether acridine orange was present or absent from the solution. Since continuous exposure of cells to UV for approximately 1 min stopped host cytoplasmic streaming and caused fluorescence of haustoria, haustoria were only checked for fluorescence immediately and then either every 5 min after the addition of an inhibitor, or when streaming had ceased as set out in Section 9.2.3.

Table 9:1 Response of Host Cells and Haustoria to the Application of Inhibitors

| Inhibitor | Concentration (μM) | After Addition of Inhibitor | | | | | | After Removal of Inhibitor | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Cytoplasmic Streaming | Time | Host Vacuolar Fluorescence | Time | Haustorial Cytoplasmic Fluorescence | Time | Cytoplasmic Streaming | Host Vacuolar Fluorescence | Haustorial Cytoplasmic Fluorescence |
| FCCP | 10 | - | Immediate | NI | NI | ++ | Immediate | - | NI | ++ |
| FCCP | 1 | + | | + | | - | | + | + | - |
| DNP | 300 | + | | + | | - | | + | + | - |
| DES | 150 | - | 5 min | - | 5-6min | + | 5-6min | - | - | + |
| Vanadate | 300 | + | | + | | - | | + | + | - |
| Vanadate | 600 | + | | + | | - | | + | + | - |
| Continuous exposure to UV | | - | 1 min | - | 1-2min | + | 1-2min | - | - | + |

NI = No Information Recorded

It was therefore difficult to determine accurately when streaming ceased as in most cases it gradually slowed and so times stated for cessation of streaming and fluorescence of haustoria are approximate.

Haustorial fluorescence was never observed until host cytoplasmic streaming had stopped. FCCP at a concentration of 10μM was the most rapidly acting inhibitor of streaming and immediately caused the brightest haustorial fluorescence observed in these experiments. Streaming was also stopped after 5 min by DES (150μM) by which time haustorial fluorescence had occurred. Streaming was not inhibited and haustoria did not fluoresce after the addition of 1μM FCCP, 300μM or 600μM sodium orthovanadate

## 9.4 Discussion

The results show ATPase activity within barley coleoptile cells at the plasmalemma of epidermal cells, but after infection it is absent from the invaginated PM. The uneven distribution of precipitates on the wall-lining PM, was similar in both infected and uninfected cells and thus not caused by infection. This suggests that the reaction is influenced by differences in the wall or that parts of the host PM are stabilised and not completely fluid. The higher ATPase activity in the anticlinal region suggests that uptake of solutes from the apoplast may occur especially in this region of the host PM and, in the absence of plasmadesmata, transfer of solutes between adjacent host cells may be effected in this way.

The presence of ATPase activity on the host wall-lining regions of the host PM and its absence from the invaginated region is in agreement with a previous investigation of the powdery mildew *E. pisi* (Spencer-Phillips & Gay, 1981). However, although detection of βGPase activity on the haustorial PM is also consistent with the findings

for E. pisi, the absence of ATPase activity from this membrane is not. Similarly, ATPase activity has not been detected on the haustorial plasmalemmas of the dikaryon rust Uromyces appendiculatus (Spencer-Phillips & Gay, 1981), the monokaryon rust P. poarum (Section 7) and the Oomycete A. candida (Woods & Gay, 1983). There is a possibility that inappropriate conditions for detection of fungal ATPases may have been employed in investigations of all the infections mentioned above. However, apart from prior fixation, the conditions employed are similar to those used in investigations of PM ATPase activity in Neurospora crassa (Scarborough, 1976) and thus it is likely that the result is valid.

The rate of transport into haustoria of E. pisi from host cells has been calculated to approach the rates expected as a result of active or facilitated processes. This conclusion has been reached in investigations of the rate of $^{14}C$ accumulation in mycelia on plants supplied with $^{14}CO_2$ (Manners, 1979). Investigations of the distribution of ATPase activity in haustorium-infected cells (Spencer-Phillips & Gay, 1981; Woods & Gay, 1983) have enabled two alternative transport mechanisms to be proposed. Each is based on the theory that in plants and fungi, ATPases in plasma membranes generate an electrochemical gradient across the membrane by extrusion of protons or ions and that solute influx is coupled to the equilibration of this gradient (Baker, 1978). Both proposed mechanisms involve a sealed 3 compartment - 3 membrane system consisting of the host cytoplasm, extrahaustorial matrix and haustorial cytoplasm and the host wall-lining, invaginated and haustorial plasmalemmas. The two hypotheses are as follows:-

i) If, as in E. pisi, ATPases are active on both the fungal and host wall-lining plasmalemmas, solutes enter the host cell via an electrochemical gradient and solutes within the extrahaustorial matrix are

similarly transported across the haustorial PM. Thus, a high concentration gradient is maintained across the invaginated PM enabling diffusion or facilitated diffusion to occur. In addition, it has been suggested that interaction of the complementary products of the electrogenic pumps at the membranes with ATPase activity may activate efflux of solutes across the invaginated PM (Spencer-Phillips & Gay, 1981).

ii) If ATPase pumps are active only on the uninvaginated PM, solutes are still transported into the host cytoplasm as proposed above. However, in the absence of electrogenic pumps on both the invaginated PM and the haustorial PM, diffusion or facilitated diffusion are the most likely mechanisms for transport across both of these membranes since assimilation of soluble translocates into non-diffusable compounds within the haustoria and mycelium would maintain a concentration gradient between the host and haustorial cytoplasms (Woods & Gay, 1983).

In both the mechanisms proposed above an electropotential gradient would exist between the haustorial cytoplasm and the exterior. Application of uncouplers of transmembrane potential to bacteria and higher plant cells reverses the polarity of solute transport (Humphreys, 1977). Therefore, if the proposed transport into haustoria is linked to a potential gradient across the host wall-lining PM, depolarisation of this membrane by application of uncouplers or inhibitors of ATPase activity would be expected to affect the potential across the host invaginated and/or haustorial plasmalemmas. If an electrogenic pump exists on the haustorial PM, depolarisation of the host PM would increase the $H^+$ (or cation) concentration only within the host cytoplasm. Alternatively, if there is no pump on the haustorial PM, depolarisation of the host PM would lead to an increased $H^+$ concentration within both the host and haustorial cytoplasms. Such changes can be monitored using fluorescent probes sensitive to membrane potential or the pH of compartments.

Acridine orange, used in the present preliminary investigation, exists below pH 6 as a cation to which membranes are relatively impermeable. The monomer fluoresces green but at high concentrations red fluorescing dimers and trimers are formed. Between pH 6 and 12 both the cation and the undissociated basic molecule (permeant to membranes) are present. The latter fluoresces green when combined with phenols and similar compounds (Stadelmann & Kinzel, 1972).

In the experiments described above, after addition of acridine orange to infected cells, the haustorial and host cytoplasms did not fluoresce, but the host and haustorial vacuoles fluoresced green. The haustorial cytoplasm fluoresced only when host cytoplasmic streaming, indicative of metabolic activity, had ceased following the application of FCCP, DES or sodium orthovanadate or exposure to UV. Since streaming did not resume on removal of the inhibitors, it is assumed that host cells had died.

The absence of haustorial fluorescence prior to application of inhibitors indicates the absence of acridine orange at concentrations above that in the applied solution,within the haustorial cytoplasm. However, the dye did accumulate within the host and haustorial vacuoles. Accumulation of acridine orange is normally related to physiochemical processes and not to the metabolic activities of a cell. Thus, the undissociated basic molecule, which is membrane permeant, becomes trapped within vacuoles by binding to substances or dissociating to form impermeant cations due to a low pH (Stadelmann & Kinzel, 1972). Although accumulation of the ionized dye may lead to red fluorescence, the very low extracellular concentration employed in the present study may have been too low for dimer formation within the vacuole. Thus, fluorescence of host and haustorial vacuoles may have been caused by either binding or ionization of the dye. The loss of host vacuolar fluorescence after death of the host cell suggests that the dye within

this vacuole was ionized. Similarly, fluorescence caused by accumulation of acridine orange within the haustorial cytoplasm after host cell death may be due to either binding or ionization of the dye. If the dye was bound it is presumed to have reacted with substances previously unavailable within the cytoplasm, indicating haustorial death and disorganization of the cytoplasm. A similar accumulation of the basic vital stain neutral red within non-viable haustoria of E. graminis has been observed by Bushnell et al. (1967) in experiments using the same microculture system. If, alternatively, acridine orange had become trapped due to ionization, it indicates that on death of the host cell protons move into the haustorial cytoplasm, decreasing the pH. This suggests that there is a depolarisation of the electrochemical gradient which exists between the extracellular medium and the haustorium, as proposed in the transport mechanisms described above. The latter explanation is more likely in the case of the immediate fluorescence of haustoria when FCCP was applied to coleoptiles, as haustorial death and disorganization of the cytoplasm is unlikely to have occurred so rapidly. It is also unlikely that host cell death occurred immediately and, thus, the fluorescence observed was probably due to destruction of electrochemical gradients by the uncoupler. Subsequent cell death is probably due to inhibition of oxidative phosphorylation. It is not possible to determine with certainty across which plasmalemmas the electrochemical gradient may have existed as the FCCP may have penetrated through the host cytoplasm to the haustorial PM. As FCCP can bind to proteins it may remain within cell membranes after removal of the inhibitor from the bathing solution. This may explain the absence of resumed cytoplasmic streaming and thus cell death, despite rapid substitution of bathing solutions.

In contrast to FCCP, the delayed fluorescence of haustoria after addition of DES or exposure to UV is more likely to have been caused

directly by haustorial death. Although stilbene derivatives have been shown to inhibit ATPase activity (Lin, 1981; Mettler, Mandala & Taiz, 1982) they are relatively impermeant to membranes (Lin, 1981). Thus, DES is unlikely to inhibit metabolic activities within the cell and therefore cessation of cytoplasmic streaming and subsequent haustorial fluorescence may have been caused by excessive exposure to UV during experiments with this inhibitor.

The absence of a response to the application of sodium orthovanadate may be due to the relative impermeability of membranes to this inhibitor and the location of the ATPase binding site on the cytoplasmic side of the host PM (Simons, 1979), although depolarisation of the transmembrane potential of germinated radish seeds has been demonstrated after the application of 200-500μM Na orthovanadate (Cocucci, Ballarin-Denti & Marre, 1980). However, the inability of the uncoupler DNP to inhibit cytoplasmic streaming cannot be explained, as it usually initiates a response similar to that of FCCP. Although FCCP is a more potent uncoupler than DNP, concentrations as low as 10μM DNP have been used to uncouple oxidative phosphorylation (Blackman, Parke & Gorton, 1955).

Thus, in summary, preliminary fluorescence experiments with acridine orange suggest that the ionophore FCCP may initiate fluorescence of the haustorial cytoplasm due to an increase in $H^+$ ion concentration caused by destroying a proton gradient across the host and/or haustorial plasmalemmas. In as much as ATPase activity has not been demonstrated at the haustorial PM, it is probable that the host plasmalemma is primarily involved. However, this result cannot be separated with certainty from the result obtained after haustorial death.

Several problems concerning the inhibitors and dyes used have been highlighted by these preliminary studies. In order to avoid interference with the haustorial PM and the metabolic activities of host and haustorial

cytoplasms it is necessary to apply reagents which are either specific to or do not pass beyond the host PM. Varying the external pH (and therefore the proton gradient across the host PM) and application of a plant PM-specific chemical such as fusicoccin, which hyperpolarizes the membrane, are two possible experimental approaches. To determine the presence or absence of an electrogenic pump across the haustorial PM, it would be advantageous to apply inhibitors directly to haustoria by incising host cells. However, it has been shown by Sullivan, Bushnell & Rowell (1974) and by attempts during the present investigation that, after incision, a thin layer of host cytoplasm remains around undamaged haustoria. Cytoplasmic streaming continues within this layer and thus, in addition to the extrahaustorial membrane, an intact host membrane may remain between the haustorium and the bathing solution. Thus, experiments cannot be carried out directly on the haustorium with certainty. Haustorial complexes, as isolated by Manners & Gay (1978), offer an experimental approach but the results of such experiments would have to be interpreted with respect to the extrahaustorial and haustorial plasma membranes.

The accumulation of acridine orange within vacuoles and the fluorescence of both the dissociated and the chemically bound undissociated forms of the molecule present severe disadvantages to accurate determination of potential changes between compartments. However, the fluorescent probes, such as oxonol, which fluoresce only in the ionized form and are also membrane permeant, offer a solution to the problem since fluorescence would depend on the distribution of the ion in accordance with electropotential, and would not be related to the binding of an electroneutral molecule. Oxonol exists as an anion at neutral or basic pH and its fluorescence is quenched as it accumulates within positively charged compartments (Smith, Russ, Cooperman & Chance, 1976). Complementary studies using this dye and a cationic fluorescent probe such as cyanine should yield more definite information about potential gradients.

## Plates 9:1 and 9:2

Section of an haustorium of a coleoptile epidermal cell of H. vulgare infected by an haustorium (h) of E. graminis hordei, prepared from material incubated with ATP and magnesium ions at pH 7.2. Plate 9:1 shows flocculent precipitates, indicating ATPase activity, present on the host plasmalemma adjacent to the anticlinal host wall (aw) and also on the tonoplast. Less flocculent precipitates are present on the region of the host plasmalemma adjacent to the periclinal host wall (pw) shown in Plate 9:2. Reaction product is absent from the region of the host plasmalemma invaginated around the haustorium and from the fungal plasmalemma. em, extrahaustorial matrix. Plate 9:1, x36950; Plate 9:2, x19800.

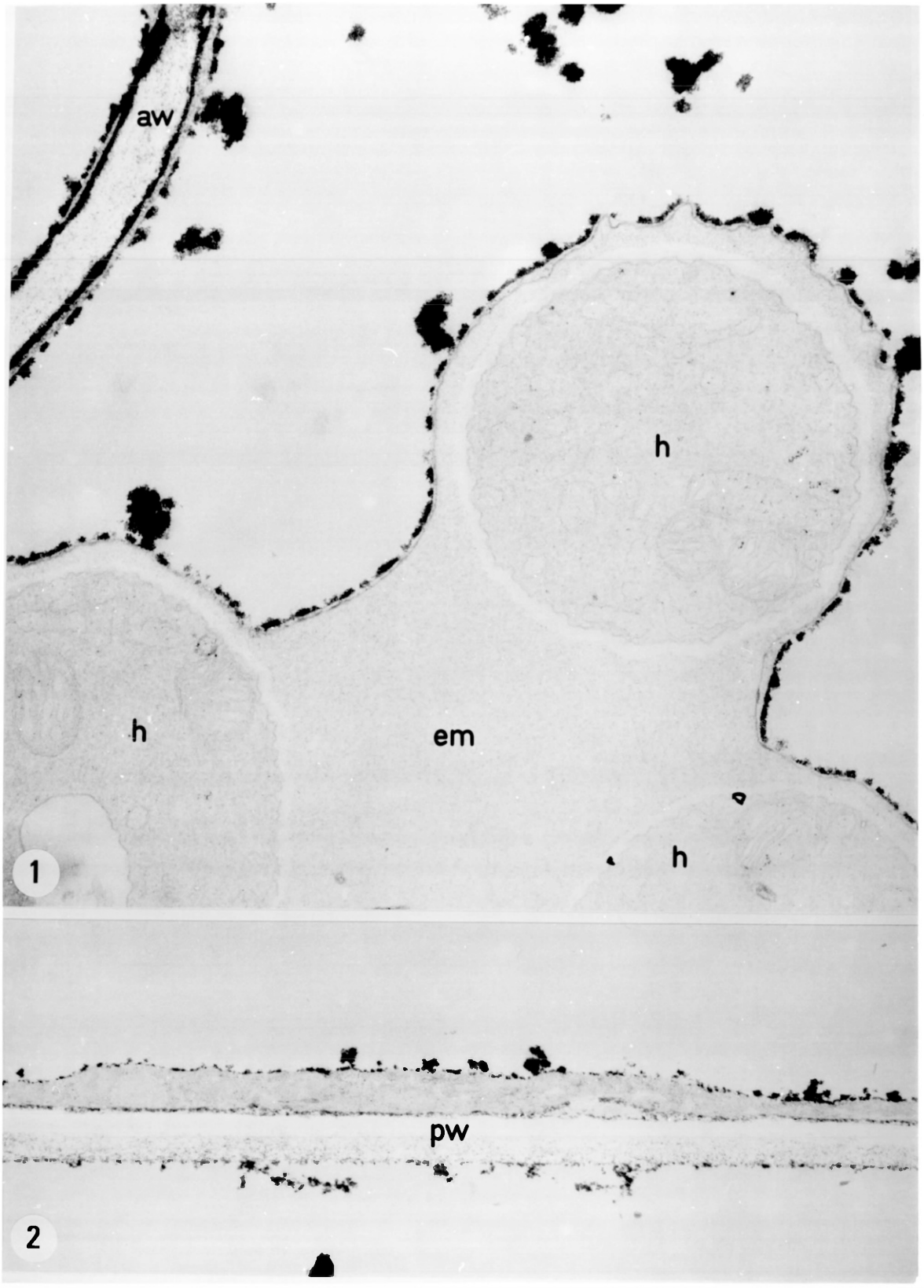

aw
h
h
em
h
1
pw
2

Plate 9:3

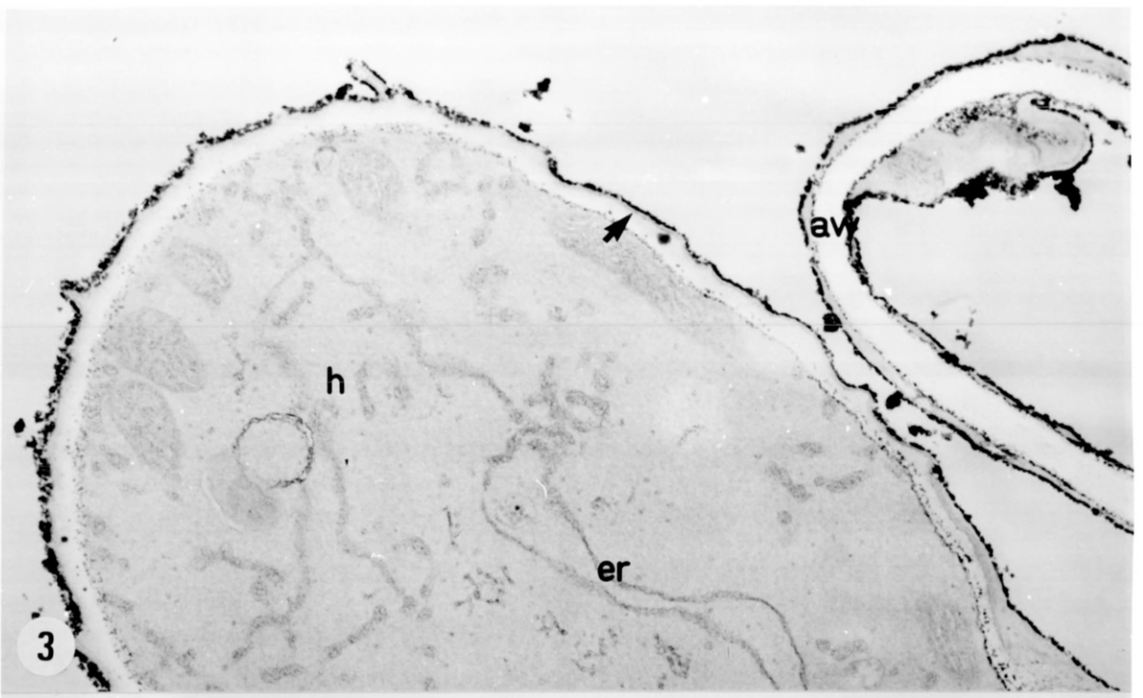
Section of an haustorium (h) of E. graminis hordei infecting a coleoptile epidermal cell of H. vulgare, prepared from material incubated with βGP and magnesium ions at pH 7.2. Granular precipitates indicating general phosphatase activity are present on the region of the host plasmalemma lining the anticlinal wall (aw) and on the fungal plasmalemma. More flocculent precipitates, indicating greater activity, can be seen on the host tonoplast. Reaction product is absent from the region of the host plasmalemma invaginated around the haustorium (arrowed). A fine lead precipitate, also observed in no substrate controls, is present within cisternae of the fungal endoplasmic reticulum (er). X19800.

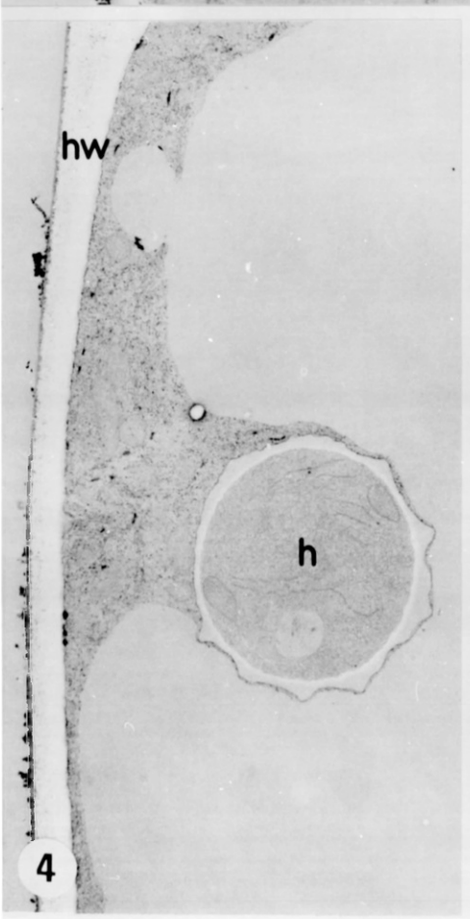
Plate 9:4

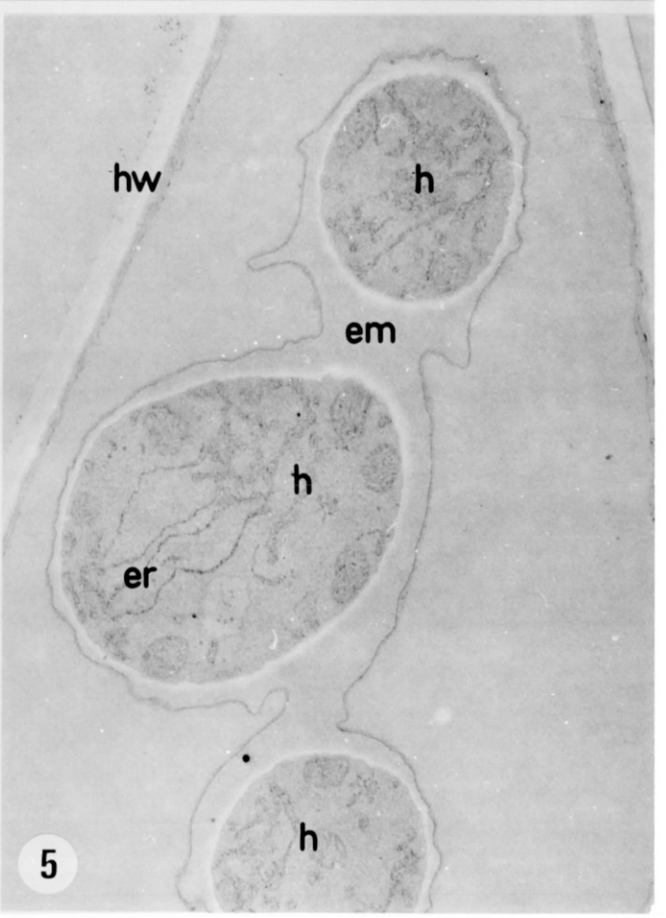
Section of an haustorium (h) of E. graminis hordei infecting a coleoptile epidermal cell of H. vulgare, prepared from material incubated with ATP, magnesium ions and an ATPase inhibitor, sodium orthovanadate, at pH 7.2. Precipitates are almost absent from the wall (hw) lining region of the host plasmalemma and are completely absent from the invaginated host plasmalemma, the host tonoplast and the fungal plasmalemma. X9350.

Plate 9:5

Section of an haustorium (h) of E. graminis hordei infecting a coleoptile epidermal cell of H. vulgare prepared from material incubated in a reaction medium without substrate at pH 7.2. Precipitates are absent apart from a fine lead precipitate within the cisternae of the fungal endoplasmic reticulum (er). em, extrahaustorial matrix; hw, host wall. X11700.


av
h
er
3


hw
h
4


hw
h
em
h
er
h
5

## 10. General Summary and Conclusions

The present investigation has shown that the region of host PM invaginated around haustoria of A. candida, P. poarum and B. lactucae is physiologically and/or structurally distinct from the uninvaginated region (Sections 3,7,8).

The origin of the specialisation of the extrahaustorial membrane is uncertain. The membrane may be modified by the fungus, as seems likely in infections by B. lactucae where the host PM has been shown to proliferate prior to fungal penetration (Ingram et al., 1976), or it may be formed from an abnormal source such as the ER. The latter alternative has been investigated in cells infected by A. candida (Section 4), but, although frequent close associations between the ER and the extrahaustorial matrix occur, the lumen of the ER and the extrahaustorial matrix are distinct and therefore unlikely to be a spacial continuum. Thus, the results are inconclusive.

The invaginated region of the host PM remains distinct from the uninvaginated part in cells infected by haustoria of A. candida, but it is highly likely that haustoria in monokaryotic infections of P. poarum become surrounded by a normal PM as the haustorium ages. Conversely, in cells infected by B. lactucae it is probable that where 2 PM domains have been established these are permanent and in cells in which the whole host PM has been altered, the modification arises independently. The differing degrees of permanence of the 2 PM domains is further demonstrated by the mechanism of encasement or enclosure of old haustoria. In cells in which the regions are permanently distinct, i.e. cells infected by A. candida (observed during the present investigation and by Berlin & Bowen, 1964) and B. lactucae, the haustoria are encased by an extension of the collar formed adjacent to the host PM proximal to the specialised region. In contrast, the haustoria in cells in which

the 2 PM regions are not permanent, i.e. cells infected by the monokaryotic stage of P. poarum, are enclosed by host wall-like material produced adjacent to the invaginated region.

According to the fluid mosaic model of membranes (Singer & Nicholson, 1972) the molecules of two regions of PM are free to diffuse laterally and so mix freely and any long range order arises from conditions external to the membrane or an additive effect of intrinsic short range interactions forming an unusually extensive array. Thus, the observation of 2 PM domains can be interpreted in terms of prevention or reduction of lateral diffusion of membrane components. This may be effected either extrinsically or intrinsically and may be restricted to the junction of the 2 PM domains or affect the whole specialised region. For example, both the neckband equivalent detected in the haustorial neck wall of A. candida and the proposed adherence of the invaginated PM to the outer haustorial wall of B. lactucae may form an extrinsic barrier to membrane fluidity, although it is suggested that in A. candida infections adherence is restricted to the neck region whereas in B. lactucae infections the whole invaginated PM is affected. It should be noted that the PACP positive regions of the extrahaustorial membrane in A. candida infections indicate that the fluidity of this membrane may also be restricted in regions other than the neck. In both infections the 2 PM domains remain permanently distinct. In contrast, the proposed decreased fluidity of the PM invaginated around M. haustoria of P. poarum,which may be effected either intrinsically or extrinsically, may decrease, but not prevent, lateral diffusion of components of the whole invaginated PM and thus the 2 PM domains would not be permanent.

Experiments have indicated the existence of a barrier in the haustorial wall of A. candida to diffusion of solutes between the extrahaustorial matrix and the general apoplast (Section 5). This barrier

is most likely to be situated at the neck region B. It has been demonstrated by X-ray microanalysis that silicon, present at the neckband of P. coronata avenae (Chong & Harder, 1980), is absent from this neck region and also the neckband of E. pisi (Section 6). Thus impermeability does not depend on the presence of silicon. The absence of such an apoplastic diffusion barrier around M-haustoria of P. poarum is indicated by the gradual enclosure of the haustoria by host wall-like material and by the absence of any region of abnormal composition within the haustorial wall. Similarly, if solute transport from host to haustorium takes place, it is unlikely that a barrier to apoplastic diffusion exists within the haustorial wall of B. lactucae unless it is restricted to an undetectably abnormal region of the haustorial wall equivalent to a neckband.

It has been shown that specialisation of the invaginated region of the host PM is a feature common to haustorial infections and is therefore likely to be essential for nutrient uptake by haustoria. According to the transport hypotheses (Spencer-Phillips & Gay, 1981; Woods & Gay, 1983) the absence of ATPase activity on the invaginated PM polarises host nutrient transport towards the haustorium. Although results are not conclusive, preliminary experiments with fluorescent probes of membrane potential support this theory, since depolarisation of the host PM potential may increase the $H^+$ ion concentration within the haustorial cytoplasm (Section 9). However, in addition to specialisation of the invaginated PM, both the transport hypotheses presuppose that the host invaginated and haustorial plasmalemmas are sealed together by a barrier to apoplastic diffusion. In the absence of such a barrier, it is expected that transport is less efficient, as discussed in Section 5:4.

In summary, in the haustorial infections examined, specialisation of the invaginated PM, which is considered to be essential for nutrient

transport, may be maintained in a variety of ways by localised or general annulment or reduction of membrane fluidity, effected either extrinsically or intrinsically. Thus a neckband is not essential for nutrient transport. However, decrease of membrane fluidity alone would not permanently maintain 2 distinct PM regions and, in addition, only a restricted specialised region within the haustorial wall (i.e. a neckband) would provide a barrier to apoplastic diffusion. Thus, although it is not essential, a neckband, which permanently maintains 2 PM domains and acts as an apoplastic seal, is expected to maximise the uptake of solutes from the host by the haustorium.

From the results of ultrastructural investigations, a range of specialisation can be seen. These vary from temporarily specialized haustorial interfaces of the monokaryon rusts, to those of the Peronosporaceae, to the highly developed haustorial interfaces with neckbands in powdery mildews, dikaryon rusts and the Albuginaceae (Table 10:1). Thus, the formation of haustoria surrounded by an abnormal host PM is common to infections by the Erysiphales, Uredinales and Peronosporales and within each of these groups a neckband has evolved. Successful biotrophy depends on many factors in addition to nutrient uptake, such as minimising damage to host organelles, translocation of host nutrients from uninfected to infected sites and adaptation of the parasitic life cycle to that of the host. However, the parallel evolution of key features of the host-parasitic interface of 3 different groups of biotrophic fungi indicates that these features are important to successful biotrophy. It may be argued that these features all relate to infections of angiosperms and may therefore represent the response of a single group of plants to fungal invasion. However, the presence of a neckband in one member of the Mucorales parasitic on another (Jeffries & Young, 1976) shows that this adaptation has also evolved in diverse hosts. Detailed ultrastructural investigations of host-pathogen inter-

Table 10:1 The degree of specialization of haustoria of members of the Peronosporales, Uredinales and Erysiphales

| Organism | Specialized Invaginated Host PM (IPM) | Tight Junction Between IPM and Haustorial Wall | Visible Neck-band | Apoplastic Diffusion Barrier | References |
|---|---|---|---|---|---|
| Monokaryotic Uredinales | | | | | |
| Puccinia poarum | + | - | - | - | Section 7 |
| Puccinia coronata avenae | + | - | - | NI | Chong, Harder & Rohringer, 1981 |
| Uromyces phaseoli vignae | + | - | - | NI | Heath, cited in Littlefield & Heath, 1979 |
| Peronosporaceae, Peronosporales | | | | | |
| Bremia lactucae | + | + | - | NI | Section 8; Ingram, Sargent & Tommerup, 1976 |
| Peronospora pisi | + | + | - | NI | Hickey & Coffey, 1978 |
| Pseudoperonospora humuli | + | + | - | NI | Pares & Greenwood, 1979 |
| Albuginaceae, Peronosporales | | | | | |
| Albugo candida | + | + | + | + | Woods & Gay, 1983 |
| Dikaryotic Uredinales | | | | | |
| Melampsora lini | + | + | + | + | Littlefield & Bracker, 1972; Allen, Coffey & Heath, 1979 |
| Uromyces phaseoli vignae | + | + | + | + | Heath, 1976; Heath cited in Littlefield & Heath, 1979 |
| Uromyces appendiculatus | + | + | + | NI | Spencer-Phillips & Gay, 1981 |
| Puccinia sorghi | NI | + | + | + | Heath, 1976 |
| Puccinia coronata avenae | + | + | + | NI | Chong & Harder, 1980; Chong, Harder & Rohringer, 1981 |

Cont'd.

| Organism | Specialized Invaginated Host PM (1PM) | Tight Junction Between 1PM and Haustorial Wall | Visible Neck-band | Apoplastic Diffusion Barrier | References |
|---|---|---|---|---|---|
| Erysiphales | | | | | |
| Erysiphe pisi | + | + | + | + | Gil & Gay, 1977; Manners & Gay, in preparation; Spencer-Phillips & Gay, 1981 |
| Erysiphe graminis hordei | + | + | + | + | Section 9; Hirata & Kojima, 1962, cited in Bushnell & Gay, 1978; Bracker, 1968 |

+, present; -, absent; NI, no information available

faces of, for example, infected ferns would further clarify this point. The formation of a neckband seems more likely to be a characteristic of the invading fungus than a universal response of the plant since a single host may be invaded by different fungi, one of which forms a neckband and another which does not, e.g. Pisum sativum may be infected by Erysiphe pisi or Peronospora pisi. The presence of a neckband may indicate haustoria which are specifically adapted for nutrient transport. It has been explained in Section 1 that although the importance of haustoria for nutrient uptake by the Erysiphales has been demonstrated, there is less evidence of the role of haustoria formed by members of the Uredinales and Peronosporales. The majority of the Erysiphales grow on the surface of their hosts and haustoria are the only interface between host and pathogen. Thus, it is expected that haustoria of this group would be highly specialized for efficient nutrient absorption and would therefore form a neckband. Since members of the Uredinales and Peronosporales form an extensive intercellular mycelium within the host, nutrient absorption may not depend solely on haustoria. Thus, the formation of a neckband may not be as essential as indicated by its absence around monokaryotic rust and downy mildew haustoria. However, the differences which exist between M- and D-haustoria of the same rust species are difficult to explain. It has been suggested that the two types of intracellular structure may have evolved separately from one macrocyclic ancestor which would explain the greater similarity between D-haustoria of different genera than between M- and D-haustoria of the same species (Littlefield & Heath, 1979). Thus, the haustoria of the dikaryon may have become more highly adapted to biotrophy than those of the monokaryon, although the lower efficiency of M-haustoria may be compensated for by other factors as indicated by the invasion of vascular tissue by intercellular hyphae and M-haustoria of P. poarum.

In conclusion, the ultrastructural evidence presented has identified key features for proposed mechanisms of nutrient absorption by haustoria and mechanisms, other than a neckband, for maintaining two host PM domains have been suggested. Further physiological investigations, such as experiments with fluorescent probes on intact living systems and on isolated haustorial complexes, and isolation and characterisation of extrahaustorial membranes, are required to provide more direct evidence which may confirm or modify the current hypotheses.

## References


Akutsu, K., Doi, Y. & Yora, K. (1980) Elementary analysis of papillae and cytoplasmic vesicles formed at the penetration site by Erysiphe graminis f.sp. hordei in epidermal cells of barley leaves. Ann. Phytopath. Soc. Japan 46, 667-671.

Al-Khesraji, T.O. (1981) Comparative anotomy and histochemistry of the association of Puccinia poarum with its alternate hosts. Ph.D. thesis, University of Sheffield.

Al-Khesraji, T.O. & Lösel, D.M. (1981) The fine structure of haustoria, intracellular hyphae and intercellular hyphae of Puccinia Poarum. Physiol. Plant Path. 19, 301-311.

Al-Khesraji, T.O., Lösel, D.M. & Gay, J.L. (1980) The infection of vascular tissue in leaves of Tussilago farfara L. by pycnial-aecial stages of Puccinia poarum Niel. Physiol. Plant Path. 17, 193-197.

Allen, R.F. (1935) A cytological study of Puccinia malvacearum from the sporidium to the teliospore. J. Agric. Res. (Washington DC) 51, 801-818.

Allen, F.H.E., Coffey, M.D. & Heath, M.C. (1979) Plasmolysis of rusted flax: a fine-structural study of the host-pathogen interface. Can. J. Bot. 57, 1528-1533.

Andrews, J.H. (1975) Distribution of label from $^{3}$H-glucose and $^{3}$H-leucine in lettuce cotyledons during the early stages of infection with Bremia lactucae. Can. J. Bot. 53, 1103-1115.

Ayres, P.G. (1976) Patterns of stomatal behaviour, transpiration and $CO_2$ exchange in pea following infection by powdery mildew (Erysiphe pisi). J. Exptl. Bot. 27, 1196-1205.

Baker, D.A. (1978) Proton co-transport of organic solutes. New Phytol. 81, 485-497.

Barber, D.A. & Shone, M.G.T. (1966) The absorption of silica from aqueous solutions by plants. J. Exptl. Bot. 17, 569-578.

Bentwood, B.J. & Cronshaw, J. (1978) Cytochemical localisation of adenosine triphosphatase in the phloem of Pisum sativum and its relation to the function of transfer cells. Planta 140, 111-120.

Berlin, J.D. & Bowen, C.C. (1964) The host-parasite interface of Albugo candida on Raphanus sativus. Am. J. Bot. 51, 445-452.

Blackman, G.E., Parke, M.H. & Gorton, G. (1955) The physiological activity of substituted phenols. II. Relationships between physical properties and physiological activity. Arch. Biochem. Biophys. 54, 55.

Borland, J. & Mims, C.W. (1980) An ultrastructural comparison of the aecial and telial haustoria of the autoecious rust Puccinia podophylli. Mycologia 72, 767-774.

Bracker, C.E. (1968) Ultrastructure of the haustorial apparatus of Erysiphe graminis and its relationship to the epidermal cell of barley. Phytopath. 58, 12-30.

Browning, A.J., Hall, J.L. & Baker, D.A. (1980) Cytochemical localisation of ATPase activity in phloem tissues of Ricinus communis. Protoplasma 104, 55-65.

Bushnell, W.R. (1972) Physiology of fungal haustoria. Ann. Rev. Phytopath. 10, 151-176.

Bushnell, W.R., Dueck, J. & Rowell, J.B. (1967) Living haustoria and hyphae of Erysiphe graminis f.sp. hordei with intact and partly dissected host cells of Hordeum vulgare. Can. J. Bot. 45, 1719-1732.

Bushnell, W.R. & Gay, J.L. (1978) Accumulation of solutes in relation to the structure and function of haustoria in powdery mildews. In: The Powdery Mildews (ed. D.M. Spencer), Academic Press, London, pp. 183-235.

Chard, J.M. & Gay, J.L. (1984) Characterization of the parasitic interface between Erysiphe pisi and Pisum sativum using fluorescent probes. Physiol. Plant Path. in press.

Chong, J. & Harder, D.E. (1980) Ultrastructure of haustorium development in Puccinia coronata avenae. I. Cytochemistry and electron probe X-ray analysis of the haustorial neck ring. Can. J. Bot. 58, 2496-2505.

Chong, J., Harder, D.E. & Rohringer, R. (1981) Ontogeny of mono- and dikaryotic rust haustoria: Cytochemical and ultrastructural studies. Phytopath. 71, 975-983.

Cocucci, M., Ballarin-Denti, A. & Marre, M.T. (1980) Effect of orthovanadate on $H^+$ secretion, $K^+$ uptake, electrical potential difference and microsomal ATPase of higher plant tissues. Plant Sci. Lett. 17, 391-400.

Coffey, M.D. (1975) Ultrastructural features of the haustorial apparatus of the white blister fungus Albugo candida. Can. J. Bot 53, 1285-1299.

Coffey, M.D. (1976) Flax rust resistance involving the K-gene: an ultrastructural study. Can. J. Bot. 54, 1443-1457.

Coffey, M.D. (1983) Cytochemical specialization at the haustorial interface of a biotrophic fungal parasite, Albugo candida. Can. J. Bot. 61, 2004-2014.

Coffey, M.D., Palevitz, B.A. & Allen, P.J. (1972) The fine structure of two rust fungi, Puccinia helianthi and Melampsora lini. Can. J. Bot. 50, 231-240.

Colley, R.H. (1918) Parasitism, morphology and cytology of Cronartium ribicola. J. Agric. Res. 15, 619-659.

Courtoy, R. & Simar, L.J. (1974) Importance of controls for demonstrating carbohydrates in electron microscopy with silver methanamine or the thio-carbohydrazide-silver proteinate methods. J. Microscopy 100, 199-211.

Demel, R.A., Geurts Van Kessel, W.S.M. & Van Deenen, L.L.M. (1972) The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. Biochim. Biophys. Acta 266, 26-40.

Dekhuijzen, H.M. (1966) The isolation of haustoria from cucumber leaves infected with powdery mildew. Neth. J. Plant Path. 72, 1-11.

Dupont, F.M., Giorgi, D.L. & Spanswick, R.M. (1982) Characterization of a proton-translocating ATPase in microsomal vesicles from corn roots. Plant Physiol. 70, 1694-1699.

Ehrlich, M.A. & Ehrlich, H.G. (1966) Ultrastructure of the hyphae and haustoria of Phytophthora infestans and hyphae of P. parasitica. Can. J. Bot. 44, 1495-1503.

Ehrlich, M.A. & Ehrlich, H.G. (1971) Fine structure of the host-parasite interfaces in mycoparasitism. Ann. Rev. Phytopath. 9, 155-184.

Fraymouth, J. (1956) Haustoria of the Peronosporales. Trans. Br. Mycol. Soc. 39, 79-107.

Gay, J.L. & Gil, F. (1975) The fungus-host interface. In: Proceedings of the First Intersectional Congress of IAMS (ed. Takezi Hasegawa), Science Council of Japan, Tokyo, Vol. 2, pp. 125-130.

Gay, J.L. & Manners, J.M.. Permeability of the parasitic interface in powdery mildews. In Preparation.

Gil, F. (1976) Ultrastructural and physiological properties of haustoria of powdery mildews and their host interfaces. Ph.D. Thesis, University of London.

Gil, F. & Gay, J.L. (1977) Ultrastructural and physiological properties of the host interfacial components of haustoria of Erysiphe pisi in vivo and in vitro. Physiol. Plant Path. 10, 1-12.

Glidewell, D.C. & Mims, C.W. (1979) Ultrastructure of the haustorial apparatus in the rust fungus Kunkelia nitens. Botanical Gazette 140, 148-152.

Gold, R.E. & Littlefield, L.J. (1978) Light and scanning electron microscopy of the telial, pycnial and aecial stages of Melampsora lini. Can. J. Bot. 57, 629-638.

Gold, R.E., Littlefield, L.J. & Statler, G.D. (1978) Ultrastructure of the pycnial and aecial stages of Puccinia recondita. Can. J. Bot. 57, 74-86.

Haigler, C.H., Brown, R.M. Jr. & Benziman, M. (1980) Calcofluor white ST alters the *in vivo* assembly of cellulose microfibrils. Science 210, 903-906.

Hanchey, P. & Wheeler, H. (1971) Pathological changes in ultrastructure: Tobacco roots infected with *Phytophthora parasitica* var. *nicotianae*. Phytopath. 61, 33-39.

Harder, D.E. (1978) Comparative ultrastructure of the haustoria in uredial and pycnial infections of *Puccinia coronata avenae*. Can. J.Bot. 56, 214-224.

Harder, D.E. & Mengden, K. (1982) Filipin-sterol complexes in bean rust- and oat crown rust-fungal/plant interactions: freeze-etch electron microscopy. Protoplasma 112, 46-54.

Harder, D.E., Rohringer, R., Samborski, D.J., Kim, W.K. & Chong, J. (1978) Electron microscopy of susceptible and near-isogenic (sr6/Sr6) lines of wheat infected by *Puccinia graminis tritici*. I. The host-pathogen interface in the compatible (sr6/P6) interaction. Can. J. Bot. 56, 2955-2966.

Hardwick, N.V., Greenwood, A.D. & Wood, R.K.S. (1971) The fine structure of the haustorium of *Uromyces appendiculatus* in *Phaseolus vulgaris*. Can. J. Bot. 49, 383-390.

Harris, N. & Chrispeels, M.J. (1980) The endoplasmic reticulum of mung-bean cotyledons: Quantitative morphology of cisternal and tubular endoplasmic reticulum during seedling growth. Planta 148, 293-303.

Heath, M.C. (1971) Haustorial sheath formation in cowpea leaves immune to rust infection. Phytopath. 61, 383-388.

Heath, M.C. (1972) Ultrastructure of the host and nonhost reactions to cowpea rust. Phytopath. 62, 27-38.

Heath, M.C. (1976) Ultrastructural and functional similarity of the haustorial neckband of rust fungi and the Casparian strip of vascular plants. Can. J. Bot. 54, 2484-2489.

Heath, M.C. (1979) Partial characterization of the electron-opaque deposits formed in the non-host plant, French bean, after cowpea rust infection. Physiol. Plant Path. 15, 141-148.

Heath, M.C. (1981a) The suppression of the development of silicon-containing deposits in French bean leaves by exudates of the bean rust fungus and extracts from bean rust-infected tissue. Physiol. Plant Path. 18, 149-155.

Heath, M.C. (1981b) Insoluble silicon in necrotic cowpea cells following infection with an incompatible isolate of the cowpea rust fungus. Physiol. Plant Path. 19, 273-276.

Heath, M.C. & Bonde, M.R. (1983) Ultrastructural observations of the rust fungus *Physopella zeae* in *Zea mays*. Can. J. Bot. 61, 2231-2242.

Heath, M.C. & Heath, I.B. (1971) Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. Physiol. Plant Path. 1, 277-287.

Hepler, P.K. (1980) Membranes in the mitotic apparatus of barley cells. J. Cell Biol. 86, 490-499.

Herth, W. (1980) Calcofluor white and Congo red inhibit chitin microfibril assembly in Poteriochromonas: evidence for a gap between polymerisation and microfibril formation. J. Cell Biol. 87, 442-450.

Hewitt, H.G. & Ayres, P.G. (1975) Changes in $CO_2$ and water vapour exchange rates in leaves of Quercus robur infected by Microsphaera alphitoides. Physiol. Plant Path. 7, 127-137.

Hickey, J.L. & Coffey, M.D. (1978) A cytochemical investigation of the host-parasite interface in Pisum sativum infected by the downy mildew fungus Peronospora pisi. Protoplasma 97, 201-220.

Houslay, M.D. & Stanley, K.K. (1982) Dynamics of Biological Membranes. Influence on Synthesis, Structure and Function; John Wiley & Sons, Chichester, New York.

Humphreys, T. (1977) Dinitrophenol-induced efflux of sucrose from maize scutellum cells. Phytochem. 16, 1359-1364.

Ingram, D.S., Sargent, J.A. & Tommerup, I.C. (1976) Structural aspects of infection by biotrophic fungi. In: Biochemical Aspects of Plant-Parasite Relationships (ed. J. Friend & D.R. Threlfall), Academic Press, London, pp. 43-78.

Jeffries, P. & Young, T.W.K. (1976) Ultrastructure of infection of Cokeromyces recurvatus by Piptocephalis unispora (Mucorales). Arch. Microbiol. 109, 277-288.

Jones, L.H.P. & Milne, A.A. (1963) Studies of silica in the oat plant. I. Chemical and physical properties of the silica. Plant and Soil 182, 207-220.

Kimmelberg, H.K. & Paphadjopoulus, D. (1972) Phospholipid requirements for $(Na^+ + K^+)$-ATPase activity: Head-group specificity and fatty acid fluidity. Biochim. Biophys. Acta 282, 277-292.

Kunoh, H. & Ishizaki, H. (1975) Silicon levels near penetration sites of fungi on wheat, barley, cucumber and morning glory leaves. Physiol. Plant Path. 5, 283-287.

Kunoh, H. & Ishizaki, H. (1976a) Accumulation of chemical elements around the penetration sites of Erysiphe graminis hordei on barley leaf epidermis. II. Level of silicon in papilla around the haustorial neck. Ann. Phytopath. Soc. Jpn. 42, 30-34.

Kunoh, H. & Ishizaki, H. (1976b) Accumulation of chemical elements around the penetration sites of Erysiphe graminis hordei on barley leaf epidermis: (III) micromanipulation and X-ray microanalysis of silicon. Physiol. Plant Path. 8, 91-96.

Kunoh, H., Takamatsu, S. & Ishizaki, H. (1978) Cytological studies of early stages of powdery mildew in barley and wheat. III. Distributions of residual calcium and silicon in germinated conidia of Erysiphe graminis hordei. Physiol. Plant Path. 13, 319-325.

Lewin, J. & Reimann, B.E.F. (1969) Silicon and plant growth. Ann. Rev. Plant Physiol. 20, 289-304.

Lin, W. (1981) Inhibition of anion transport on corn root protoplasts. Plant Physiol. 68, 435-438.

Littlefield, L.J. & Bracker, C.E. (1970) Continuity of host plasma membrane around haustoria of Melampsora lini. Mycologia 62, 609-614.

Littlefield, L.J. & Bracker, C.E. (1972) Ultrastructural specialisation at the host-pathogen interface of rust-infected flax. Protoplasma 74, 271-305.

Littlefield, L.J. & Heath, M. (1979) Ultrastructure of Rust Fungi; Academic Press, New York and London

Luft, J.H. (1961) Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9, 409-414.

Maclean, D.J. & Tommerup, I.C. (1979) Histology and physiology of compatability and incompatability between lettuce and the downy mildew fungus, Bremia lactucae Regel. Physiol. Plant Path. 14, 291-312.

Maeda, H. & Ishida, N. (1967) Specificity of binding of hexapyranosyl polysaccharides with fluorescent brightener. J. Biochem. 62, 276.

Manners, J.M. (1979) Physiology of fungal haustoria (Erysiphales). Ph.D. Thesis, University of London.

Manners, J.M. & Gay, J.L. (1977). The morphology of haustorial complexes isolated from apple, barley, beet and vine infected with powdery mildews. Physiol. Plant Path. 11, 261-266.

Manners, J.M. & Gay, J.L. (1978) Uptake of $^{14}C$ photosynthates from Pisum sativum by haustoria of Erysiphe pisi. Physiol. Plant Path. 12, 199-209.

Manners, J.M. & Gay, J.L. (1980) Autoradiography of haustoria of Erysiphe pisi. J. Gen. Microbiol. 116, 529-533.

Manners, J.M. & Gay, J.L. (1983) The host-parasite interface and nutrient transfer in biotrophic parasitism. In: Biochemical Plant Pathology (ed. J.A. Callow), John Wiley & Sons, Chichester, pp. 163-195.

Meier, H., Buchs, L., Buchala, A.J. & Homewood, T. (1981) (1-3)-B-D-glucan (callose) is a probable intermediate in biosynthesis of cellulose of cotton fibres. Nature 289, 821-822.

Mettler, I.J., Mandala, S. & Taiz, L. (1982) Characterization of *in vitro* proton pumping by microsomal vesicles isolated from corn coleoptiles. Plant Physiol. 70, 1738-1742.

Morré, D.J. & Mollenhauer, H.H. (1974) The endomembrane comcept: A functional integration of endoplasmic reticulum and Golgi apparatus. In: Dynamics of Plant Ultrastructure (ed. A.W. Robards), McGraw-Hill Book Co., New York, pp. 84-137.

Morré, D.J. & Ovtracht, L. (1977) Dynamics of the Golgi apparatus: Membrane differentiation and membrane flow. In: International Review of Cytology, Supplement 5 (ed. G.H. Bourne & J.F. Danielli), Academic Press, New York, pp. 61-188.

Mount, M.S. & Ellingboe, A.H. (1969) $^{32}P$ and $^{35}S$ transfer from susceptible wheat to *Erysiphe graminis* f.sp. *tritici* during primary infection. Phytopath. 59, 235.

Onoe, T., Tani, T. & Haito, N. (1973) The uptake of labelled nucleosides by *Puccinia coronata* grown in susceptible oat leaves. Rep. Tottori Mycol. Inst. 10, 303-312.

Pares, R.D. & Greenwood, A.D. (1979) The perihaustorial membrane in hop cells infected by *Pseudoperonospora humuli*. New Phytol. 83, 473-477.

Perera, R. & Gay, J.L. (1976) The ultrastructure of haustoria of *Sphaerotheca pannosa* (Wallroth ex Fries) Léveillé and changes in infected and associated cells of rose. Physiological Plant Pathology 9, 57-65.

Peterson, C.A., Emanuel, M.E. & Wilson, C.E. (1982) Identification of a Casparian band in the hypodermis of onion and corn roots. Can. J. Bot. 60, 1529-1535.

Peyton, G.A. & Bowen, C.C. (1963) The host-parasite interface of *Peronospora manshurica* on *Glycine max*. Am. J. Bot. 50, 787-797.

Pfenninger, K.H. & Bunge, R.P. (1974) Freeze-fracturing of nerve growth cones and young fibres. A study of developing plasma membrane. J. Cell Biol. 63,180-196.

Pick, U., Gounaris, K., Admon, A. & Barber, J. (1984) Activation of the $CF_o$-$CF_1$, ATP synthase from spinach chloroplasts by chloroplast lipids. Biochim. Biophys. Acta 765, 12-20.

Rattee, I.D. & Breur, M.M. (1974) The Physical Chemistry of Dye Adsorption, Academic Press, New York.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208-212.

Rice, M.A. (1927) The haustoria of certain rusts and the relation between host and pathogene. Bull. Torrey Bot. Club 54, 63-153.

Rijkenberg, F.H.J. & Truter, S.J. (1973) Haustoria and intracellular hyphae in the rusts. Phytopath. 63, 281-286.

Robb, J., Harvey, A.E. & Shaw, M. (1975) Ultrastructure of tissue cultures of Pinus monticola infected by Cronartium ribicola. II. Penetration and post-penetration. Physiol. Plant Path. 5, 9-18.

Roland, J.C., Lembi, C.A. & Morré, D.J. (1972) Phosphotungstic acid - chromic acid as a selective electron dense stain for plasma membranes of plant cells. Stain Technol. 47, 195-200.

Sangster, A.G. & Parry, D.W. (1971) Silica deposition in the grass leaf in relation to transpiration and the effect of dinitrophenol. Ann. Bot. 35, 667-677.

Sangster, A.G. & Parry, D.W. (1976) Endodermal silicon deposits and their linear distribution in developing roots of Sorghum bicolor (L.) Moench. Ann. Bot. 40, 361-372.

Sargent, C. & Gay, J.L. (1977) Barley epidermal apoplast structure and modification by powdery mildew contact. Physiol. Plant Path. 11, 195-205.

Sargent, J.A. (1981) The fine structure of the downy mildews. In: The Downy Mildews (ed. D.M. Spencer), Academic Press, London, pp. 183-236.

Satir, B., Sale, W.S. & Satir, P. (1976). Membrane renewal after dibucaine deciliation of Tetrahymena. Exp. Cell. Res. 97, 83-91.

Scarborough, G.A. (1976) The Neurospora plasma membrane ATPase is an electrogenic pump. Proc. Nat. Acad. Sci. USA 73, 1485-1488.

Schnepf, E., Hausmann, K. & Herth, W. (1982) The osmium tetroxide-potassium ferrocyanide (OsFeCN) staining technique for electron microscopy: A critical evaluation using ciliates, algae mosses and higher plants. Histochem. 76, 261-271.

Scurfield, G., Anderson, C.A. & Segnit, E.R. (1974) Silica in woody stems. Austral. J. Bot. 22, 211-229.

Sexton, R. & Hall, J.L. (1978) Enzyme cytochemistry. In: Electron Microscopy and Cytochemistry of Plant Cells (ed. J.L. Hall), Elsevier/North Holland Biomedical Press, Amsterdam, pp. 63-147.

Simons, T.J.B. (1979) Vanadate - a new tool for biologists. Nature 281, 337-338.

Singer, S.J. & Nicholson, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

Slesinski, R.S. & Ellingboe, A.H. (1971) Transfer of $^{35}S$ from wheat to the powdery mildew fungus with compatible and incompatible parasite/host genotypes. Can. J. Bot. 49, 303-310.

Smith, G. (1900) The haustoria of the Erysipheae. Bot. Gazette 29, 153-184.

Smith, J.C., Russ, P., Cooperman, B.S. & Chance, B. (1976) Synthesis, structure determination, spectral properties and energy-linked spectral responses of the extrinsic probe Oxonol V in membranes. Biochem. 15, 5094-5105.

Spencer-Phillips, P.T.N. & Gay, J.L. (1981) Domains of ATPase in plasma membranes and transport through infected plant cells. New Phytol. 89, 393-400.

Stadelmann, E.J. & Kinzel, H. (1972) Vital staining of plant cells. In: Methods in Cell Physiology (ed. D.M. Prescott), Academic Press Inc., New York, London, vol. V, pp. 325-372.

Staehelin, L.A. & Kiermayer, O. (1970) Membrane differentiation in the Golgi complex of Micrasterias denticulata Bréb. visualized by freeze-etching. J. Cell Sci. 7, 787-792.

Sullivan, T.P., Bushnell, W.R. & Rowell, J.B. (1974) Relations between haustoria of Erysiphe graminis and host cytoplasm in cells opened by microsurgery. Can. J. Bot. 52, 987-998.

Thatcher, F.S. (1942) Further studies of osmotic and permeability relations in parasitism. Can. J. Res. (ser. C) 20, 283-311.

Thiéry, J.P. (1967) Mise en evidence des polysaccharies sur coupes fines en microscopie electronique. J. Microscopie (Paris) 6, 987-1018.

Van Der Kamp, B.J. (1969) Peridermium pini (Pers.) Lev. and the resin-top disease of Scots Pine.III. Infection and lesion development. Forestry 43, 73-88.

Van der Woude, W.J. (1973) Significance of the specific staining of plant plasma membranes by treatment with chromic acid - phosphotungstic acid. Plant Physiol. 51, 15 (suppl).

Van Dyke, C.G. & Hooker, A.L. (1969) Ultrastructure of host and parasite in interactions of Zea mays with Puccinia sorghi. Phytopath. 59, 1934-1946.

Waggoner, A. (1976) Optical probes of membrane potential. J. Membrane Biol. 27, 317-334.

Walles, B. (1974) Ultrastructure of the rust fungus Peridermium pini (Pers.) Lev. Studia Forestalia Suecica 122, 1-30.

Walz, B. (1982) Sequestering smooth endoplasmic reticulum in an invertebrate photoreceptor. I. Intracellular topography as revealed by OsFeCN staining and in situ Ca accumulation. J. Cell. Biol. 93, 839-848.

Woods, A.M. & Gay, J.L. (1983) Evidence for a neckband delimiting structural and physiological regions of the host plasma membrane associated with haustoria of Albugo candida. Physiol. Plant Path. 23, 73-88.

Zeyen, R.J. & Bushnell, W.R. (1979) Papilla response of barley epidermal cells caused by Erysiphe graminis: rate and method of deposition determined by microcinematography and transmission electron microscopy. Can. J. Bot. 57, 898-913.

Zeyen, R.J., Carver, T.L.W. & Ahlstrand, G.G. (1983) Relating cytoplasmic detail of powdery mildew infection to presence of insoluble silicon by sequential use of light microscopy, SEM and X-ray microanalysis. Physiol. Plant Path. 22, 101-108.

*Physiological Plant Pathology* (1983) **23,** 73–88

# Evidence for a neckband delimiting structural and physiological regions of the host plasma membrane associated with haustoria of *Albugo candida*

A. M. Woods and J. L. Gay

*Department of Pure and Applied Biology, Imperial College of Science and Technology, London SW7 2BB*

(*Accepted for publication January 1983*)

The infection of *Cardamine hirsuta* by *Albugo candida* was investigated by transmission electron microscopy, using a lead precipitation technique to determine sites of ATP-ase activity at pH 7·2 in the presence of magnesium ions and section-staining methods specific for plasma membranes and polysaccharides.

ATP-ase activity was only observed on the host plasmalemma lining the wall, but was absent from the region invaginated around haustoria and the haustorial plasmalemma. The polysaccharide stain reacted intensely with the fungal and host plasmalemmas except in part of the neck region (region B) where staining was slight. The fungal wall in this region appeared electron-transparent after this stain, but showed slight granularity elsewhere. The plasmalemma-specific stain reacted intensely with the wall-lining region and region B of the host plasma membrane, but only slightly to moderately with most of the invaginated part, excepting the extremities of peripheral membranes of the matrical network around mature haustoria. Intense staining was shown by the fungal plasmalemma and region B of the fungal wall.

The properties of region B are discussed in relation to the neckbands of powdery mildew and rust haustoria and a mechanism of solute transport into haustoria of *A. candida* is proposed.

## INTRODUCTION

Considerable evidence has accumulated to show continuity of the host plasma membrane around haustoria of rusts [*7*, *10*, *15*, *20*, *21*], powdery mildews [*12*], and downy mildews [*3*, *9*, *13*, *24*, *26*]. However, the invaginated region of the membrane has been found to be structurally distinct from the wall-lining region in both powdery mildew [*12*, *22*] and rust [*21*] infections. In addition, recent research has demonstrated a physiological difference between the two domains of the host plasma membrane in both types of infection, and this has provided a basis for an hypothesis for nutrient transport into haustoria [*31*]. Wherever distinct domains have been observed the transition regions are marked by structures, termed neck bands, visible in and around the haustorial neck wall. Since, according to the fluid mosaic model for membranes [*30*], the molecules of two regions of a plasmalemma are free to diffuse laterally and so mix freely, the evidence indicates that the neckband forms a stabilizing structure which maintains the two distinct domains of the host plasma membrane. This accounts for the lack of modification shown by the wall-lining region of the host plasma membrane and it has been proposed that this feature is essential for the transport of nutrients into haustoria [*31*].

0048–4059/83/040073 + 16 $03.00/0 © 1983 Academic Press Inc. (London) Limited

In Oomycete infections only Hickey & Coffey [*19*] with *Peronospora pisi*, and Pares & Greenwood [*24*] with *Pseudoperonospora humuli* have demonstrated structural distinctness of the invaginated from the uninvaginated regions of host plasma membranes, but neither physiological differences nor structures corresponding to neck bands have been described. Therefore, a thorough investigation of an *Albugo candida* infection has been made using three techniques to distinguish structural and physiological characteristics of the membranes, and special structures associated with the transition.

*Albugo candida* is an Oomycete classified in the Peronosporales and was chosen for this study since it is one of the most biotrophic of this group and therefore seemed likely to have structures comparable to those of rust and powdery mildew infections.

## MATERIALS AND METHODS

*Fixation and embedding of material for differential staining*

Naturally infected leaves and stems of *Cardamine hirsuta* bearing young blisters of *A. candida* (Pers.) Kuntze which had not erupted were cut into 1 mm cubes under a fixative solution of 2·5% glutaraldehyde and 0·1 M sodium cacodylate buffer at pH 7. The cubes were left in the fixative for 24 h at room temperature and then washed in the buffer three times, each for 20 min. The specimens were post-fixed in 1% osmium tetroxide in 0·1 M sodium cacodylate for 1–2 h at room temperature. This was followed by dehydration in a graded ethanol series and the specimens were then processed for embedding as follows: successive 10-min periods in ethanol/epoxypropane (2 : 1), ethanol/epoxypropane (1 : 2), 100% epoxypropane, epoxypropane/epon (70 : 30) for 1 h with the specimen enclosed and then open to the atmosphere overnight at room temperature to allow the epoxypropane to evaporate. The epon resin used was obtained from TAAB Laboratory Equipment Ltd, Reading, U.K. The specimens were then transferred to fresh resin on four occasions over 2 days before polymerization at 60 °C for 60 h in a flat PVC embedding dish. Sections of the embedded material were cut using a diamond knife and a Reichert ultramicrotome. These were then mounted on 200 mesh gold grids.

*Cytochemical staining methods*

*Phosphotungstic acid–chromic acid technique (PACP).* This method was adapted from Roland, Lembi & Morré [*27*]. Grids were immersed in 1% periodic acid for 30 min, washed in distilled water, stained for 15 min in an aqueous solution of 10% chromic acid and 1% phosphotungstic acid (PTA) and then again washed in distilled water.

For a control, treatment with chromic acid and PTA was omitted.

*Periodic acid–thiocarbohydrazide–silver proteinate technique (PATCHSP).* This method was adapted from Thiéry [*32*]. Sections on grids filmed with Formvar were immersed in 1% periodic acid for 30 min, washed in distilled water and then transferred to 0·2% thiocarbohydrazide (TCH) in 20% acetic acid for 3 days. After washing in a series of acetic acid solutions (20, 15, 10 and 5%) for 5 min at each concentration and finally in distilled water, the grids were transferred to an aqueous solution of 1% silver proteinate for 30 min in the dark. Freshly-prepared solutions of TCH and silver proteinate were always used.

Control treatments, first suggested by Courtoy & Simar [*8*], omitted either periodic acid, TCH or silver proteinate treatments.

*Localization of ATP-ase activity*

The method employed was the same as used by Spencer-Phillips & Gay [*31*] which had been adapted from Bentwood & Cronshaw [*2*]. Infected leaves were immersed in fixative and the lower epidermes were removed and pieces (0·5–1·0 mm²) excised to aid the permeation of the tissue. The fixative was a mixture of glutaraldehyde (0·5%) and formaldehyde (4%) in sodium cacodylate buffer (0·1 M) at pH 7·2. After 1·5–2 h the specimens were washed in Tris–maleate buffer and then incubated for 2·5 h in a mixture of ATP (2 mM), magnesium nitrate (2 mM) and lead nitrate (2 mM) in Tris–maleate buffer (0·1 M) at pH 7·2. Control treatments were carried out without substrate, with β-glycerophosphate (β-GP) replacing ATP, or with sodium fluoride included in the medium. After incubation, the specimens were washed in sodium cacodylate buffer, post-fixed in osmium tetroxide, dehydrated and embedded according to the procedure described above.

Ultrathin sections were cut using a diamond knife and a Reichert ultramicrotome. These were then mounted on Formvar-coated 100 mesh copper grids and examined either unstained or after immersion in 2% aqueous uranyl acetate for 30 min at 60 °C, rinsing in distilled water, transferring to lead citrate for 10 min and finally rinsing again in distilled water.

All sections were observed with a Philips 301G or an AEI 6B electron microscope.

## RESULTS

Observation with the electron microscope showed the collar, penetration jacket, haustorium and extrahaustorial matrix within the infected cells, as described in previous investigations of *A. candida* [*3*, *6*], except that the penetration jacket was often ill-defined and variable in extent making it impossible to detect its limits with certainty. Continuity of the wall-lining region of the host plasma membrane with invaginations around individual haustoria was traced and shown to follow the contours of the collar, the haustorial neck distal to the collar, and the haustorial body (Fig. 2). Both host and fungal plasma membranes were very closely associated with the fungal wall in a part of the neck which is referred to as region B [Figs 1(a), (b); 2; 4]. Many vesicles and membranous cisternae could be seen in the host cytoplasm around the bodies of mature haustoria [Figs 2; 3(a)–(c); 4(a)]. Since their contents always appeared identical to the extrahaustorial matrix and connections could often be seen between their membranes and the extrahaustorial membrane, so that an extensive network was sometimes present, they probably represent extensions of the matrix. Also, cisternae with contents staining differently from the matrix, and representing host endoplasmic reticulum were often seen in very close association with the extrahaustorial membrane, but few definite connections were seen [Figs 3(b), (c); 5].

The special staining and enzyme localization techniques employed, selectively stained structures of the host–parasite interface to reveal various domains in the host and fungal plasma membranes and regions of the fungal cell wall.

In the experiments where ATP was used as a substrate, precipitates ranging from slightly granular, to granular, to flocculent were observed and indicated different degrees of ATP-ase activity. Deposits heavier than those obtained in the controls without substrate and the $\beta$-GP controls were confined to the wall-lining region of the host plasma membrane and were absent from the invagination (Fig. 1). $\beta$-GP gave precipitates at the host tonoplast. The sodium fluoride controls sometimes gave deposits on the wall-lining plasma membrane very similar to those obtained without sodium fluoride in the medium. The mesophyll cells usually showed relatively low ATP-ase activities on their plasma membranes [Fig. 1(b)] but occasionally high levels were observed [Fig. 1(a)]. Infection did not alter the level of ATP-ase activity of the wall lining plasma membrane. No activity above the levels in any controls was observed on the fungal plasma membrane in haustoria (Fig. 1) but occasionally a reaction was shown in intercellular hyphae.

Additional evidence of differences between invaginated and wall-lining regions of the host plasma membrane was obtained when their reaction to the PACP staining technique was observed (Figs 2, 3). The host plasma membrane of uninfected cells and the wall-lining region of infected cells were usually heavily stained although there was some variation of intensity within the same cell (Figs 2, 3). However, the extrahaustorial region was only lightly to moderately stained (Figs 2, 3). The only exceptions were the membranes of the cisternae and the periphery of the matrical network around mature haustoria which were frequently as electron dense as the wall-lining region (Figs 2, 3). Thus, two distinct types of invaginated plasmalemma can be identified—the normal and the cisternal-network periphery. In both the intercellular hyphae and the haustoria, the fungal plasma membrane was more intensely stained than any part of the host plasma membrane.

When material had been treated with the PATCHSP method both the wall-lining and most of the invaginated host plasma membrane appeared moderately granular (Figs 4, 5). However, the part of the host plasmalemma in the neck region was distinguished by being only lightly stained (Fig. 4). The fungal plasma membrane within this region was unstained in contrast to its intense granular staining elsewhere (Fig. 4).

Thus, distinctions between different regions of both host and fungal plasma membranes have been observed with all methods. In each case the transition occurred in region B. The ATP-ase activity of the host plasma membrane ends abruptly where the membrane comes into close contact with the wall of the haustorial neck (Fig. 1); the transition in staining characteristics of the host plasmalemma observed with the PACP technique occurs at the other edge of this wall-associated band (Fig. 2); and both the host and fungal plasma membranes in this annular region are distinguished by the PATCHSP technique [Fig. 4(b)]. In addition, within region B the fungal wall stains differently with both the PACP and PATCHSP methods. This region of special staining tapers towards its edges at the outer surface of the fungal wall. One edge is about one-third of the way along the neck from the penetration site and the other is just beyond the junction of the neck with the haustorial body. After treatment with the PACP technique, region B of the fungal wall is more densely stained than in other regions (Fig. 2). However, with the PATCHSP method the wall in region B appeared electron-transparent in contrast to the lightly stained walls of the

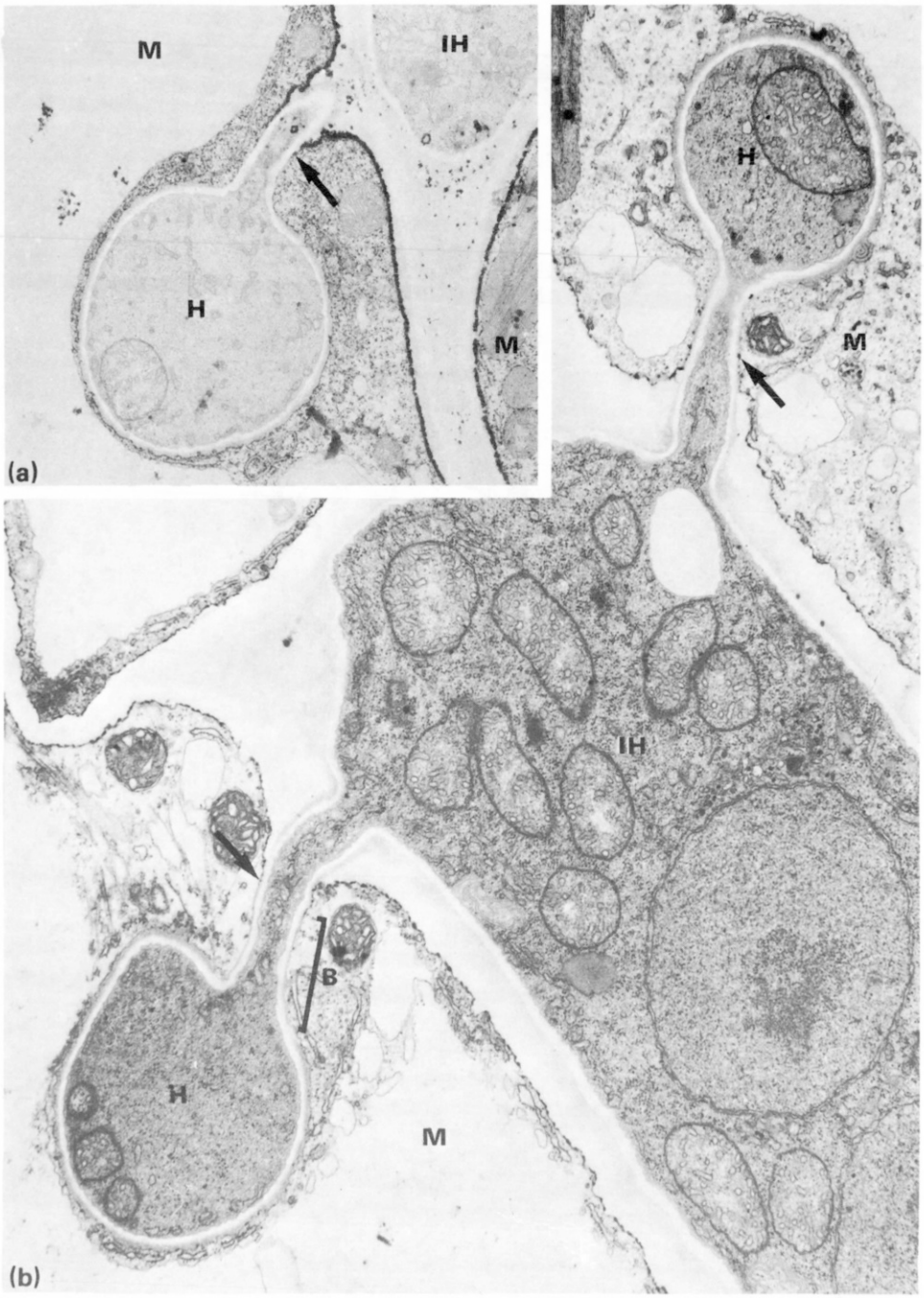



FIG. 1. (a), (b) Sections of mesophyll cells of *Cardamine hirsuta* infected with haustoria (H) of *Albugo candida* prepared from material incubated with ATP and magnesium ions at pH 7·2. The sections were stained with uranyl acetate and lead citrate. Precipitates indicating ATP-ase activity are present on the wall-lining host plasma membrane of the infected and adjacent cells; these are unusually dense in (a). Precipitates are absent from the invaginated host plasma membrane and the fungal plasma membrane. The transition in activity on the host plasmalemma occurs where the membrane comes into close contact with the fungal wall one-third to half-way along the haustorial neck (arrowed). Region B [bracketed in (b)] of the haustorial neck wall is only just discernable as slightly darker than other regions of the fungal wall after staining with uranyl acetate and lead citrate. IH, intercellular hypha; M, mesophyll cell. (a) × 11 800; (b) × 15 700.

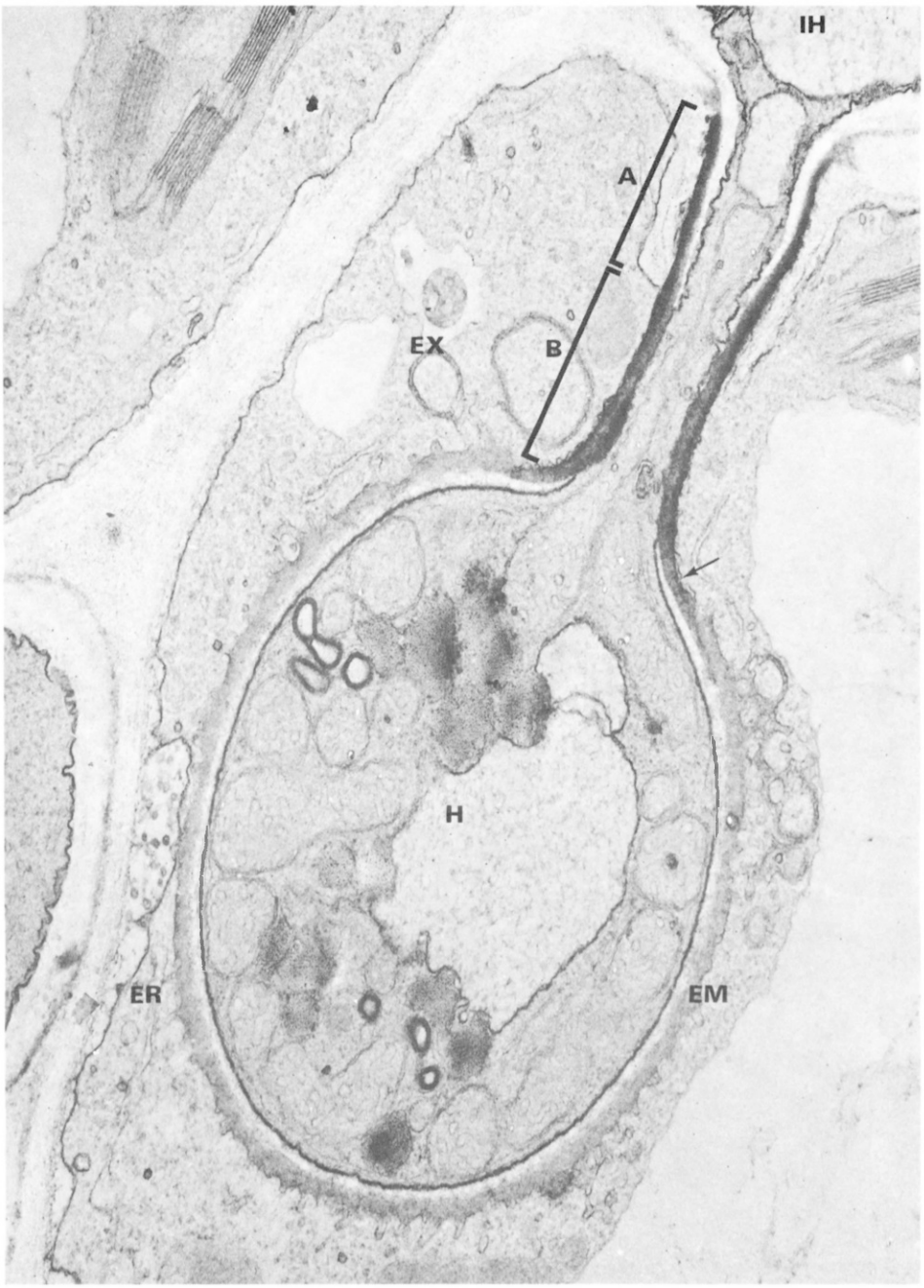



FIG. 2. Median section of haustorium (H) stained using the PACP technique. The haustorial neck wall shows region A, which is lightly stained as is the wall of the intercellular hypha (IH) and haustorial body, and B, which is intensely stained. The host plasmalemma is intensely electron-dense where it lies adjacent to the host cell wall and the haustorial neck region B, but is mostly only lightly stained around the haustorial body. The transition in the staining occurs at the edge of region B (arrowed). Extensions (EX) of the extrahaustorial matrix (EM) are limited by darkly stained membranes, unlike most of the membrane surrounding the haustorial body. Smooth and rough endoplasmic reticulum (ER) cisternae have lightly stained membranes × 27 200.

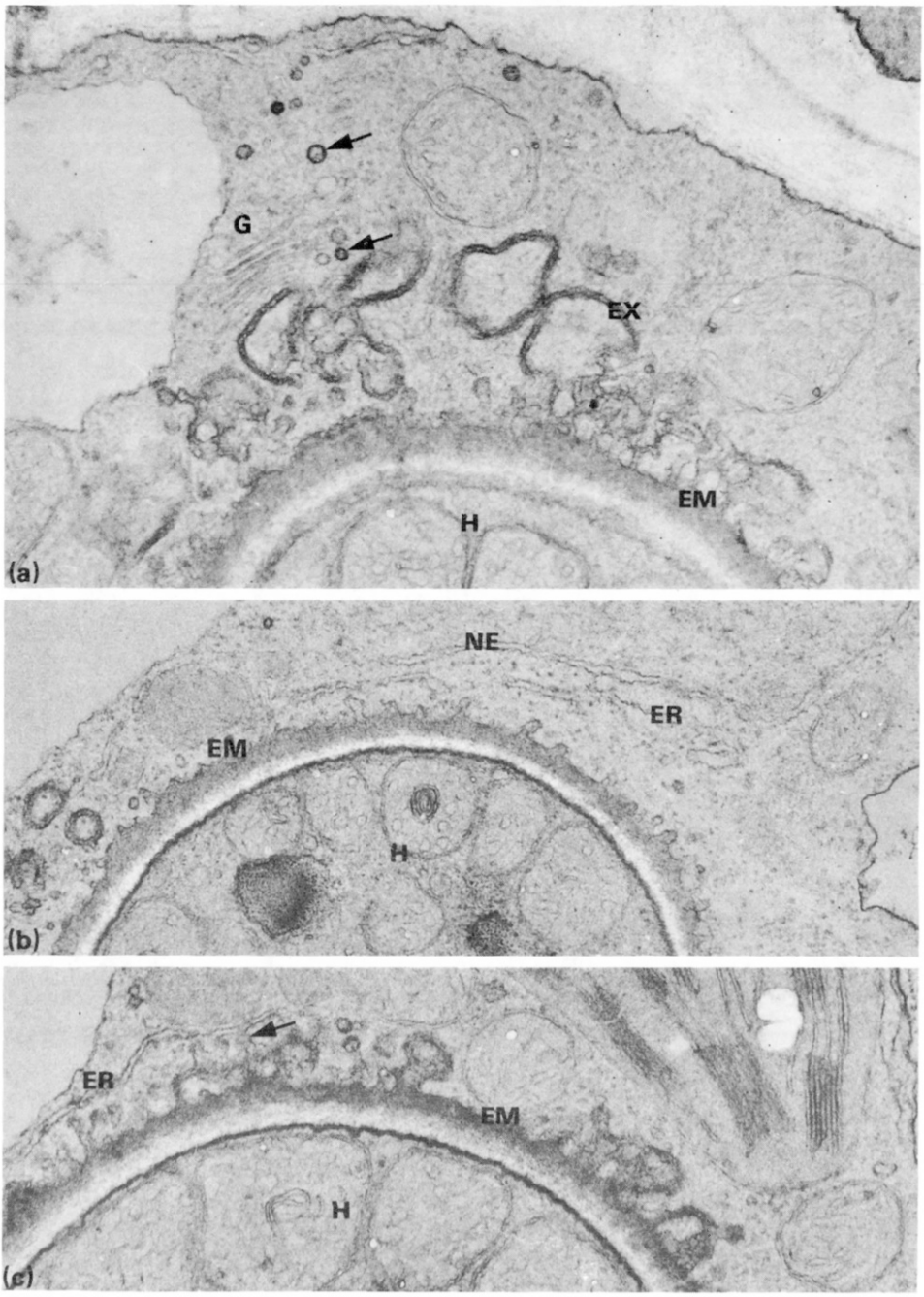



FIG. 3. Sections of margins of haustoria (H) stained using the PACP procedure. (a) shows intensely stained membraneous cisternae (EX) connected to the light to moderately electron-dense parts (EM) of the extrahaustorial membrane. The membranes of the Golgi body (G) within the host cytoplasm are very lightly stained although vesicles (arrowed) which may have been derived from the Golgi are bounded by membranes with similar electron density to the membraneous cisternae and the wall lining host plasmalemma. × 32 400. (b) Illustrates the similarity of staining shown by the normal regions of the extrahaustorial membrane (EM), the host endoplasmic reticulum (ER) and the nuclear envelope (NE). × 27 200. (c) Shows a probable connection (arrowed) between the normal extrahaustorial membrane (EM) and the smooth host endoplasmic reticulum (ER). × 36 950.

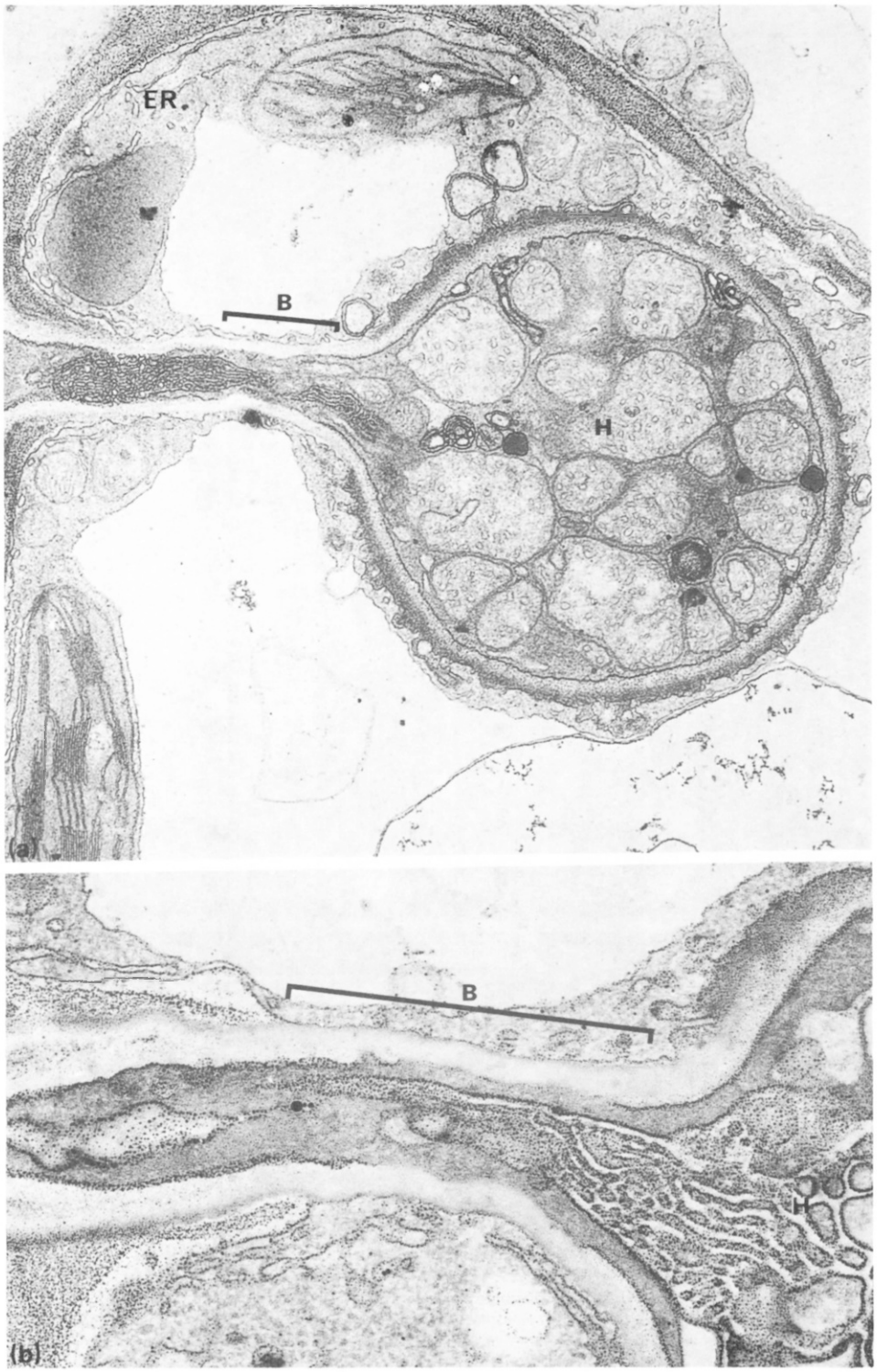



FIG. 4. (a), (b) Median sections of haustoria (H) stained by the PATCHSP method. Region B of the haustorial neck wall is virtually unstained in contrast to the granularity of the fungal wall elsewhere. Both host and fungal plasmalemmas are almost unstained in this region; this is shown especially clearly in (b). Elsewhere, the host plasmalemma is moderately granular, as is the host endoplasmic reticulum (ER), and the fungal plasma membrane shows intense granularity. (a) × 19 850; (b) × 48 600.

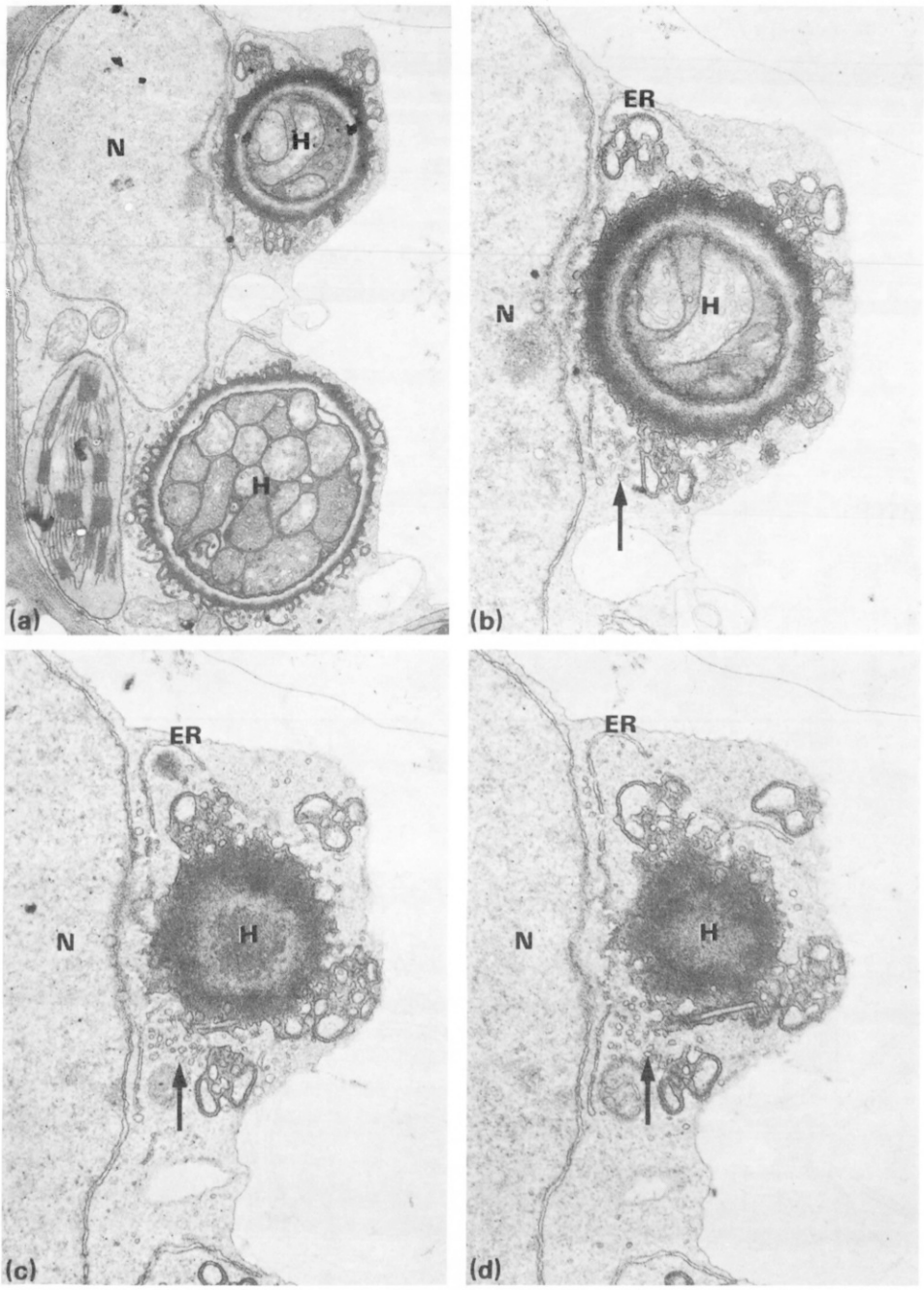
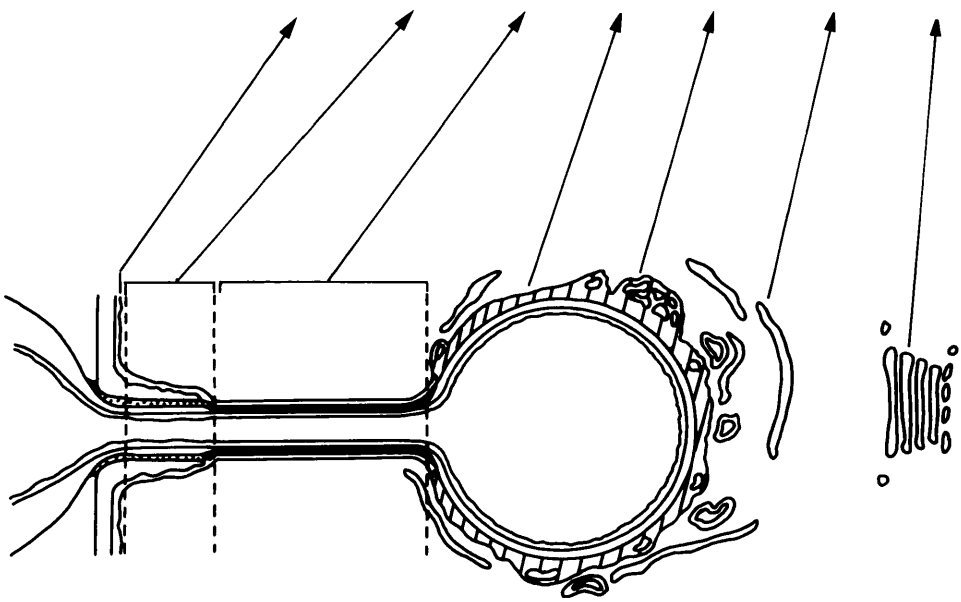



FIG. 5. Serial sections, stained by the PATCHSP method, of an haustorium (H) adjacent to the host nucleus (N), showing the very close relationship between the nuclear envelope, endoplasmic reticulum (ER) and extrahaustorial membrane (EM), and also the sponge-like elaborations of the latter. Profiles resembling transition vesicles can be seen between the endoplasmic reticulum and the extrahaustorial membrane (arrowed). (a) × 9900; (b)–(d) × 18 500.

TABLE 1

*Reaction of host membranes and fungal wall to specific treatments*

| Treatment | | Wall lining region of host plasmalemma | Haustorial neck region | | Haustorial body region | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A | B | Normal | Cisterna/ network periphery | Endoplasmic reticulum | Golgi body |
| ATP-ase | Host membrane | +/++ | +/++ | − | − | − | − | − |
| PACP | Host membrane | +++ | +++ | +++ | +/++ | +++ | +/++ | + |
| | Fungal wall | NA | + | +++ | + | + | NA | NA |
| PATCHSP | Host membrane | ++ | ++ | −[a] | ++ | ++ | ++ | ++ |
| | Fungal wall | NA | + | − | + | + | NA | NA |
| Uranyl acetate lead citrate | Host membrane | + | + | + | + | + | + | + |
| | Fungal wall | NA | + | +/++ | + | + | NA | NA |



[a] The fungal plasmalemma lacks stain in this region only.
−, No reaction; +, slight reaction; ++, moderate reaction; +++, intense reaction; NA, not applicable.

Diagram indicates regions examined.

intercellular hypha and haustorial body (Fig. 4). In uranyl acetate–lead citrate-stained material, this part of the neck wall can just be detected as slightly darker than normal (Fig. 1). All these observations are summarized in Table 1.

Other features of infected cells were also differentially stained. After treatment by the PACP method the membranes of the ER and the nuclear envelope were lightly to moderately stained, similar to the normal type of extrahaustorial membrane with which they were closely associated [Fig. 3(b)]. All the membranes of the Golgi stack were only lightly stained [Fig. 3(a)]. The PATCHSP technique, however, did not distinguish between the endoplasmic reticulum, nuclear envelope or Golgi membranes, all being moderately to intensely stained as described above for the invaginated and wall-lining host plasma membrane.

The extrahaustorial matrix was moderately electron-dense after both the PACP and PATCHSP procedures, as were the contents of the extrahaustorial extensions and similar profiles for which connections with the extrahaustorial matrix could not be seen [Figs 2, 3, 4(a)]. Usually, the contents of the endoplasmic reticulum were only lightly stained with both procedures, although some portions which were in very close association with the haustorium were more electron-dense (Fig. 5). With both the PACP and PATCHSP methods the Golgi stack showed a gradation in staining with unstained contents at one face and contents of moderate electron density, similar to the extrahaustorial matrix, at the other [Fig. 3(a)].

*Controls for PACP and PATCHSP staining*

All membranes were unstained in control sections of the PACP technique where chromic acid and PTA were omitted, illustrating the removal of osmium by the periodic acid. When periodic acid was omitted from the PATCHSP procedure, silver was deposited wherever osmium was present, due to the argentophilic nature of osmium. When either TCH or silver proteinate was omitted there was no deposition of silver.

## DISCUSSION

The types of deposits obtained in the ATP-ase localization experiments on *A. candida* were the same as those obtained in previous investigations [*4*, *31*]. Sometimes the addition of sodium fluoride to the incubation medium did not alter the level of deposit on the wall-lining region of the host plasmalemma. However, it has been shown that sodium fluoride is neither a specific nor an efficient inhibitor of ATP-ase activity [*29*]. Furthermore, it is to be expected that non-enzymic hydrolysis of the substrate by lead would result in similar deposits with ATP and $\beta$-GP as substrates. This was not found and therefore the activity shown on the wall-lining plasma membrane is almost certainly due to a specific ATP-ase. The lack of enzymic activity at the extrahaustorial membrane cannot be attributed to the non-penetration of the lead trapping agent because, with $\beta$-GP, precipitates were observed at the host tonoplast. The types of staining obtained with the PACP and PATCHSP techniques and their controls agree with those previously observed on comparable material [*21*, *19*].

The application of these methods has demonstrated both physiological and

structural differences between the wall-lining and the invaginated regions of the host plasma membrane in cells infected by *A. candida* with abrupt transitions occuring at the haustorial neck (region B). Thus, the results of this investigation indicate the presence of a stabilizing structure maintaining the two distinct domains of a continuous host plasma membrane. Region B may therefore be comparable to the neck bands of powdery mildews and rusts. The neckbands of powdery mildews and rusts do not show precisely the same morphology and differences even exist between the neckbands of individual rust species. Also, powdery mildews possess two neckbands, A and B [*12*], while rusts generally possess only one, although two adjacent bands, $\alpha$ and $\beta$, have been observed in *Puccina coronata* var. *avenae* [*5*]. However, common characteristics are shared by rust and mildew neckbands as listed below and the properties of region B of the haustorial neck wall of *A. candida* will be discussed in relation to each of them:

*(i)* The composition of the fungal wall is abnormal within the neckband (or neck ring) as indicated by its different staining properties [*5*, *7*, *10*, *12*, *15*, *16*, *18*, *22*, *25*, *33*].

Both the A and B bands of the powdery mildews and the neck band(s) of rusts are more electron-dense than elsewhere in the fungal wall after fixation including osmium tetroxide treatment and staining with uranyl and lead salts. Region B of the haustorial neck wall of *A. candida* is not especially electron-dense after uranyl acetate–lead citrate staining of osmium fixed material, but staining above the level in other regions of the fungal wall can just be discerned.

The neck ring of rusts (or the $\beta$ band when two bands are observed) is preferentially etched away by periodic acid, sometimes leaving a hole in the section, to give an electron-transparent region when stained by the PACP [*21*] or PATCHSP and PACP methods [*5*]. Region B of *A. candida* also appeared electron-transparent after staining with PATCHSP, but was electron-dense after PACP. Thus, unlike the rust neck ring, this region of the wall was not completely removed by periodic acid. These results indicate that during the PATCHSP procedure osmium was removed by the periodic acid to give electron transparency, but aldehyde groups were not generated; and that PTA and chromic acid preferentially stain substances which are present in region B but absent elsewhere.

Thus, although region B is like the rust and powdery mildew neckbands in being structurally different from the normal fungal wall, it differs from them in composition.

*(ii)* Only in the region of the neckband are both the host and fungal plasma membranes closely associated, without undulation, with the surfaces of the neck wall [*12*, *15*, *21*].

The host and fungal plasma membranes of *A. candida* infections are more closely associated with region B of the neck wall than in other regions. However, there is some undulation of the membranes indicating that the junction may not be as "tight" as in the other pathogens.

*(iii)* The neckband marks the point of transition in the characteristics of the host plasma membrane [*12*, *21*, *31*].

The transitions of ATP-ase activity, PACP- and PATCHSP-staining occurred at one or other edge of region B of the haustorial neck wall of *A. candida* as described in the results.

*(iv)* The neckband forms an impermeable barrier preventing direct diffusion of solutes between the extrahaustorial matrix and the apoplast [*11*, *17*].

This is an important criterion of a neck band which has yet to be investigated in *A. candida* infections.

The above comparisons provide strong evidence that region B in the haustorial neck wall of *A. candida* represents a neckband.

The findings of the present work agree with results of earlier investigations of *A. candida* [*3*, *6*] although the interpretations differ. Berlin & Bowen [*3*] noted the change of staining characteristics of the fungal wall at region B, but considered this to indicate the total absence of fungal wall. They suggested that the material present in its place was continuous with regions which are now commonly referred to as the extrahaustorial matrix and the penetration jacket. Coffey [*6*] established that the fungal wall was continuous around the haustorium, but the differently stained band was not reported. However, it can just be detected in his micrographs.

The electron transparency of region B observed after PATCHSP-staining may be due to the absence of polysaccharides or to their impregnation by additional substances which prevent them from reacting. The latter phenomenon was indicated in barley when, after staining for polysaccharide, clear halos were seen in epidermal cell walls in contact with *Erysiphe graminis* [*28*]. This was due to the deposition of silicon and cuticular lipids within the wall. Similarly, X-ray microanalysis has demonstrated the presence of silicon, phosphorous, chlorine and iron in the PATCHSP-negative $\beta$ neckband of *Puccina coronata* var. *avenae* [*5*]. However, the $\alpha$ neckband of *P. coronata* was PATCHSP-positive despite containing silicon.

No evidence has yet emerged indicating the cause of differences between the invaginated and wall-lining regions of the host plasma membrane in biotrophic infections. Possibly the fungus exerts some direct influence on the host plasma membrane with which it is associated, so changing its structure and function. Alternatively, the invaginated plasma membrane may be formed in a different way from the normal host plasma membrane. According to the endomembrane concept [*23*], membrane derived from the nuclear envelope passes to the endoplasmic reticulum, then to transition vesicles which coalesce to form the cisternae of the Golgi body. Subsequently, secretory vesicles separate from the mature face of the Golgi and travel through the cytoplasm to fuse with the plasma membrane. However, in *A. candida* the frequently observed close association of the endoplasmic reticulum with the extrahaustorial membrane and the similar staining reaction of these two types of membrane with the PACP technique suggest that the extrahaustorial membrane may be formed directly from the endoplasmic reticulum. A similar suggestion has been made for rust infections of wheat [*14*] where frequent close associations and occasional connections between the endoplasmic reticulum and the invaginated plasma membrane have been observed. There, a much more extensive endoplasmic reticulum network (termed the endoplasmic reticulum complex) was present and it was suggested that it represented a

modified Golgi-like structure. However, this hypothesis does not explain the intense staining of regions of the extrahaustorial membrane with PACP. Since these portions of the membrane react with PACP in the same way as the wall-lining plasma membrane, unlike any other host membrane, they may be derived either from the wall-lining plasma membrane or the same source as the latter. The common source explanation seems more likely since close associations of the membrane around the haustorial body with Golgi were more frequent than associations with the wall-lining plasma membrane. In addition, the PACP-positive regions were not present within the extrahaustorial membrane around young (as defined by Coffey [*6*]) haustoria and therefore were unlikely to be derived from the original invagination of the host plasma membrane. The presence around the haustorial body of two different types of membrane which are continuous indicates that restriction is imposed on the fluidity within the extrahaustorial membrane. However, unlike in the neck region, a structure associated with the distinction is not apparent.

The localization of ATP-ase activity on the wall-lining plasma membrane suggests the presence of an ATP-energized proton pump such as that proposed by Baker [*1*]. The hydrolysis of ATP at the membrane is thought to result in the extrusion of protons from the cell, thus maintaining an electrochemical gradient of protons across the membrane, down which organic solutes are transported into the cell. The level of activity in infected and uninfected mesophyll cells was usually low but similar to that observed in this tissue in pea [*2*, *31*]. This is probably because they contain chloroplasts and so manufacture their own photoassimilates, unlike epidermal cells which possess higher levels of ATP-ase activity on their plasma membrane. This presumably enables them to scavenge the apoplast for photosynthates.

The distribution of ATP-ase in plasma membranes of haustorium-infected cells of *Cardamine hirsuta* is similar to that described for powdery mildew and rust infections [*31*]. The *Albugo* and rust infections investigated are also similar in the absence of ATP-ase activity from the haustorial plasma membranes, but differ from haustoria of *Erysiphe pisi* in this respect. Thus, the mechanism proposed for solute uptake into haustoria of *E. pisi* [*31*] cannot be directly applied to haustoria of *A. candida* and *Uromyces appendiculatus*. In these infections the possible forces which may be implicated in solute influx into haustoria are:

(i) influx into infected cells by the ATP-ase driven pumps in the wall-lining region of the host plasma membrane;

(ii) increase of solute concentration by photosynthesis in infected cells;

(iii) decrease in concentration of soluble translocates by their incorporation into non-diffusable compounds, e.g. polysaccharide and protein, within the haustoria and intercellular mycelium.

In the *Albugo* and *Uromyces* infections haustorial uptake is thus likely to depend largely on active processes of the host and, for these to be effective, the absence of ATP-ase pumps from the extrahaustorial membrane and a neckband seal preventing loss from the extrahaustorial matrix to the leaf apoplast of solutes undergoing transport are essential. As pointed out above, the neckband, or its equivalent in *A. candida* is also essential in preventing the intermixing of components of the invaginated and wall-lining domains of the plasma membrane. The mixing would probably be detrimental to the host cell. Thus, the presence of an efficient and permanent neckband

is likely to be a key factor determining biotrophy and its period of operation in haustorial infections.

We are grateful to Miss Marion Martin for technical assistance and to the Science and Engineering Research Council for a postgraduate research studentship (A.M.W.) and a Research Grant.

## REFERENCES

1. Baker, D. A. (1978). Proton co-transport of organic solutes. *New Phytologist* **81,** 485–497.
2. Bentwood, B. J. & Cronshaw, J. (1978). Cytochemical localisation of adenosine triphosphatase in the phloem of *Pisum sativum* and its relation to the function of transfer cells. *Planta* **140,** 111–120.
3. Berlin, J. D. & Bowen, C. C. (1964). The host–parasite interface of *Albugo candida* on *Raphanus sativus*. *American Journal of Botany* **51,** 445–452.
4. Browning, A. J., Hall, J. L. & Baker, D. A. (1980). Cytochemical localisation of ATP-ase activity in phloem tissues of *Ricinus communis*. *Protoplasma* **104,** 55–65.
5. Chong, J. & Harder, D. E. (1980). Ultrastructure of haustorium development in *Puccinia coronata avenae*. I. Cytochemistry and electron probe X-ray analysis of the haustorial neck ring. *Canadian Journal of Botany* **58,** 2496–2505.
6. Coffey, M. D. (1975). Ultrastructural features of the haustorial apparatus of the white blister fungus *Albugo candida*. *Canadian Journal of Botany* **53,** 1285–1299.
7. Coffey, M. D., Palevitz, B. A. & Allen, P. J. (1972). The fine structure of two rust fungi, *Puccinia helianthi* and *Melampsora lini*. *Canadian Journal of Botany* **50,** 231–240.
8. Courtoy, R. & Simar, L. J. (1974). Importance of controls for demonstrating carbohydrates in electron microscopy with silver methenamine or the thio-carbo-hydrazide-silver proteinate methods. *Journal of Microscopy* **100,** 199–211.
9. Ehrlich, M. A. & Ehrlich, H. G. (1966). Ultrastructure of the hyphae and haustoria of *Phytophthora infestans* and hyphae of *P. parasitica*. *Canadian Journal of Botany* **44,** 1495–1503.
10. Ehrlich, M. A. & Ehrlich, H. G. (1971). Fine structure of the host-parasite interfaces in mycoparasitism. *Annual Review of Phytopathology* **9,** 155–184.
11. Gay, J. L. & Manners, J. M. (1983). Permeability of the parasitic interface in powdery mildews. *Physiological Plant Pathology* (in press).
12. Gil, F. & Gay, J. L. (1977). Ultrastructural and physiological properties of the host interfacial components of haustoria of *Erysiphe pisi in vivo* and *in vitro*. *Physiological Plant Pathology* **10,** 1–12.
13. Hanchey, P. & Wheeler, H. (1971). Pathological changes in ultrastructure: Tobacco roots infected with *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* **61,** 33–39.
14. Harder, D. E., Rohringer, R., Samborski, D. J., Kim, W. K. & Chong, J. (1978). Electron microscopy of susceptible and resistant near-isogenic (sr 6/Sr 6) lines of wheat infected by *Puccinia graminis tritici*. I. The host-pathogen interface in the compatible (sr 6/P6) interaction. *Canadian Journal of Botany* **56,** 2955–2966.
15. Hardwick, N. V., Greenwood, A. D. & Wood, R. K. S. (1971). The fine structure of the haustorium of *Uromyces appendiculatus* in *Phaseolus vulgaris*. *Canadian Journal of Botany* **49,** 383–390.
16. Heath, M. C. (1972). Ultrastructure of the host and nonhost reactions to cowpea rust. *Phytopathology* **62,** 27–38.
17. Heath, M. C. (1976). Ultrastructural and functional similarity of the haustorial neckband of rust fungi and the Casparian strip of vascular plants. *Canadian Journal of Botany* **54,** 2484–2489.
18. Heath, M. C. & Heath, I. B. (1971). Ultrastructure of an immune and a susceptible reaction of cowpea leaves to rust infection. *Physiological Plant Pathology* **1,** 277–287.
19. Hickey, E. L. & Coffey, M. D. (1978). A cytochemical investigation of the host-parasite interface in *Pisum sativum* infected by the downy mildew fungus *Peronospora pisi*. *Protoplasma* **97,** 201–220.
20. Littlefield, L. J. & Bracker, C. E. (1970). Continuity of host plasma membrane around haustoria of *Melampsora lini*. *Mycologia* **62,** 609–614.
21. Littlefield, L. J. & Bracker, C. E. (1972). Ultrastructural specialisation at the host-pathogen interface of rust-infected flax. *Protoplasma* **74,** 271–305.
22. Manners, J. M. & Gay, J. L. (1977). The morphology of haustorial complexes isolated from apple, barley, beet and vine infected with powdery mildews. *Physiological Plant Pathology* **11,** 261–266.
23. Morré, D. J. & Mollenhauer, H. H. (1973). The endomembrane concept: A functional integration of endoplasmic reticulum and Golgi apparatus. In *Dynamics of Plant Ultrastructure*, Ed. by A. W. Robards, pp 84–137. McGraw-Hill Book Company, New York.

24. Pares, R. D. & Greenwood, A. D. (1979). The perihaustorial membrane in hop cells infected by *Pseudoperonospora humuli*. *New Phytologist* **83,** 473–477.
25. Perera, R. & Gay, J. L. (1976). The ultrastructure of haustoria of *Sphaerotheca pannosa* (Wallroth ex Fries) Léveillé and changes in infected and associated cells of rose. *Physiological Plant Pathology* **9,** 57–65.
26. Peyton, G. A. & Bowen, C. C. (1963). The host-parasite interface of *Peronospora manshurica* on *Glycine max*. *American Journal of Botany* **50,** 787–797.
27. Roland, J. C., Lembi, C. A. & Morré, D. J. (1972). Phosphotungstic acid-chromic acid as a selective electron-dense stain for plasma membranes of plant cells. *Stain Technology* **47,** 195–200.
28. Sargent, J. A. & Gay, J. L. (1977). Barley epidermal apoplast structure and modification by powdery mildew contact. *Physiological Plant Pathology* **11,** 195–205.
29. Sexton, R. & Hall, J. L. (1978). Enzyme cytochemistry. In *Electron Microscopy and Cytochemistry of Plant Cells*, Ed. by J. L. Hall, pp. 63–147. Elsevier/North Holland Biomedical Press, Amsterdam.
30. Singer, S. J. & Nicholson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science* **175,** 720–731.
31. Spencer-Phillips, P. T. N. & Gay, J. L. (1981). Domains of ATP-ase in plasma membranes and transport through infected plant cells. *New Phytologist* **89,** 393–400.
32. Thiéry, J. P. (1967). Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *Journale de Microscopie (Paris)* **6,** 987–1018.
33. Van Dyke, C. G. & Hooker, A. L. (1969). Ultrastructure of host and parasite in interactions of *Zea mays* with *Puccinia sorghi*. *Phytopathology* **59,** 1934–1946.